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Development of an on-line high-performance liquid chromatography method for the rapid detection of boronic acids in complex mixtures

Report of Master Thesis ORC-80436 & ORC-80433

Puspita Ayu Wardani (790920928070)

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A rapid on-line method for detecting the fluorescent complex of Boronic Acids and Alizarin was established using an alkalic-acetonitrile of alizarin. The HPLC-separated analytes react post-column with the alizarin solution, and the fluorescent adduct is detected by an fluorescence detector at the excitation and emission wavelength respectively 469 nm and 610nm. An optimized instrumental setup is introduced. The method is applicable for both isocratic and gradient HPLC runs with mobile-phase compositions ranging from 10% to 90% organic solvent in water or acidic water. The method is simple, has abroad applicability, and uses common instruments, inexpensive and stable reagents, and a time-saving and non-laboratory experimental protocol. The method was applied to several derivatives of boronic acid .The minimum detectable concentration (MDC) were 0.03-33 µM which is depending on the compound tested.

Table of Contents

List of Figures

List of Table

List of Chromatogram

1. Introduction

1.1. Boronic acids

Boronic acids are widely used in synthesis organic chemistry as essential intermediate in Suzuki[1], Chan-Lam[2] and Liebeskind–Srogl[3] coupling reaction due to their particular belongings as a weak Lewis acid and . Their reversible covalent binding with sugar are also involved in a smart sensors and separation tools[4-6]. Moreover, boronic acids are also potential drugs as anticancer, antiviral and antibacterial [7]. Due to the widely usage of boronic acids, the rapid, sensitive and selective detection method of boronic acids needs to be developed to speed up purification process or to monitor the reaction progress.

This study is focused on developing method for boronic acid detection in complex mixtures using inexpensive reagent by means RP-HPLC to do separation on the mixtures.

1.2. On-line HPLC

The On-line HPLC is a tool that can be utilized to measure the product purity in near real time which can reduce product variability, increase process efficiency, and enable automation and control. This method is done simultaneously from one process to another in one running time analysis.

1.3. Current detection of Boronic acids during syntesis

The latest detection method for boronic acid was established in 2013 by Kumar and his team. To analyze boronate esters, Kumar proposed an analytical method using C18 column and no pH modifier to minimize on-column hydrolysis.[8] His study focused on the factors which influenced the hydrolysis occurance during analysis by means RP-HPLC using UV detector.

In 2012, Duval and her coworkers successfully developed the TLC method to monitor the boronic acids during synthesis reaction through developing silica plate in certain eluent followed by dipping it in alizarin solution[9]. Figure 1 describes the reaction that occurs when the TLC plate was dipped in alizarin solution. This TLC method has been successfully published and usefull for determining the end up of a reaction in synthesis involving boronic acids. In analogy with other HPLC on-line assays, e.g. for antioxidants, which were developed from TLC detection,[6,10-13] a new on-line analytical method to detect boronic acids is able to be established to selectively detect boronic acids in (preparative) LC eluates. This eluates will be reacted by fluorescent reporter as a post column reaction.

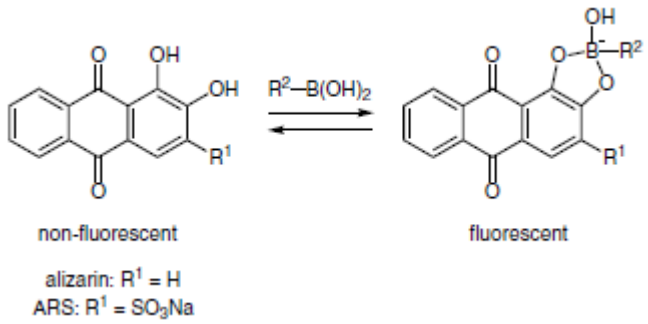


Figure 1. The reaction between alizarin/ARS with boronic acid[9]

1.4. Research Question

Can the detection method of boronic acid derivatives by means a TLC method with alizarin post-treatment be converted to an online HPLC system which the same principle as a detection method for radical scavengers developed by Koleva [10,11] and antioxidants detection proposed by Dapkevicius [12,13]?

1.5. Aim

The aim of this study is to establish such an online HPLC method, using alizarin, to detect and evaluate boronic acids that able to be applied in many practices.

1.6. Research Principle

The boronic acid derivatives are separated by RP-HPLC with UV detector and reacted with Alizarin as a post column reaction to form fluorescence complex which are detected by Fluorescence detector.

2. Experimental Section

2.1. Instrument Setup

The objective of this research was executed by two steps, namely offline and online experiment which subsequently done. The offline study was intended to investigate the factors that correspond to the online system. It is known that the interaction between boronic acids and diols are highly influenced by pH, buffer and solvent[14]. Therefore the effect of those three factors in the complex of boronic acids and alizarin were studied under both offline and online experiments.

2.1.1. The offline experiment

This system was carried out by fluorescence spectrometer from Edinburgh Instrument F900. The boronic acid and alizarin or ARS solutions were mixed, placed in a quartz cuvette (Suprasil® quartz, 10x10 mm path length, Hellma 101-QS) and scanned for both excitation and emission modes

2.1.2. The online experiment

This experiment was performed by HPLC system using two detectors, respectively UV and fluorescence detector. The tested compound was injected into HPLC system which equipped with pump from Knauer (K-1001 WellChrom; solvent organizer K-1500), injector with a 10µL loop from Must HP 6 (Multiport Streamswitch-Spark Holland) while the separation occurred in the Alltima column (5µ, 150mm x 3.0mm i.d.) and detected at 270 nm by UV detector from Waters (2487 dual λ-absorbance detector). The outlet of UV detector was connected by a microvolume connector- tees, to a super-loop for delivery of alizarin solution and a reaction coil for the reaction betwen BA-alizarin taking place. In the reaction coil, the fluorescent complex of BA and alizarin was formed and detected by a fluorescence detector from JASCO (Model FP-1520 Inteligent Fluorescence Detector, P/N: 0302-0378A, made in Japan) at the emission and the excitation wavelength respectively, 469nm and 610nm. The reaction coil was placed in a water bath which equipped with hot plate by Ikamag (ret no. 0515489, AFS 03485MR, 200-250V ~ 50/60Hz, 620W 0-110 1/min) and temperature controller by Ikatron™ (ETS-D2 temperature controller - IKA-Werke GmbH & Co. KG) to enhance the reaction rate of boronic acid and alizarin. The signals obtained from both detectors were processed by Agilent interface 35900E. The detailed online setup and the tubings used are described Figure 2 and Table 1.

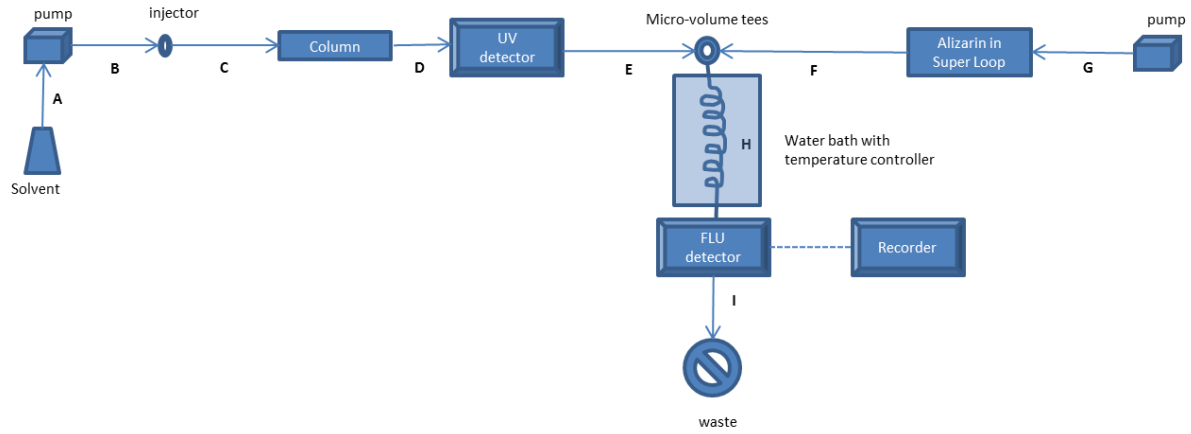


Figure 2. Instrumental set up for the online HPLC with Alizarin system

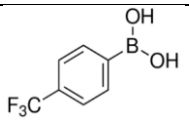
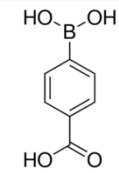
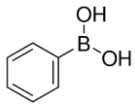
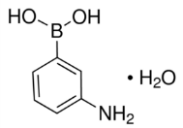
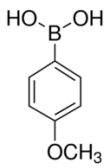
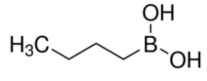
Table 1. List of tubing used in on-line setup

Tubing	Specification	P/N	Length (cm)
A	DIL 40x Tubing Inlet PTFE 20um Filter	Gilson 3645357	90
B	1/16" OD PEEK tubing; 0.005" i.d.	JR-T-6007	20
C	Alltech-Grace; stainless steel 0.0625" o.d. x 0.010" i.d.	2106936	30
D	Alltech-Grace; stainless steel 0.0625" o.d. x 0.010" i.d.	2106936	10
E	1/16" OD PEEK tubing; 0.01" i.d.	JR-T-6009	15
F	1/16" PTFE Tubing 0.76 mm i.d – Grace Davison Discovery Science	35670	15
G	1/16" PTFE Tubing 0.76 mm i.d – Grace Davison Discovery Science	35670	85
H	Reaction coil PEEK Tubing 3.5m x 0.01" i.d	JR-T-6009	350
I	1/16" PTFE Tubing 0.76 mm i.d – Grace Davison Discovery Science	35670	100

2.2. Materials

All solvents used were degassed by ultrasonic to remove air bubbles, before being employed in the online system. For the fluorescent reporter Alizarin Red S (ARS) certified by the Biological Stain Commission by Sigma Aldrich (P/N A5533-25G) and Alizarin made in Acros Organics (P/N 15369-1000) were utilized during this study. Besides boronic acids, the non-boronic acid compounds with various functional groups were also tested for the selectivity test purpose. The list of BAs, non BAs, solvents and reagents are presented, respectively in the Table 2, Table 3 and Table 4.

Table 2. List of Boronic acid (BA) derivatives

Name	Specification	Structure
4-(Trifluoromethyl)phenylboronic acid TFM-PBA	Sigma Aldrich Cat No. : 439320-1G	
4-Carboxyphenylboronic acid C-PBA	Sigma Aldrich Cat No. : 456772-10G	
Phenylboronic acid PBA	Fluka Cat No. : 78181-1G	
3-aminophenylboronic acid monohydrate A-PBA	Sigma Aldrich Cat No. : 287512-5G	
4-Methoxyphenylboronic acid M-PBA	Sigma Aldrich Cat No. : 417599-1G	
Butylboronic acid BBA	1-Butane boronic acid ; Sigma Aldrich Cat No. : 163244-1G	

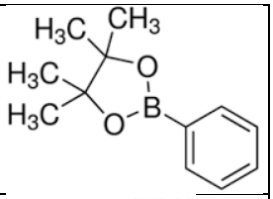
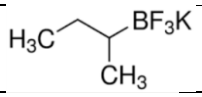
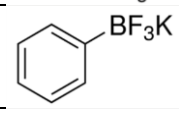
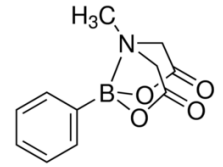
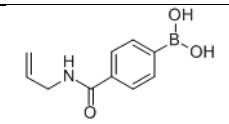
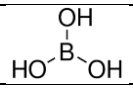
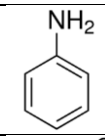
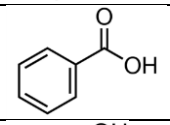
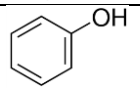
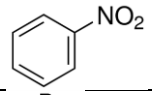
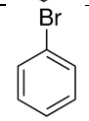
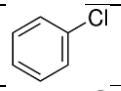
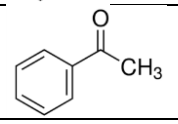
Phenylboronic acid pinacol ester PBA-PE	Sigma Aldrich Cat No. : 647098-5G	
Potassium Butyltrifluoroborate PBFB	Sigma Aldrich Cat No. : 660094-1G	
Potassium Phenyltrifluoroborate PPFB	Sigma Aldrich Cat No. : 563951-1G	
Phenylboronic acid MIDA ester 95% PBA-MIDA ester	Sigma Aldrich Cat No. : 698032-1G	
(4-Allylaminocarbonyl)phenylboronic acid AAC-PBA	ABCR Gmbh&Co.KG (Karlsruhe) Cat No. : AB150488.1g	
Boric acid	Sigma Aldrich Cat No. : B0394	

Table 3. List of non-BA used for selectivity test

Name	Specification	Structure
Aniline 99%	Reagen Plus®; Sigma Aldrich Chemie B.V. Cat No : 242284	
Benzoic acid ≥99.5%	ACS Reagent; Sigma Aldrich Chemie B.V Cat No. : 242381	
Phenol	unstabilized, reagent Plus ≥99% ; Sigma Aldrich Chemie B.V. Cat No : 185450	
Nitrobenzene	Sigma Aldrich Chemie B.V Cat No. : 252379	
Bromobenzene ≥99.5% GC, puriss	Sigma Aldrich Chemie B.V Cat No. : 16350	
Chlorobenzene, ACS, puriss. p.a.	Sigma Aldrich Chemie B.V Cat No. : 23570	
Acetophenone ≥99.0% (GC)	Sigma Aldrich Chemie B.V Cat No. : 00790-250mL	

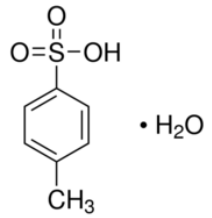
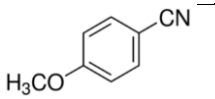
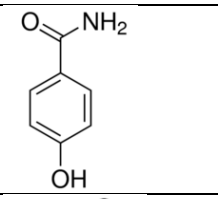
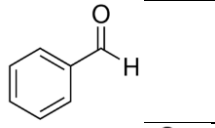
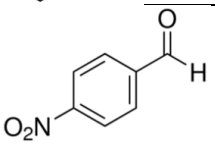
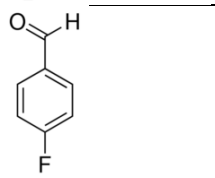
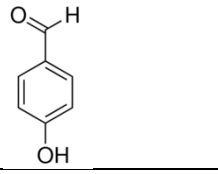
p-toluensulfonic acid monohydrate, ACS reagent, ≥98.5%	Sigma Aldrich Chemie B.V Cat No. : 402885	
4-methoxybenzonitrile 99%	Sigma Aldrich Chemie B.V Cat No. : 132470	
4-hydroxybenzamide 98%	Sigma Aldrich Chemie B.V Cat No. : 270253	
Benzaldehyde	Fisher Scientific Cat. No. : 105220010	
4-nitrobenzaldehyde	Sigma Aldrich Chemie B.V Cat No. : 130176	
4-fluorobenzaldehyde 98%	VWR International Cat. No. : A15383.14	
4-hydroxybenzaldehyde	Sigma Aldrich Chemie B.V Cat No. : 144088	

Table 4. List of solvents and reagents

Name	Specification
Milli-Q Integral 3 system	Molsheim, France
PBS (Phosphate Buffered Saline)	Sigma Aldrich P3813 pH 7.48
Acetonitrile HPLC-S	Biosolve BV; Cat No. : 01200702
Methanol CHROMASOLV®, for HPLC, ≥99.9%	Sigma Aldrich; Cat No. : 34860-2.5L
Acetone CHROMASOLV®, for HPLC, ≥99.8%	Sigma Aldrich; Cat No. : 34850-2.5L
THF [Tetrahydrofuran] (unstabilized) HPLC-S	Biosolve BV; Cat No. : 20220702
TEA (triethylamine) 99%	Acros Organics; Cat No. : 15791-0010
Acetic acid ACS Reagent ≥99.7%	Sigma Aldrich; Cat No. : 695092-100mL
Ethanol absolute	AnalaR NORMAPURE VWR; P/N: 20821.321
DMPA (2,2-Dimethoxy-2-phenylacetophenone) 99%	Sigma Aldrich; Cat No. : 19,611-8
Boc-cysteine	N-alpha-t-Buthyloxycarbonyl-L-cysteine 99% ABCR (Karlsruhe, Germany); Cat No. : AB 155598
HCl	Hydrochloride acid for analysis, fuming , 37%, solution in water; Acros Organics ; Code : 124630010

2.3. Methods

The objective of this research was executed by two steps, namely offline and online experiment which subsequently done. The offline study was intended to investigate the factors that correspond to the online system. It is known that the interaction between boronic acids and diols are highly influenced by pH, buffer and solvent[14]. Therefore the effect of those three factors in the complex of boronic acids and alizarin were studied under both offline and online experiments.

2.3.1. Preliminary Test

The preliminary test was mainly addressed to obtain the data about the solubility and the precipitation of boronic acid and fluorescent transporter in the solvents commonly used in RP-HPLC, namely: water, methanol and acetonitrile. Since not many information about the solubility of both compounds in acetonitrile, therefore this preliminary study was absolutely needed. The solubility test was done by dissolving the compound in the solvent by which little by little solvent addition and ended up with saturated concentration. In the study of solubilty, a sonicator without controlled temperature was applied for 5 minutes after solvent addition . Furthermore, the precipitation test was employed by mixing the water or organic solvent to the saturated concentration yielded from the solubility test.

2.3.2. Offline Experiment

In this study, the offline experiment was purposed to establish the optimum conditions for the online experiment that applied on the online experiment. The following experiments were sequentially done under this offline experiment:

1. Determination of the optimum excitation and emission wavelength of ARS-boronic acid
2. Determination of the ratio of alizarin and boronic acids for the optimum fluorescence
3. Determination of the minimum concentration of ARS for inducing a detectable fluorescence
4. Scope of detection method
5. Comparison of ARS and Alizarin
6. Influence of acid and base on the fluorescent complex of Alizarin-boronic acid
7. The effect of the concentration of the base on the fluorescence intensity of the complex
8. The strength of the base to neutralize the acid condition
9. Kinetic Test of the reaction between Alizarin and Boronic acids
10. Determination of the UV wavelength for the detection of all boronic acid derivatives.

2.3.3. On-line Flow Injection Analysis experiment

The study done by Flow Injection Analysis was performed by HPLC system as the setup on Figure 2 but the column placed before injector, which emphasized the physical factors of the reaction to optimize the fluorescent complex of alizarin and boronic acids. The setup without column was intended to shorten the run time of the analysis.

The physical parameters which related to the reaction rate between alizarin and boronic acids were observed by doing the experiments below:

1. The influence of the dimension of the reaction coil on the fluorescent intensity of boronic acid
2. The influence of the temperature on the fluorescent intensity of boronic acid

2.3.4. On-line experiment with alizarin system

The online system with alizarin was carried out by using instrumental setup as Figure 2 which purposed to establish a good analytical method for boronic acids detection. Therefore, the following researches were done under this system:

1. Optimization of the flow rate of Superloop
2. Optimization of the alizarin concentration as the reactant for PBA
3. Auto-fluorescence test for boronic acids
4. Scope of analytical method
5. Determine MDC (minimum Detectable Concentration) and MDA (minimum Detectable Amount)
6. Selectivity test

2.3.5. Applications of the online experiment

The aim of this study was to test the analytical method which established from on-line experiments using alizarin. These applications were focused on monitoring the reactions that boronic acid derivatives took a part as in the reaction below:

1. Monitoring esterification of 4-carboxyphenylboronic acid (C-PBA) with ethanol
2. Monitoring reaction of (4-Allylaminocarbonyl)boronic acid with Boc-cysteine

3. Result and Discussion

3.1. Preliminary test

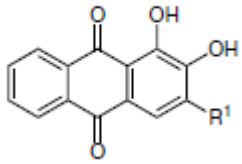
3.1.1. Solubility test

The solubility studies of phenylboronic acid and fluorescent reporter in the solvents which commonly used for RP-HPLC were shown in Table 5.

Table 5. The solubility test of alizarin, ARS, and PBA in water, methanol and acetonitrile.

Solvent	Saturated concentration		
	Alizarin (mM)	ARS (mM)	PBA (mM)
Water	hardly soluble	3	9
Methanol	5	1	> 40
Acetonitrile	4	Less than 0.1	15

The result of the solubility test are presented in Table 5.



Alizarin : $R^1 = H$
ARS : $R^1 = SO_3Na$

Figure 3. Basic structure of Alizarin and Alizarin Red S. (ARS) [9]

The result of this solubility study also conforms with the investigation done by Derksen and team in 2002 [15]. In her research, it was reported that alizarin was almost insoluble in water, but well dissolved in alcohol, ether and alkaline solution. Alizarin and ARS are the derivatives of anthraquinone with SO_3Na as modified group for ARS (Figure 3). It is clearly seen that the sodium sulfonic acid group in ARS plays an important role on the solubility. Therefore ARS is soluble in water, but not in organic solvent due to its salt form.

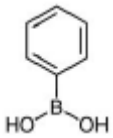


Figure 4. Structure of phenylboronic acid (PBA)

Phenylboronic acid (PBA) has the Lewis acidity behavior which is together with the hydrogen bond donating capacity of their hydroxyl groups, lead this compound to a polar character[16]. Hence, it is clear that this polar character induces PBA to dissolve well in water and organic solvent as the result shown in Table 5.

3.1.2. Precipitation test

The study about the precipitate formation was only emphasized for the solution of the fluorescent transporters, namely alizarin and ARS, to get to know their interaction with water or organic solvent. Since the alizarin is hardly soluble in water and ARS has a solubility problem in acetonitrile, hence the test was done to observe the possibility of precipitation formation as the effect of adding water to the organic solution of alizarin and as well as the ARS solution in water merges with acetonitrile.

Table 6 has shown that the alizarin in methanol easily precipitated by adding a few amount of water, less than one third of its concentrated solution (v:v). Moreover, the alizarin solution in acetonitrile was able to be mixed with more water with its twice higher volume to form precipitate. In contrast, the addition of acetonitrile into the ARS solution in water did not induce precipitate formation.

The result on Table 6 proves that alizarin solution in methanol tends to form prespitate more easily than its solution in acetonitrile. Since the hidrogen bondings that induced by the two lone pairs electron from the OH group play important role on its solubilty in methanol, therefore their bondings change in the present of water. The OH group from water molecules replace the OH group from methanol due to the polarity strength of the water molecule. Thus, the interaction between alizarin-methanol breaks up and yield the free molecule of alizarin. Consequently, the presipitation is observed on alizarin in the mixture of methanol and water.

Table 6. Precipitation test on the saturated solution of alizarin and ARS

Solution	Saturated Conc. (mM)	Other solvent addition	additional volume (ratio v:v)	Precipitate
Alizarin in methanol	5	Water	0.3	+
Alizarin in acetonitrile	4	Water	2	+
ARS in water	3	Acetonitrile	25	-

From the preliminary test, we conclude that ARS is more suitable to be utilized as the fluorescent reporterfor offline experiment due to its solubility in water and organic solvent, as well as its compatibility with organic solvents which means not form a precipitate. the formation of the fluorescent adduct between phenyl boronic acid (PBA) and ARS was studied in the off-line experiments.

3.2. Offline experiment

The offline experiment was done before the on-line experiment in order to optimize the fluorescent agent and the fluorescent adduct formed when boronic acids react with this agent. Based on the conclusion from the preliminary test, alizarin red S (ARS) and PBA were utilized in the screening experiment of the fluorescent complex, respectively as the fluorescent reporter and the representative of boronic acid derivatives.

3.2.1. Determination of the optimum excitation and emission wavelength of ARS-boronic acid

Determine the excitation and emission wavelength of the fluorescent adduct of ARS and PBA was the first step which done in the offline experiment. The starting condition used during this study referred to the research about the optimum parameters for the complexation of ARS and PBA performed by Springsteen in 2002. His team discovered that the fluorescent complex of boronic acid and ARS reached their optimum intensity under pH 7.4 with 0.1M phosphate buffer[8]. Hence, both ARS and PBA were dissolved in the phosphate buffer solution as the same condition as the previous research done by Springsteen while the concentration of each ARS and PBA was respectively 3µM and 300µM.

The spectra of both the emission scan and the excitation scan of ARS and ARS+PBA was presented in Figure 5. On the Figure 5 (a), it was obviously seen that ARS was autofluorescence and the emission scan at the excitation wavelength of 469nm shown two significant peaks in both ARS and the complex of ARS+PBA, 557nm and 610nm. Since ARS emitted significant fluorescence, the spectrum of ARS was subtracted from the spectrum of ARS+PBA to observe the real fluorescence of the complex (Figure 5 (b)). A single peak appeared at 610nm.

Futhermore, to confirm that 469 nm was the maximum excitation wavelength of the complex of ARS-PBA, the excitation scans were investigated at both emission wavelength, 557nm and 610 nm. Figure 5(c) shows that the excitation scans at 557nm and 610 nm yielded four significant peak at the excitation wavelength of 469 nm; 480nm; 490 nm and 524nm, but it only pointed out one major peak at 469nm after subtracting the ARS excitation spectrum from the ARS+PBA excitation spectrum, as presented on Figure 5 (d).

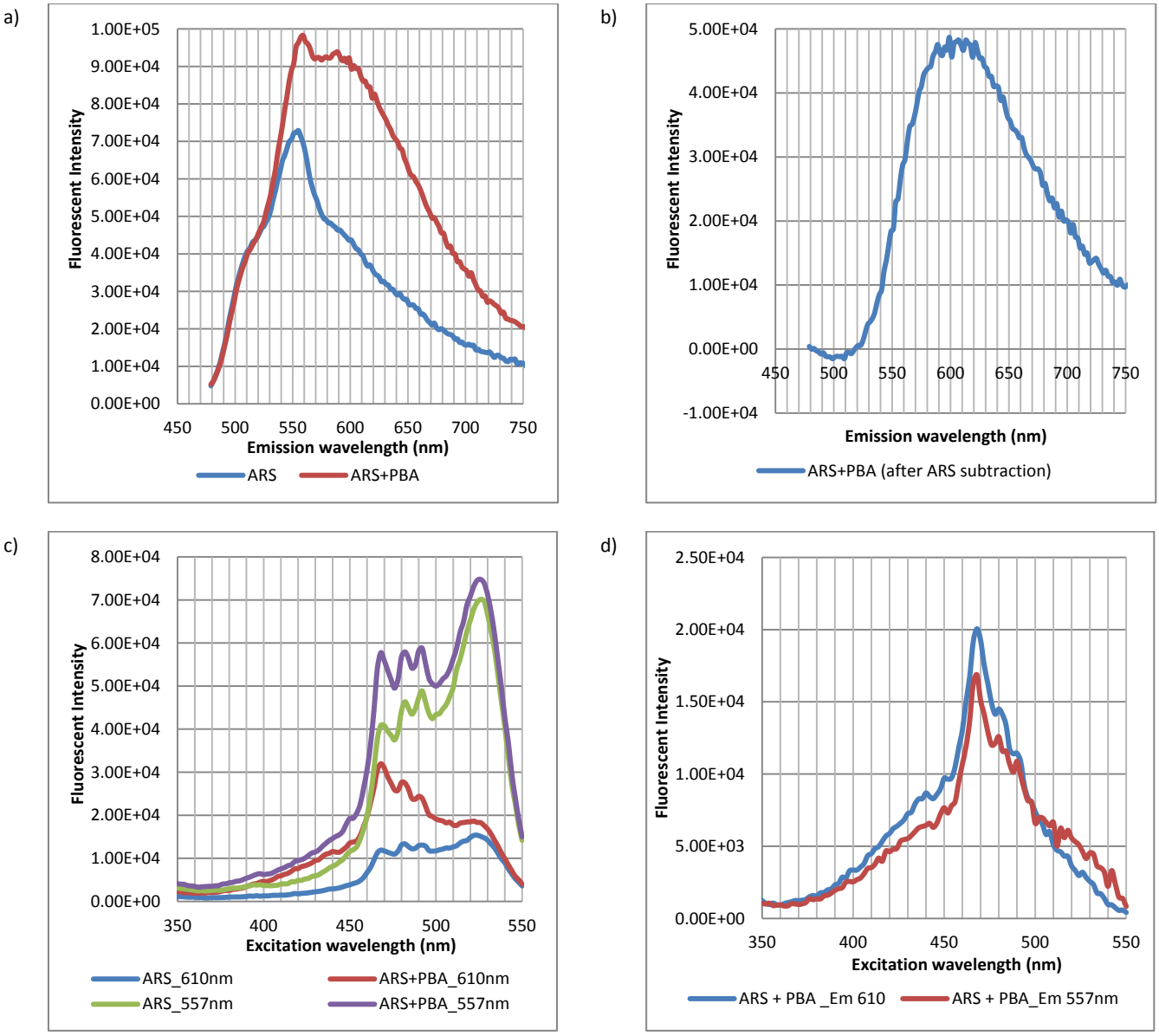


Figure 5. Spectra of the emission scans and the excitation scan of ARS+PBA. a) Emission scans of ARS and ARS+PBA at λ_{Ex} 469nm. b) Emission scans of ARS+PBA at λ_{Ex} 469nm, after ARS subtraction. c) Excitation scan ARS+PBA at λ_{Em} 557nm and λ_{Em} 610nm. d) Excitation scan ARS+PBA at λ_{Em} 557nm and λ_{Em} 610nm, after ARS subtraction.

This study brings to the conclusion that the optimum wavelength of emission and excitation for the fluorescent complex of ARS-PBA are respectively 469nm and 610nm.

3.2.2. Determination of the ratio of alizarin and boronic acids

The aim of this study is to investigate the amount of boronic acids that induce the fluorescence when they react with ARS. An experiment was designed to probe the fluorescent intensity of the complex of ARS-PBA in various ratios of PBA in which ARS was set as a controlled variable. In this study, the idea of setting ARS as a constant variable is to mimic the condition in on-line experiment whereas the solution of ARS is continuously pumped into the post-column system as a fluorescent reporter for boronic acid compounds.

Figure 6 shows that the intensity of the fluorescence has an increasing trend towards the increasing amount of boronic acids which means that to form a fluorescent complex of ARS-PBA requires the excess amount of PBA. Springsteen also observed that the high amount of PBA increased the

fluorescent intensity of ARS-PBA complex.[17] Further, the minimum ratio of one to ten for ARS towards PBA, in which resulted will be taken into account for the next investigations.

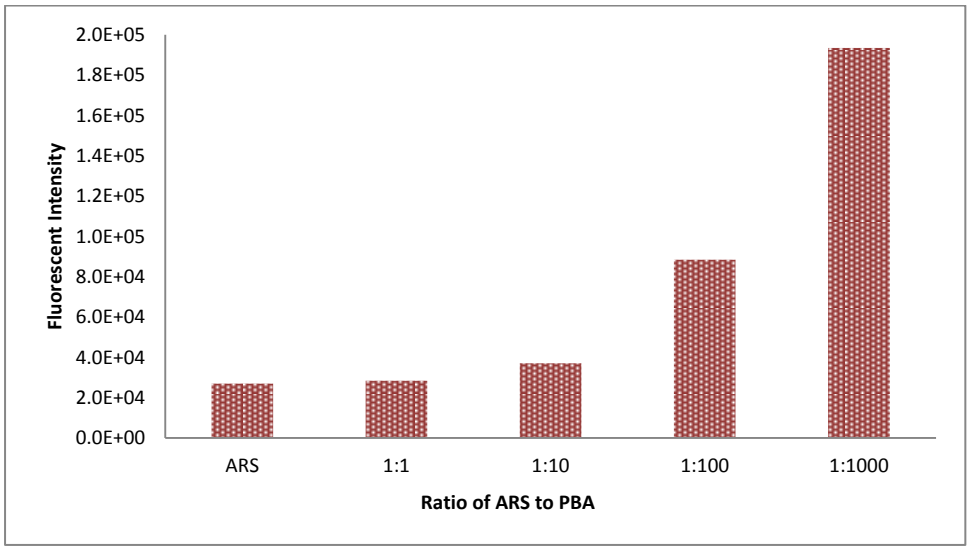


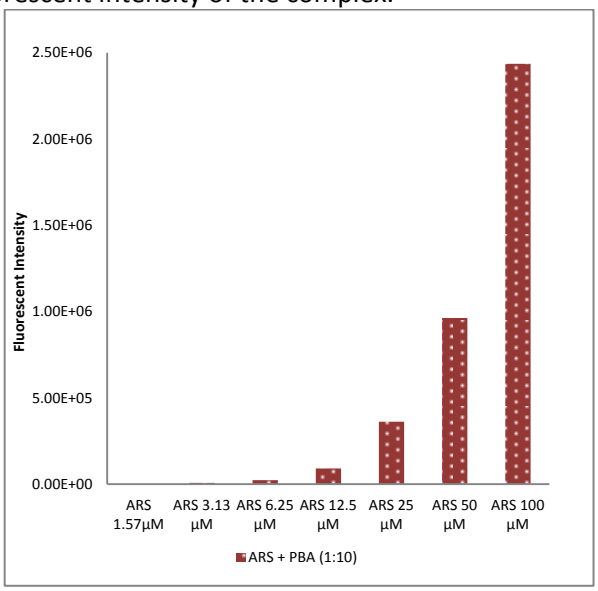
Figure 6. Profile of the fluorescent intensity of ARS+PBA in the various ratios of ARS and PBA. (solvent : phosphate buffer 0.1M pH 7.42; λ_{Ex} 469nm; λ_{Em} 610nm).

3.2.3. Determination of the minimum concentration of ARS

From the previous experiment, it is known that the excess amount of PBA was needed to form fluorescent complex of ARS-PBA (Figure 6) and also Figure 5 has proven that ARS alone emits the fluorescence. These two facts triggered the following test which done in the purpose of examining the minimum concentration of ARS that still be detected by fluorocent spectroscopy when reacts with PBA in a constant ratio of ARS-PBA at 1 to 10.

Figure 7 shows the effect of the concentration of ARS and PBA on emitting fluorescent complex by keeping the constant ratio of ARS-PBA at 1 to 10. The present of ARS in the mixture induces the fluorescent intensity of the complex.

a)



b)

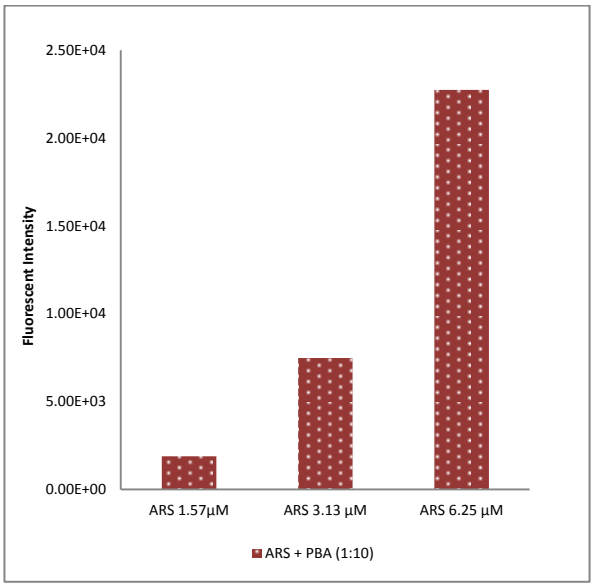


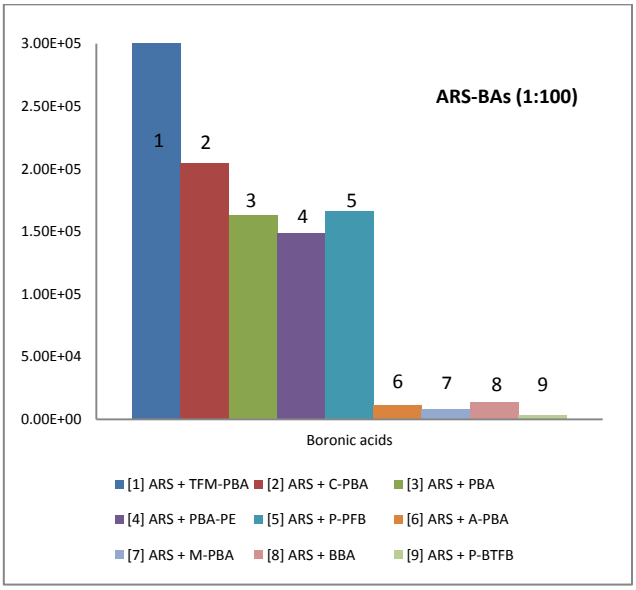
Figure 7. Profile of the fluorescent intensity of ARS-PBA in the various concentrations of ARS. a) The fluorescent intensity of ARS-PBA in the range concentration of ARS from 1.57µM - 100µM. b) The zooming version of (a), ARS concentration 1.57µM-6.25µM. (solvent : phosphate buffer 0.1M pH 7.42; λ_{Ex} 469nm; λ_{Em} 610nm)

The message of this experiment is that the concentration of ARS at 1.57µM is still able to emit the detectable fluorescence with PBA in the minimum ratio of 1 to ten.

3.2.4. Scope of the detection method

Figure 8 (a) describes the fluorescent intensity of nine boronic acid derivatives resulted from the reaction with ARS in phosphate buffer 0.1M pH 7.42. There are two unlike groups which TFM-PBA, C-PBA, PBA, PBA-PE, and P-PFB emit higher fluorescent intensity than A-PBA, M-PBA, BBA and P-BTFB (Figure 8 (a)). In this study, the boronic acids which give higher fluorescence can be simply categorized by their type of substituent functional groups, namely the boronic acids with electron withdrawing groups on its substituent which consists of fluor (TFM-PBA and P-BTFB) and carboxyl group (C-PBA) while PBA and its derivatives do not have substituent. Contrary, Figure 8 (b) shows that the last four BAs compounds on the right side emmit the low intensity of fluorescence, namely : A-PBA, M-PBA, BBA and P-BTFB that possess the electron donating groups. In general, the electron-withdrawing groups can significantly lower the pK_a of boronic acids and the electron donating groups increase the pK_a. It has been generally believed that electron-withdrawing groups can significantly lower the pK_a of boronic acids and electron donating groups increase the pK_a.[18] Furthermore, the low pKa of the boronic acids generate the higher binding affinities of ARS-boronic acids that induce the greater fluorescence intensity.

a)



b)

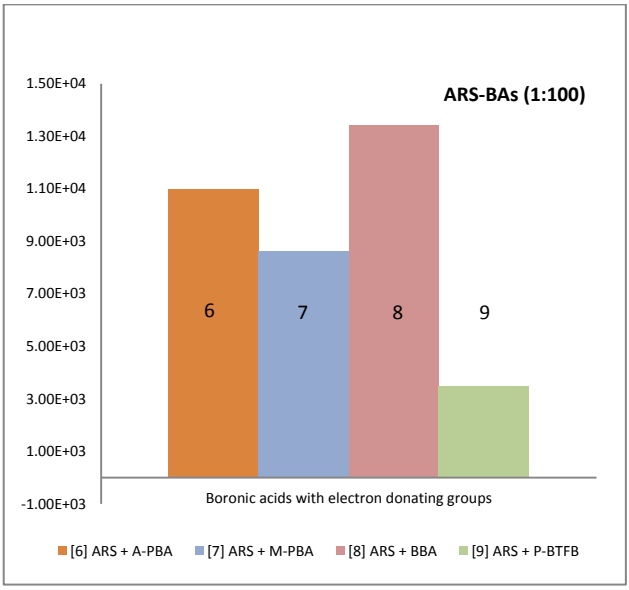


Figure 8. Fluorescent intensity of ARS-Bororonic acids. a) Fluorescent intensity of 9 boronic acid derivatives b) Fluorescent intensity of boronic acid derivatives wit electron donating groups. (solvent: phosphate buffer 0.1M pH 7.42; λ_{Ex} 469nm; λ_{Em} 610nm)

The fact observed from this study that boronic acid derivatives with electron donating properties are less fluorescence , it then led to the thought to employ alizarin as the fluorescent reporter. Further, the experimental procedures were designed to investigate the comparison of ARS and alizarin for the detection of boronic acids with electron donating groups.

3.2.5. Comparison of ARS and Alizarin

The aim of this study was to examine the fluorescent intensity between ARS and alizarin regarding to the tendency of being self-fluorescent compound. Futhermore, this study was also intended to observe the comparison of ARS and alizarin on their interaction with boronic acid derivatives which possess electron donating groups. The experiment was designed with different parameters, especially

the solvent. Since alizarin is insoluble in water as the result shown on Table 5, the 0.1M phosphate buffer pH 7.42 was no longer used in the upcoming study in order to construct the comparable condition between ARS and alizarin. Instead of phosphate buffer, organic solvents as the common solvents for RP-HPLC, particularly methanol and acetonitrile, were used during this study. By involving those organic solvents and also water, the circumstances under RP-HPLC system were covered by this experiment. Moreover, M-PBA was employed as the representative of boronic acids with electron donating groups due to its stability in a solution while APBA and BBA are poorly stable in aqueous solutions.

Figure 9 shows that in general, alizarin (ALZ) releases lower intensity in fluorescence compared to ARS in the pure organic solvent (methanol) and the mixture of water-organic solvents commonly used in RP-HPLC such as methanol-water and acetonitrile-water. The intensity of ARS in the present of water is higher than in the pure organic solvent. This may be explained by the fact that ARS is better dissolved in water than in organic solvents.

Besides, the mechanism of the auto-fluorescence on ARS is caused by the formation of the quasiaromatic character to the ring structure resulted from OH group and SO_3 group which has important role on increasing its fluorescence properties. Regarding to the theory about auto-fluorescence properties on anthraquione dyes, Dahiya and his team observed that the fluorescence properties of anthraquinon dyes, like alizarin and its derivatives, relate to the intense hydrogen bondings in intramolecular level which introduce a kind of quasiaromatic character to create rigid structure, thus increase their fluorescence intensity[19].

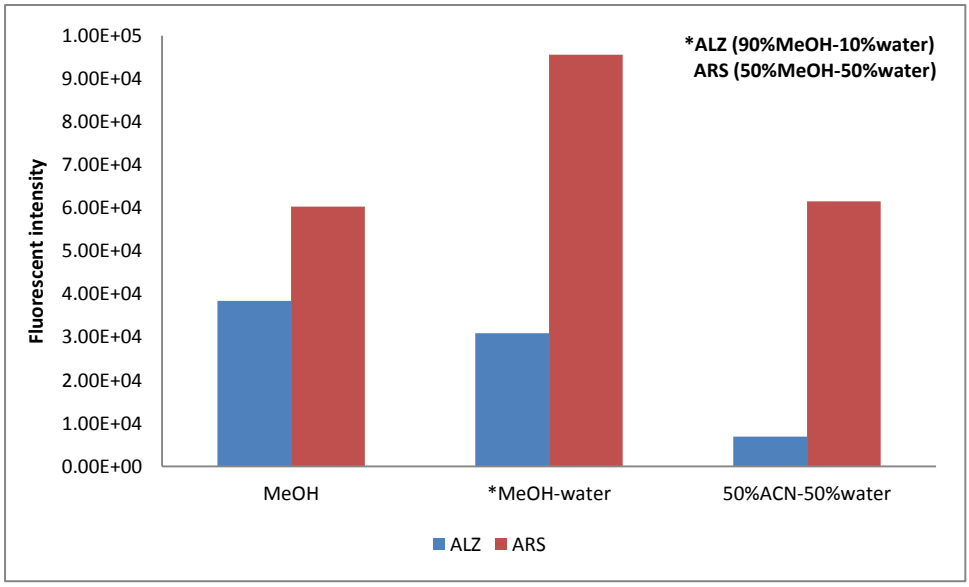


Figure 9. The fluorescent intensity of alizarin (ALZ) and ARS (λ_{Ex} 469nm; λ_{Em} 610nm)

Figure 10 describes the interaction of two fluorescent reporters, alizarin and ARS, with M-PBA as a model of electron donating group's boronic acid derivative. The comparison study about ARS and alizarin on their interaction with M-PBA prove that alizarin has much better binding with M-PBA in resulting higher fluorescent intensity than ARS+M-PBA. The intensity of alizarin+M-PBA complex reached high level in pure organic solvent, but the signal dropped when the water present in the complex system. It may relate to the tendency of alizarin to be hardly soluble in water that cause less forming of boronate ester which induce the fluorescence.

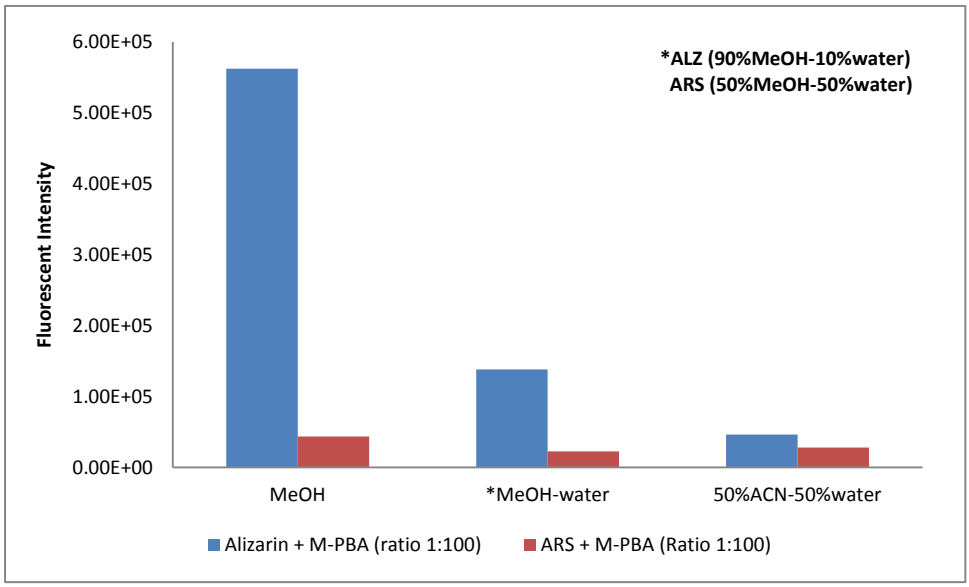


Figure 10. The comparison of fluorescence intensity in between the complex of Alizarin+M-PBA and ARS+M-PBA. M-PBA is one of the boronic acid derivatives with electron donating group. (λ_{Ex} 469nm; λ_{Em} 610nm)

The investigation on comparing alizarin and ARSgives conclusion that alizarin performs better than ARS based on its interaction with boronic acid derivatives owning electron donating groups, particularly M-PBA. Thus, alizarin would be optimized on improving the fluorescent intensity of it complex system with boronic acids. For further investigation, alizarin in acetonitrile was employed by considering its lower viscosity than methanol so that produces less bubbles when mixed with water or water-organic solvent. In addition, acetonitrile is also the best for HPLC system regarding to the fluorescent complexation of alizarin-boronic acid due to Lewis acidic properties of boronic acid which tend to form adducts with water and hydroxyl groups. Moreover, alizarin is less autofluorescent.

3.2.6. Influence of acid and base

This study employed acetic acid (HOAc) and triethylamine (TEA) to provide acidic and alkalic condition respectively. Moreover, TEA and acetic acid were chosen as the pH controller due to their ability to dissolve well in organic solvent while the percentage of both acid and base were picked at 0.1%. Further, the tested solvents involved water and organic solvent as a mimic condition in on-line sytem by assuming that in the reaction coil, alizarin from the super loop will meet up boronic acids from HPLC system in 1 to 1 ratio ((v:v).

Figure **11** shows that under the base condition, the fluorescent intensity of the complexation of alizarin-boronic acid increases compared to the neutral condition, while the intensity of the fluorescent complex significantly drops in acidic circumstance. This phenomena relate to the optimum condition of alizarin-boronic acids at neutral condition as well as the condition for ARS-boronic acids. The present of acetic acid induces the decreasing pH which causes the reduced fluorescence intensity.

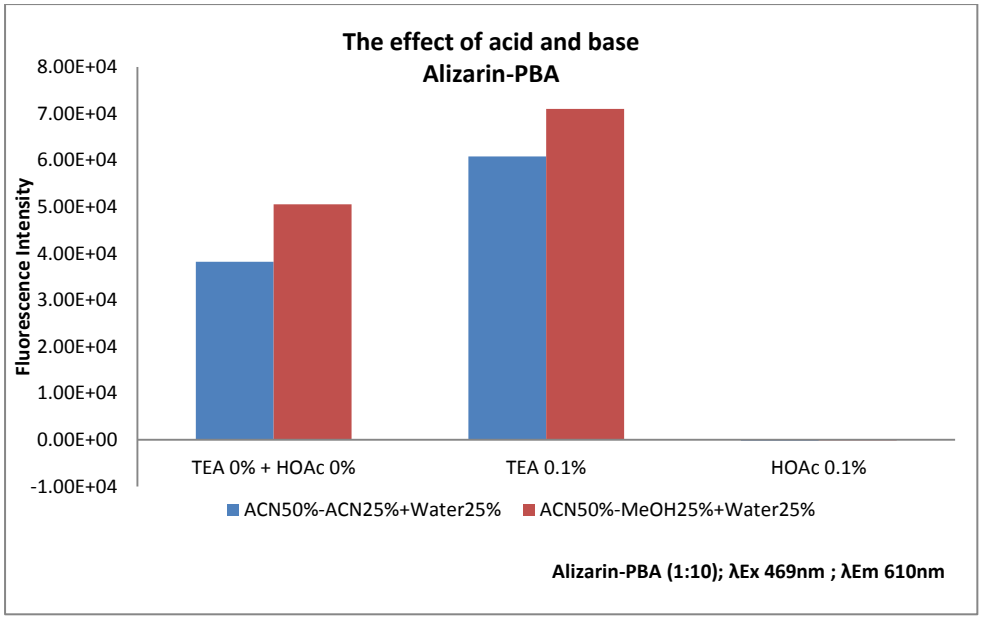


Figure 11. The effect of acid and base of alizarin-PBA at λ_{Ex} 469n; λ_{Em} 610nm; Ratio alizarin-PBA (1:10).
Solvent : mimicking the real condition in HPLC system [Acetonitrile-Acetonitrile+Water (2:1:1) and Acetonitrile-MeOH+Water (2:1:1)]. 2 part of acetonitrile represents the solvent for alizarine, while acetonitrile-water (1:1) and methanol-water (1:1) correspond to the mobile phase on HPLC system.

In conclusion, adding 0.1%TEA help the fluorescent complex of alizarin-boronic acids to reach their optimum condition on emitting the fluorescence. The further experiment is intended to investigate the effect of the base concentration on improving the fluorescence intensity.

3.2.7. The effect of the base concentration to neutralize the acid condition

The addition of base is proven on improving the fluorescent intensity of the complex of alizarin-PBA. This study is intended to observe the addition of various concentration of TEA on the fluorescent intensity of alizarin-PBA.

Figure 12 shows that in general, adding 0.1% TEA gave the highest intensity on fluorescence compared to zero, 1% and 10% concentration of TEA. It is relates to the impact given by adding percentage of TEA which induces the alkalic condition and yields the decreasing fluorescence of the complex. This result conforms to the research done by Springsteen and his co-worker about the effect of pH on the intensity of the fluorescence complex of ARS-PBA that it reached the maximum value at pH 7.4 [17].

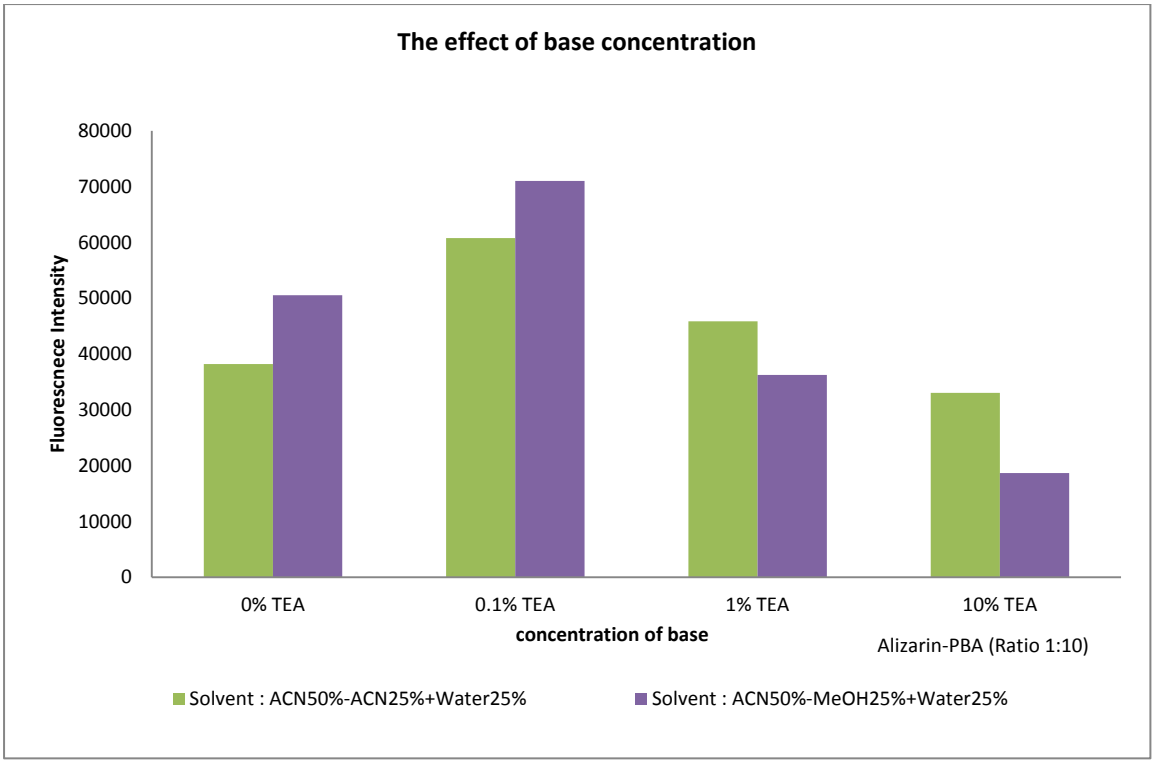


Figure 12. The effect of base concentration on the fluorescence intensity alizarin-PBA at λ_{Ex} 469n; λ_{Em} 610nm; Ratio alizarin-PBA (1:10).
Solvent : mimicking the real condition in HPLC system [Acetonitrile-Acetonitrile+Water (2:1:1) and Acetonitrile-MeOH+Water (2:1:1)]. 2 part of acetonitrile represents the solvent for alizarine, while acetonitrile-water (1:1) and methanol-water (1:1) correspond to the mobile phase on HPLC system.

This study convinces that the optimum percentage of TEA is 0.1%.

3.2.8. The strength of the base to neutralize the acid condition

The aim of the study is to find the concentration of base which is able to compensate the effect of the acid from the HPLC eluate. This acid may be required to improve the separation of boronic acids in the column, before it is reacted with alizarin in the post column reaction.

Figure 13 shows that to neutralize the acid condition from 0.1% acetic acid, it is required 1% of TEA. The intensity of 0.1% of acetic acid addition on the solvent reached the same level as the addition 0.1%TEA by adding 1%TEA on the acidic solvent.

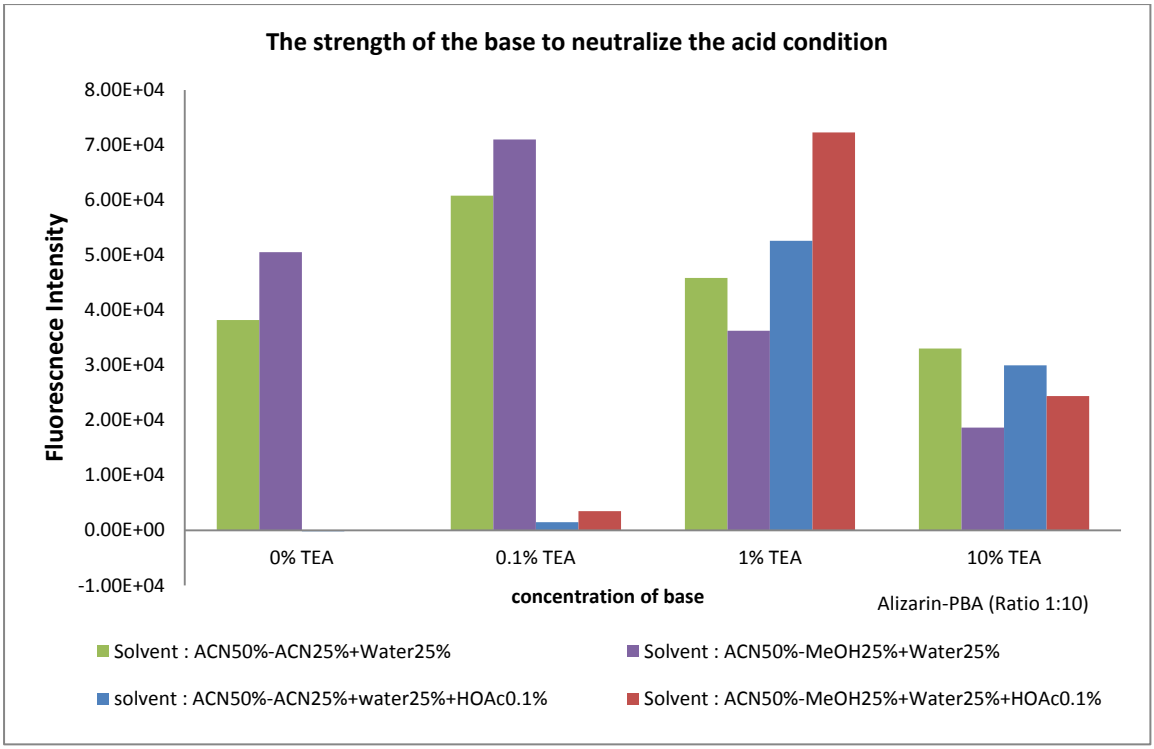


Figure 13. The strength of the base to neutralize the acid condition alizarin-PBA at λ_{Ex} 469n; λ_{Em} 610nm; Ratio alizarin-PBA (1:10).
Solvent : mimicking the real condition in HPLC system [Acetonitrile-MeOH+Water (2:1:1)]. 2 part of acetonitrile represents the solvent for alizarine, while methanol-water (1:1) corresponds to the mobile phase on HPLC system.

In summary, the study about the strength of the base to eliminate the acidic condition proves that 1%TEA is enough to keep the optimum condition for the complex of alizarin-PBA to emit the fluorescence in the acidic circumstance that caused by 0.1% acetic acid.

3.2.9. Kinetic Test of the reaction between Alizarin and Boronic acid

The aim of this study is to probe the reaction rate of the fluorescent complex formed by alizarin-PBA. This study relates to the estimation for the residence time needed by alizarin and PBA to react. Since the residence time is the reaction time of alizarin-PBA which occurs in the reaction coil, thus it is calculated as the volume of the reaction coil divided by the combined flow rate of HPLC system and the super loop. The residence time corresponds to the length of coil as the place for post column reaction between alizarin and PBA. In this experiment, the adding of various concentrations of TEA were observed.

Figure 14 shows that the reaction under 0.1%TEA has the highest starting point and reaches the highest intensity as well compared to the zero, 1% and 10% TEA. The addition of 0.1%TEA (v:v) generates the optimum condition which reffered to the optimum pH for alizarin-PBA to form the fluorescent configuration, thus the reaction starts faster than the others. From the graph, the reaction touched the maximum intensity after 5 minutes (300 seconds). It means that longer reaction coil is probably needed which is around 100 m length of reaction coil with 0.25 mm i.d. and the combined flow rate is 1.0 mL/min.

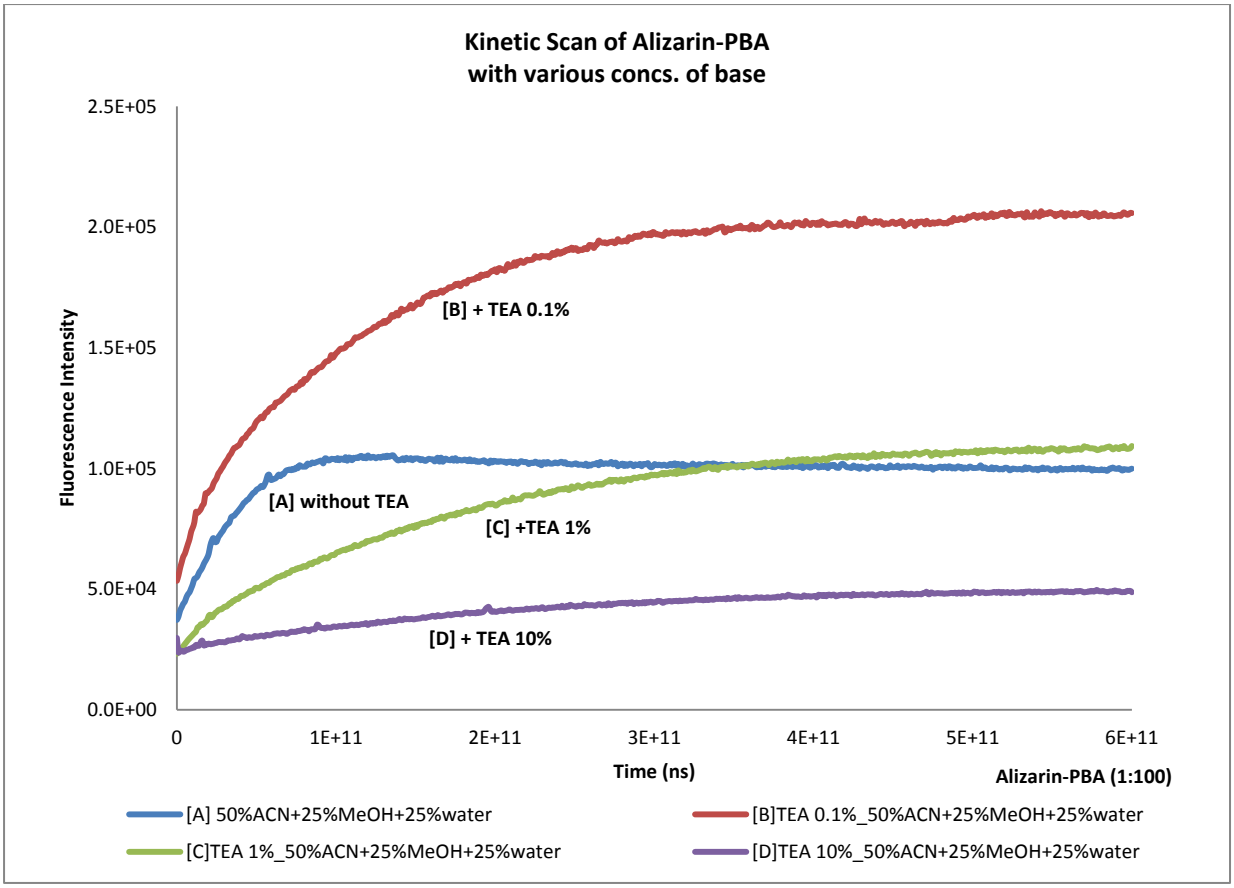


Figure 14. The kinetic scan of alizarin-PBA complex with various concentrations of TEA at λ_{Ex} 469nm; λ_{Em} 610nm; Ratio alizarin-PBA (1:100).
Solvent : mimicking the real condition in HPLC system [Acetonitrile-MeOH+Water (2:1:1)]. 2 part of acetonitrile represents the solvent for alizarine, while methanol-water (1:1) corresponds to the mobile phase on HPLC system.

As the conclusion of this experiment, adding 0.1% TEA helps to increase the reaction rate of alizarin-PBA.

3.2.10. Determination of the UV spectra of BAs derivatives

The purpose of this study is to find the optimum UV wavelength for the UV detection of all boronic acids. Nine boronic acids were examined under UV spectrophotometer.

Figure 15 shows the spectra of nine boronic acids in phosphate buffer 0.1M, pH 7.42. It is quite difficult to find the optimum UV wavelength for all the boronic acids. by loooking through the spectra, the wavelength at 270nm seems the optimum value for all, except for BBA and P-BTFB which do not have chromopore groups resulting on not UV detected.

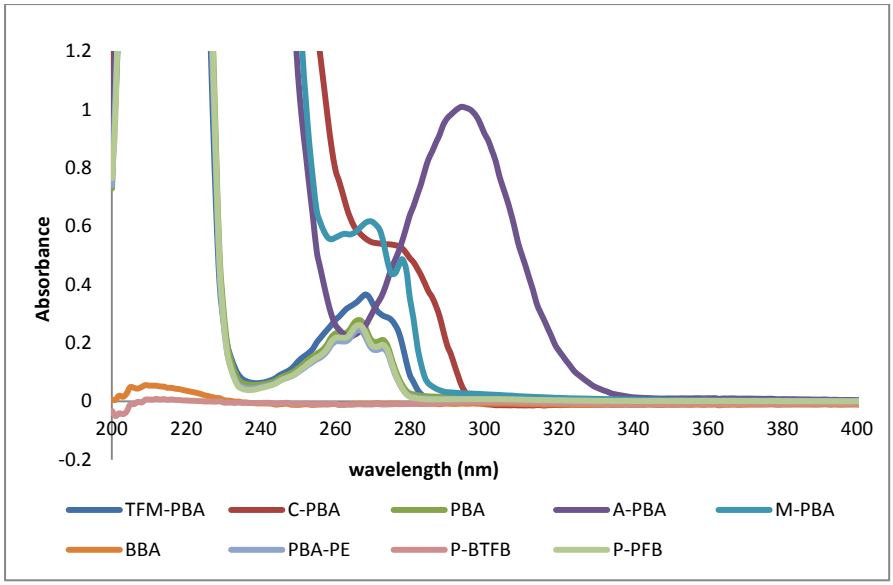


Figure 15. UV-Vis spectra of boronic acid derivatives. Concentration of each boronic acid was 625µM, solvent Phosphate Buffer 0.1M pH 7.42.

3.2.11. Conclusion for the offline experiment

Regarding to the ten experiments done under the offline experiment, we conclude that:

1. The excitation wavelength and emission wavelength are respectively 469 nm and 610 nm.
2. The UV wavelength was set up as 270 nm .
3. Alizarin 3.13 µM in acetonitrile with the addition of 0.1% TEA was chosen to be investigated in the initial on-line experiment.
4. PBA with concentration 100x higher than alizarin was employed on the on-line optimization experiment .
5. The mobile phase for HPLC system was acetonitrile-water (50:50) to avoid boronic acids to form adducts with water and hydroxyl groups regarding to their Lewis acidic properties[20]. Lorand observed that due to their Lewis properties, boronic acids tend to form the complex with water and hydroxyl groups. Therefore, acetonitrile is the best choice for this reason.

3.3. On-line Flow Injection Analysis experiment

The Flow Injection Analysis (FIA) experiment is the earliest step on the on-line experiment to try forming fluorescent complex of alizarin and boronic acids by continuous process under the on-line system. In principle, the boronic acids come out from the UV detector of HPLC system, meet up with alizarin which pumped out from superloop, and interact in the reaction coil to be detected by fluorescence detector.

As described in the experimental setup, FIA experiment was set as the same set up of the on-line system, but the column placed in between the pump and the injector as a damper pressure, thus the tested boronic acids pass through the UV detector without separation in the column. The aim of this setup is to shorten the run time analysis due to no separation in a column in order to optimize the physical parameters that relate to the formation of a fluorescent complex. The effect of reaction coil dimension and temperature were investigated under this experimental setup.

3.3.1. The influence of the dimension of the reaction coil on the fluorescent intensity

The aim of this study is to observe the effect of the reaction coil dimension on the fluorescent intensity of the complex alizarin-boronic acids. In term of HPLC system, the resulted peak reflects the fluorescent configuration of the alizarin-boronic acids, while the residence time refers to the reaction time occuring in the reaction coil. This study involved two different coil dimensions with different diameters and lengths to investigate the effect of the residence time on the peak height and the peak width.

Table 7 (a) shows the peak characters resulted from two different internal diameter of reaction coils. It is clearly seen that the longer residence time produces higher peak height which caused by giving longer time for alizarin and boronic acids to interact in the coil. Moreover, the coil with wider internal diameter tends to induce peak broadening due to diffusion effect eventhough it has low back pressure due to its wider diameter. Conversely, the narrower coil performed better on the peak height, but it induced high back pressure in the longer coil. Thus, it is not a good idea to improve the peak height by increasing the length for the narrow coil to obtain longer residence time.

Table 7. The peak height and peak width on different coil dimensions

a

Coil dimension	Peak height	peak width , s	Residence time , s
1.25 m x 0.25 mm i.d.	2.7	9	3
3.50 m x 0.25 mm i.d.	2.7	11	9
2.90 m x 0.50 mm i.d.	4.9	14	15
4.70 m x 0.50 mm i.d.	5.5	19	25

*LC : mobile phase acetonitrile-water (50:50, v:v); flow rate **0.7**mL/min ; UV detector 270 nm; Fluorescence detector λ_{Ex} 469nm; λ_{Em} 610nm
*Supeloop : Alizarin 3.13µM + 0.1%TEA (v:v) in Acetonitrile; flow rate **0.5**mL/min
*Compound tested : 313 µM in methanol *Column **:**Alltima 5µ; ID 4.6.0mm x 250mm (before injector)

b

Coil dimension	Pressure (bar)	Peak height	peak width , s	Residence time , s
3.5 m x 0.25 mm i.d.	3	8.3	18	8.6
6.0 m x 0.25 mm i.d.	6	8.8	18	14.7
10 m x 0.25 mm i.d.	9	8.9	15	24.5

*LC : mobile phase acetonitrile-water (50:50, v:v); flow rate **0.4**mL/min ; UV detector 270 nm; Fluorescence detector λ_{Ex} 469nm; λ_{Em} 610nm
*Supeloop : Alizarin 3.13µM + 0.1%TEA (v:v) in Acetonitrile; flow rate **0.8** mL/min [max pressure for super loop: 10bar)
*Compound tested : 313 µM in methanol *Column **:**Alltima C18 5µ; ID 3.0.0mm x 150mm (before injector)
*Temperature: 50^0C

Based on this study, the narrow coil, with the internal diameter of 0.25 mm and 3.5 m length was chosen to be probed for further experiment due to resulting the sharp peak.

3.3.2. Influence of the temperature

This study is intended to enhance the reaction rate by increasing the temperature. The experiment to observe the temperature effect on the fluorescence configuration was designed by placing the reaction coil inside the water bath with temperature controller which set at four different level temperatures, respectively 22^0C, 30^0C, 40^0C and 50^0C. Due to the volatility of toxic acetonitrile, the maximum tested temperatures were 50^0C. Furthermore, it is also recommended by the manual instruction of the PEEK tubing to work at the temperature below 100^0C.[21] Thus, to avoid PEEK tubing decomposition and for the safety reason, 50^0C was set as the maximum temperature for this study.

Figure 16 shows that the peak height on fluorescence detector increases as the increasing temperature. By increasing temperature, the reaction rate is increasing as well due to the change on kinetic energy.

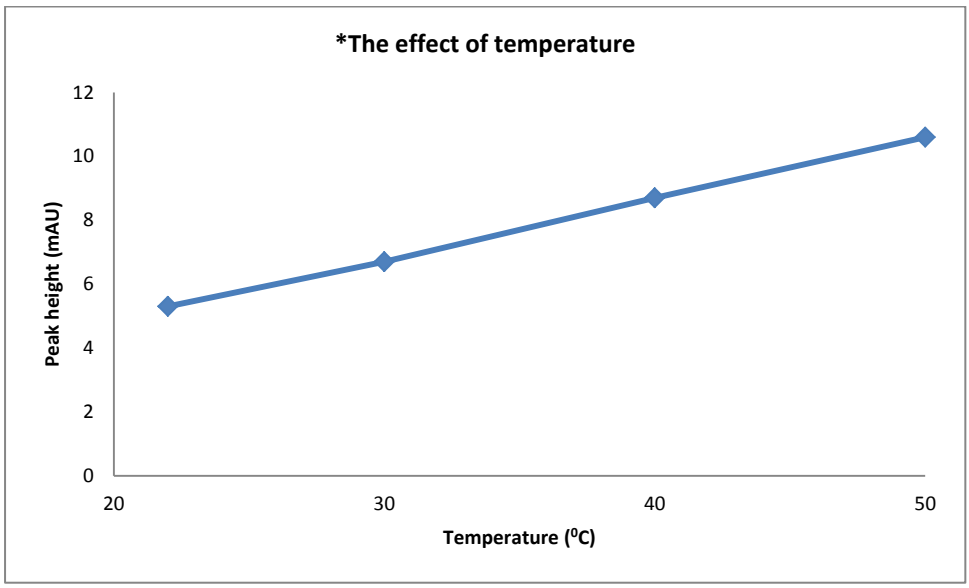


Figure 16. The effect of the temperature on the peak height of PBA in fluorescence detector.
*LC : mobile phase acetonitrile-water (50:50, v:v); flow rate 0.4mL/min ; UV detector 270 nm; Fluorescence detector λ_{Ex} 469nm; λ_{Em} 610nm
*Supeloop : Alizarin 3.13µM + 0.1%TEA (v:v) in Acetonitrile; flow rate 0.8mL/min
*Coil dimension : 3.50 m x 0.25 mm i.d.
*Compound tested : 313 µM in methanol
*Column :Alltima C18 5µ; ID 3.0mm x 150mm

To summary the study done by FIA experiment, it is clear that the narrow reaction coil ,3.50 m x 0.25 mm i.d., was chosen as the dimension of the reaction coil for further experiment which equipped by temperature controller at 50^0C.

3.4. On-line experiment

The on-line experiment is the last step to optimize the detection method for boronic acids with the real setup for on-line HPLC system with alizarin as described on Figure 2. The optimization of alizarin in the super loop, likewise the flow rate of super loop and the alizarin concentration were investigated by this experiment. Beside, the scope and the selectivity of this detection method, including the autofluorescence test, were also done via the on-line experiment.

3.4.1. Optimize the flow rate of superloop

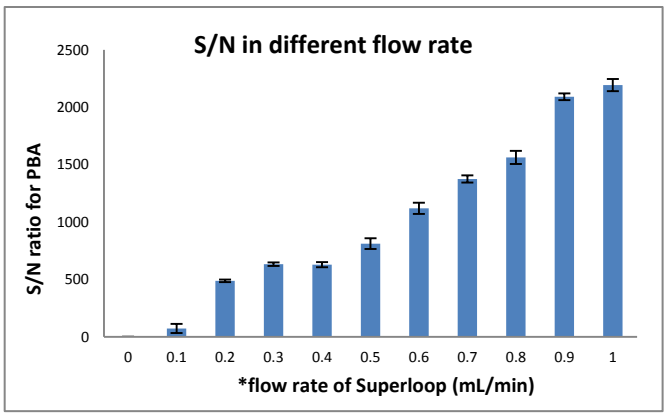
The aim of this study is to investigate the effect of the the superloop flow rate on the fluorescent intensity of alizarin-boronic acids by measuring the signal to noise ratio (S/N ratio) of every superloop flow rate. By considering the noise on the S/N ratio, the fluorescence of alizarin is corrected.

This test was designed by setting the constant flow rate of HPLC, at 0.4 mL/min as the suggested minimum flow rate for this column (Alltima 5µ; ID 3.0mm x 150mm). The HPLC flow rate was set as minimum to respect the result from the off-line experiment that the fluorescent intensity of alizarin-boronic acids increased with the excess amount of boronic acids.

Figure 17 (a) shows that the higher flow rates increase the S/N while Figure 17 (b) presents the comparison of the peak width from UV and fluorescence detector. Eventhough the greater flow rates of super loop trigger the decreasing residence time, the increment signal is caused by the increasing amount of both alizarin and acetonitrile. From the off-line experiment, it proved that the present of acetonitrile related to the increasing signal. Beside, the experiment on the Figure 17 (a) was done with the diluted concentration of alizarin, thus the result did really not represent the real effect of the flow rate. In this situation, the increasing low rate of super loop generated the amount of alizarin and acetonitrile which caused the greater fluorescent intensity.

The experience of working using superloop taught that the high flowrate of superloop introduced the high pressure in super loop which may break the super loop itself. Based on this experience, we chose the optimum flow rate which referred to the minimum flow rate that cause significant changes. When we look closely into Figure 17 (a) and (b), the lowest flow rate that causes a significant change, occurs at 0.6 mL/min. Although the signal to noise ratio at the flow rate 0.6 mL/min was not the highest, it emitted significant fluorescence which produced acceptable peak in fluorescence detector. However, setting the identical flow rate for both HPLC system and super loop is suggested to avoid the backflow and also the bubbles formation caused by the turbulence which relates to the baseline stability on the fluorescence detector. Since it was not possible to set the flow rate of super loop as identical as the HPLC flow rate due to the lower fluorescence intensity and also broader peak, therefore, 0.6 mL/min was optimum flow rate for the super loop which was only 1.5 times of the HPLC flow rate.

a)



b)

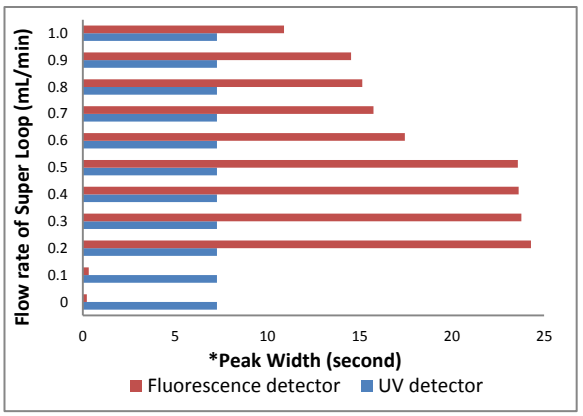


Figure 17. (a) S/N of Alizarin-PBA in various flow rates of the super loop. (b) The profile of the peak width of PBA in UV detector and fluorescence detector.
*LC: mobile phase acetonitrile-water (50:50, v:v); flow rate 0.4mL/min ; UV detector 270 nm; Fluorescence detector λ_{Ex} 469nm; λ_{Em} 610nm *Supeloop: Alizarin 3.13µM + 0.1%TEA (v/v) in Acetonitrile. *Coil dimension: 3.50 m x 0.25 mm i.d. (T 50^{0}C) *Compound tested: 313 µM in methanol. *Column: Alltima 5µ; ID 3.0mm x 150mm

In summary, the flow rate of super loop at 0.6 mL/min was chosen as the fix flow rate of super loop for further experiment.

3.4.2. Optimize the alizarin concentration as the reactant for PBA

The purpose of this study is to obtain the optimum concentration of alizarin which results in the highest sensitivity for PBA detection. In this experiment, signal to noise ratio was used to determine the optimum alizarin concentration. Six different concentrations of alizarin were tested to see the response of the signal of alizarin- PBA.

Table 8 shows the result of additional test to improve the retention time of PBA in UV detector that the peak of PBA come up after the dead volume. The ratio of acetonitrile-water (40:60, v/v) shows a very good separation between the solvent signal and PBA while the peak width of alizarin-PBA remained the same. Thus, for the the investigation about the optimize alizarin concentration, the mobile phase used was acetonitrile-water (40:60, v/v).

Table 8. The effect of the percentage of acetonitrile on the retention time of PBA

Mobile phase ratio		UV		Fluorescence	
%ACN	%Water	Rt	W 1/2h (sec)	Rt	$W_{1/2}h$ (sec)
50	50	2.61	8	3.01	18
45	55	2.84	9	3.27	21
40	60	3.17	10	3.55	19

*LC flow rate 0.4mL/min ; UV detector 270 nm; Fluorescence detector λ_{Ex} 469nm; λ_{Em} 610nm *Supeloop: Alizarin 3.13µM + 0.1%TEA (v/v) in Acetonitrile. *Coil dimension: 3.50 m x 0.25 mm i.d. (T 50^{0}C) *Compound tested: PBA 313 µM in methanol. *Column**:** Alltima 5µ; ID 3.0mm x 150mm

Figure 18 shows that high alizarin concentration produces high noise which caused by the autofluorescence of the alizarin. Moreover, Figure 19 presents the responses of the six different level PBA concentrations on the increment concentration of alizarin which shows that alizarin concentration at 75 µM gives the highest signal to noise ratio. The result on Figure 18 explains the reason of the decreasing signal to noise ratio at 100 µM and 300 µM. However, the response of two detectors, UV detector and fluorescence detector, on the peak area of PBA give a linear response as shown on Table 9 with correlation coefficient about 0.999. This linear response reflects that alizarin enhances the signal of PBA in fluorescence detector which means that boronic acid detection in fluorescence detector is more sensitive than in UV detector

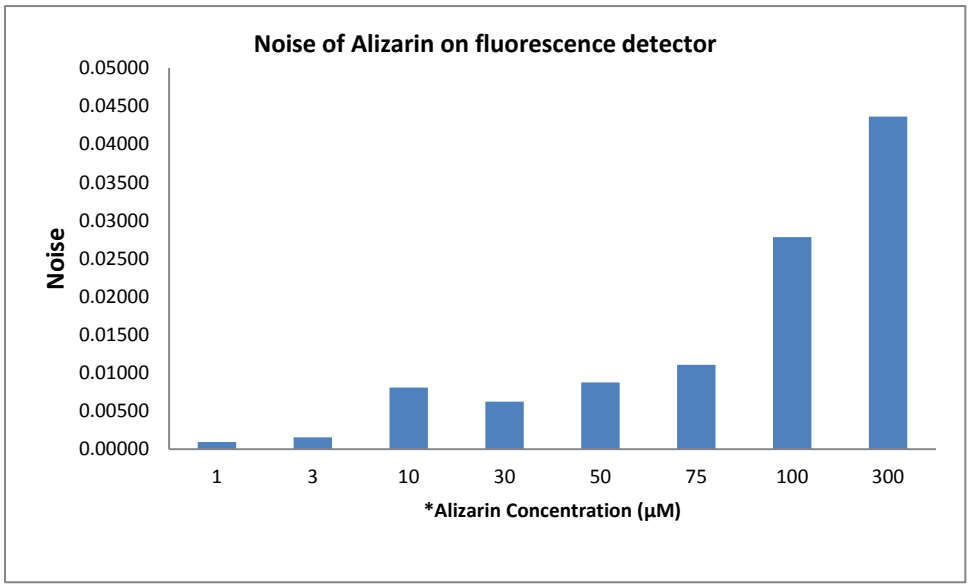


Figure 18. The noise of various concentrations of alizarin on the fluorescence detector.
*LC: mobile phase acetonitrile-water (40:60, v:v); flow rate 0.4mL/min ; UV detector 270 nm; Fluorescence detector λ_{Ex} 469nm; λ_{Em} 610nm *Supeloop: Alizarin + 0.1%TEA (v/v) in Acetonitrile. *Coil dimension: 3.50 m x 0.25 mm i.d. (T 50^{0}C) *Compound tested: solvent (blank). *Column**:** Alltima 5µ; ID 3.0mm x 150mm

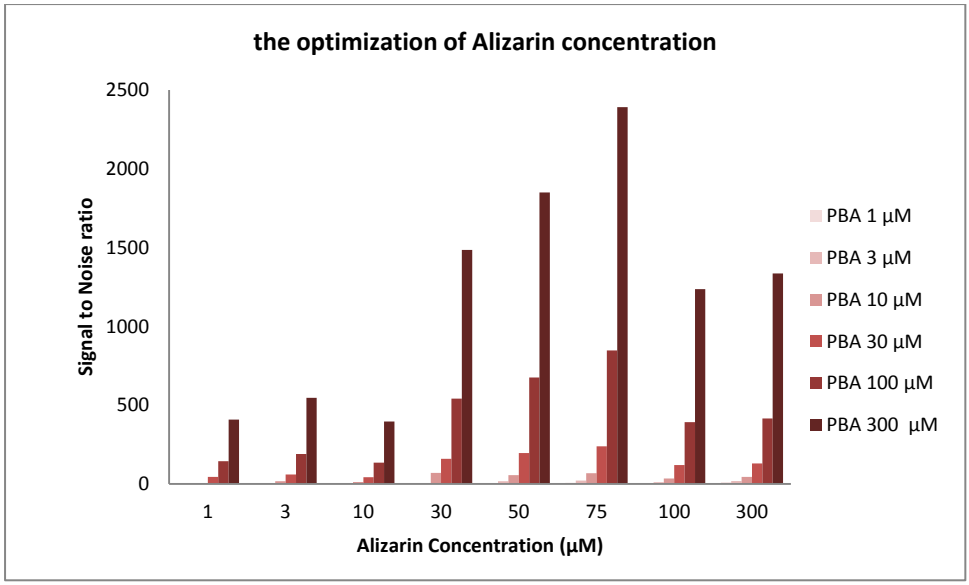


Figure 19. The optimization of alizarin concentration
*LC: mobile phase acetonitrile-water (40:60, v:v); flow rate 0.4mL/min ; UV detector 270 nm; Fluorescence detector λ_{Ex} 469nm; λ_{Em} 610nm *Supeloop: Alizarin + 0.1%TEA (v/v) in Acetonitrile. *Coil dimension: 3.50 m x 0.25 mm i.d. (T 50^{0}C). *Column**:** Alltima 5µ; ID 3.0mm x 150mm. *Compound tested: PBA 1µM, 3µM, 10µM, 30µM, 100µM and 300µM in MeOH.

Table 9. Response peak area of two detector, UV and Fluorescence.

Alizarin concentration	slope	intercept	PBA lecel conc. (n)	Correration coefficient (r)
Alizarin 1µM	0.055	-0.050	6	0.9989
Alizarin 3µM	0.145	-0.155	6	0.9998
Alizarin 10µM	0.527	-1.100	6	0.9983
Alizarin 30µM	1.775	2.659	6	0.9974
Alizarin 50µM	2.589	-2.154	6	0.9996
Alizarin 75µM	3.804	-1.906	6	0.9994
Alizarin 100µM	4.643	-4.588	6	0.9999
Alizarin 300µM	10.571	-8.578	6	0.9997

*LC: mobile phase acetonitrile-water (40:60, v:v); flow rate 0.4mL/min ; UV detector 270 nm; Fluorescence detector λ_{Ex} 469nm; λ_{Em} 610nm *Supeloop: Alizarin + 0.1%TEA (v/v) in Acetonitrile. *Coil dimension: 3.50 m x 0.25 mm i.d. (T 50^{0}C). *Column**:** Alltima 5µ; ID 3.0mm x 150mm. *Compound tested: PBA 1µM, 3µM, 10µM, 30µM, 100µM and 300µM in MeOH.

A conclusion of the experiment done under this study is that the alizarin concentration at 75 µM give the optimum signal to noise ratio, thus this concentration is used for further investigation.

3.4.3. Auto-fluorescence test for boronic acids

The aim of this study is to examine the boronic acids under the on-line HPLC system without alizarin. Therefore, the on-line system was set without superloop and the reactor coil was replaced by shorter tubing as a direct connector between the UV detector and the fluorescence detector.

Table 10 shows that among the tested boronic acid compounds, only M-PBA emit the fluorescence without alizarin. Since BBA and PBFB do not possess aromatic function, thus they can not be detected by UV detector. Further, these boronic acids were tested under the on-line HPLC with alizarin system to prove that alizarin transforms boronic acids to the fluorescent complex which are able to be detected by fluorescence detector. The chromatograms of all the tested boronic acids are presented on Appendix 3.

Table 10. The auto-fluorescence test for boronic acids

Compound	Concentration (µM)	Rt (min) UV	Rt (min) FLU	Peak height
TFM-PBA	250	12.61	-	-
C-PBA	300	1.97	-	-
PBA	300	6.63	-	-
A-PBA	1200	4.60	-	-
***M-PBA**	**300**	**6.96**	**7.14**	**42.6**
BBA	300	-	-	-
PBA-PE	300	6.6	-	-
PBFB	300	-	-	-
PPFB	300	6.34	-	-
PBA-MIDA Ester	300	6.66	-	-

*LC: mobile phase gradient program (Appendix 2), flow rate 0.4mL/min ; UV detector 270 nm; Fluorescence detector λ_{Ex} 469nm; λ_{Em} 610nm. *Coil dimension: 3.50 m x 0.25 mm i.d. (T 50^{0}C). *Column**:** Alltima 5µ; ID 3.0mm x 150mm.
*All the tested boronic acids were dissolved in acetonitrile-water (20:80, v:v)

3.4.4. Scope of HPLC-Alizarin on-line system for boronic acids detection

The aim of this study is to observe the ability of this on-line system to detect the boronic acid derivative with the fluorescence detector after forming a fluorescent complex with alizarin. The derivatives of boronic acid were injected on HPLC on-line system under optimized parameters.

Table 11 and Figure 21 (in 3.4.6) show that all the tested boronic acids are detected on fluorescence detector with a significant signal to noise ratio. Among the boronic acids with electron donating groups, namely: M-PBA, A-PBA, BBA and P-BFB; A-PBA has low number of signal to noise ratio. It might be caused by the high pKa of A-PBA in its solution which above the optimum pKa for the fluorescent complex. Contrary, M-PBA produces high signal to noise ratio due to autofluorescence. Furthermore, HPLC on-line with alizarin system is able to detect BBA and PBFB which are non UV compounds. The chromatograms of all the tested boronic acids are presented on Appendix 3.

Table 11. The result of boronic acids detection under HPLC on-line system with alizarin

No	Compound	Conc. (µM)	Rt (min) UV	Rt (min) FLU	S/N FLU	Residence time (sec)
1	TFM-PBA	250	12.85	13.18	2643	20
2	C-PBA	300	1.86	2.18	334	20
3	PBA	300	6.57	6.963	644	23
4	A-PBA	1200	3.857	4.256	36	24
5	M-PBA	300	6.97	7.32	990	21
6	BBA	300	-	6.53	383	-
7	PBA-PE	300	6.6	6.94	638	20
8	PBFB	300	-	6.49	383	-
9	PPFB	300	6.64	6.98	662	20
10	PBA-MIDA Ester	300	6.55	6.898	559	21

*LC: mobile phase gradient program (Appendix 2), flow rate 0.4mL/min ; UV detector 270 nm; Fluorescence detector λ_{Ex} 469nm; λ_{Em} 610nm *Supeloop: Alizarin 75 µM + 0.1%TEA (v/v) in Acetonitrile, flow rate 0.6mL/min
*Coil dimension: 3.50 m x 0.25 mm i.d. (T 50^{0}C). *Column**:** Alltima 5µ; ID 3.0mm x 150mm.
*All the tested boronic acids were dissolved in acetonitrile-water (20:80, v:v)

To sum up, this on-line system with alizarin successfully detects most of the boronic acid derivatives. Further, to enhance the signal to noise ratio of A-PBA, the addition of low percentage of acetic acid might be needed to neutralize the pKa which optimum to form the fluorescent complex.

3.4.5. Determine Minimum Detectable Concentration and Minimum Detectable Amount

This study is intended to determine limit of detection (LOD) of the HPLC on-line system with alizarin for boronic acids detection which done by calculating the minimum detectable concentration and minimum detectable amount. For this purpose, a dilute solution of each BA that its estimated peak height was around $5 * SD\ of\ Noise$. By using data peak height on the study about the scope of the HPLC on-line system with alizarine, the dilute concentration of each boronic acids was predicted.

Furthermore, the response peak height of this dilute solution and zero concentration in fluorescence detector was then plotted to make a curve of the concentration against the peak height. The linear equation as $y = bx + a$ for each BA was derived by which y and x respectively referred to the peak height and the concentration (µM). Moreover, from that linear equation, the MDC was derived by substituted the value of $LOD = 3 * SD\ of\ Noise$ as y. Since the MDA refers to the amount of the compound in every injection, thus, MDA was calculating from MDC multiplied by the volume of the loop (10µL).

The estimation MDC and MDA of the tested boronic acids are presented on Figure 20 and Table 12. The results seem make no sense for some compounds which the determined MDC are lower than the tested concentration, namely C-PBA, PBA, BBA, PBA-PE, and PPFB. Since the tested concentrations were prepared based on estimation that the peak height of the signal would be 5xSD of noise which should be above the peak height for LOD (LOD = 3*SD of noise). It may be caused by the error during determining the diluted concentration which close to the LOD concentration. Beside, gradient program for HPLC system induces high noise that cause the peak somehow does not appear obviously. However, these are kind of the drawbacks of MDC estimation using single concentration.

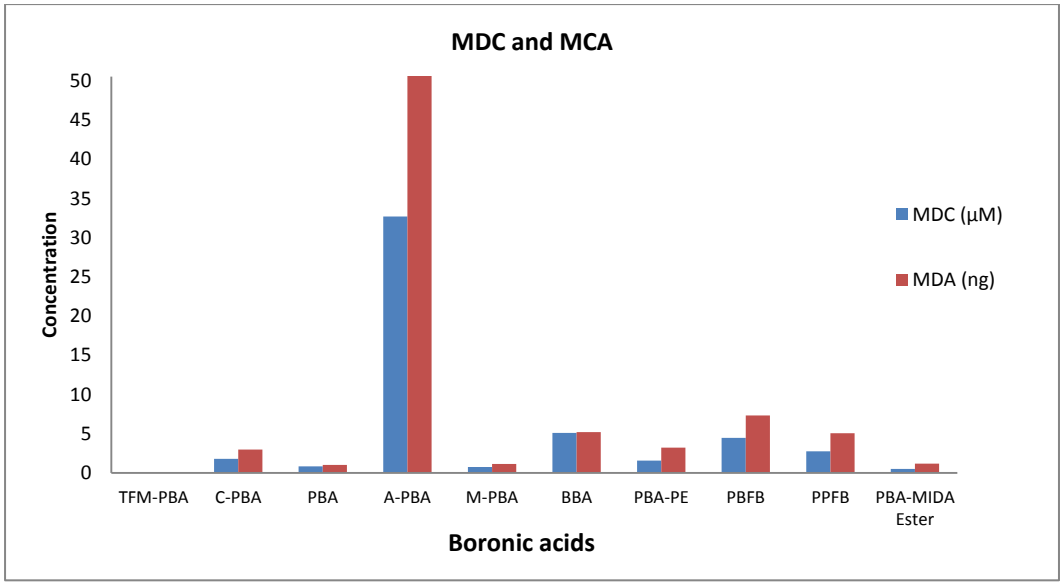


Figure 20. Minimum Detectable Concentration (MDC) and Minimum Detectable Amounts (MDA) of boronic acids in HPLC-alizarin online system under gradient condition

*LC: mobile phase gradient program (Appendix 2), flow rate 0.4mL/min ; UV detector 270 nm; Fluorescence detector λ_{Ex} 469nm; λ_{Em} 610nm *Supeloop: Alizarin 75 µM + 0.1%TEA (v/v) in Acetonitrile, flow rate 0.6mL/min *Coil dimension: 3.50 m x 0.25 mm i.d. (T 50^{0}C). *Column: Alltima C18 5µ; ID 3.0mm x 150mm.

*All the tested boronic acids were dissolved in acetonitrile-water (20:80, v:v)

Table 12. Minimum Detectable Concentration (MDC) and Minimum Detectable Amounts (MDA) of boronic acids in HPLC-alizarin online system under gradient condition

Compound	Tested conc., µM	MDC, µM	MDA, ng
TFM-PBA	0.2	0.03	0.05
#C-PBA	1.5	2	3
#PBA	0.8	1	1
A-PBA	55	33	51
M-PBA	5.7	1	1
#BBA	0.8	5	5
#PBA-PE	0.8	2	3
PBFB	5.2	4	7
#PPFB	0.8	3	5
PBA-MIDA Ester	1	1	1

*LC: mobile phase gradient program (Appendix 2), flow rate 0.4mL/min ; UV detector 270 nm; Fluorescence detector λ_{Ex} 469nm; λ_{Em} 610nm *Supeloop: Alizarin 75 µM + 0.1%TEA (v/v) in Acetonitrile, flow rate 0.6mL/min *Coil dimension: 3.50 m x 0.25 mm i.d. (T 50^{0}C). *Column**:** Alltima 5µ; ID 3.0mm x 150mm.
*All the tested boronic acids were dissolved in acetonitrile-water (20:80, v:v)

To summarize, in general, the estimation of MDC and MDA of boronic acids by this HPLC on-line system with alizarin are relatively low, except for the A-PBA by the reason as mentioned in 3.4.4.

3.4.6. Selectivity test

The selectivity test of this analytical method is intended to ensure that alizarin is the specific fluorescent reporter for boronic acid derivatives. For this purpose, other compounds with various functional groups which possess aromatic ring, were tested under the optimized analytical method for boronic acid detection. The concentration of non boronic acids were made ten times higher from the concentration of boronic acids.

Figure 21 shows that in general, alizarin does not form the fluorescent complex with non boronic acid compounds which have various functional groups. Due to autofluorescence, some non boronic acid compounds, respectively: aniline, phenol, methoxybenzonitrile, p-toluensulfonic acid and hydroxybenzamide show the high signal to noise as shown on Table 13. By applying autofluorescence test for these compounds, it is proven that the five non boronic acid compounds are autofluorescence. This result is confirmed by Bridges et.al[22] and Bass[23] who did investigation of the fluorescence, respectively on aniline and methoxybenzonitrile. In Appendix 4 (c), it is seen in the fluorescence detector that the peak resulted from the autofluorescent test without alizarin is higher than the peak resulted from the HPLC on-line system with alizarin. The absence of the super loop and the reaction coil on the instrumental setup of autofluorescence test cause the decreasing height of the peak due to no dilution by alizarin solution. For the next experiment, it is suggested that the autofluorescence test should be done with the same setup as the HPLC on-line system with alizarin, by filling the super loop with the pure solvent of alizarin instead of the alizarin solution, to consider the dilution factor of the super loop.

However, this study proves that alizarin is the specific fluorescent reporter for boronic acid compounds.

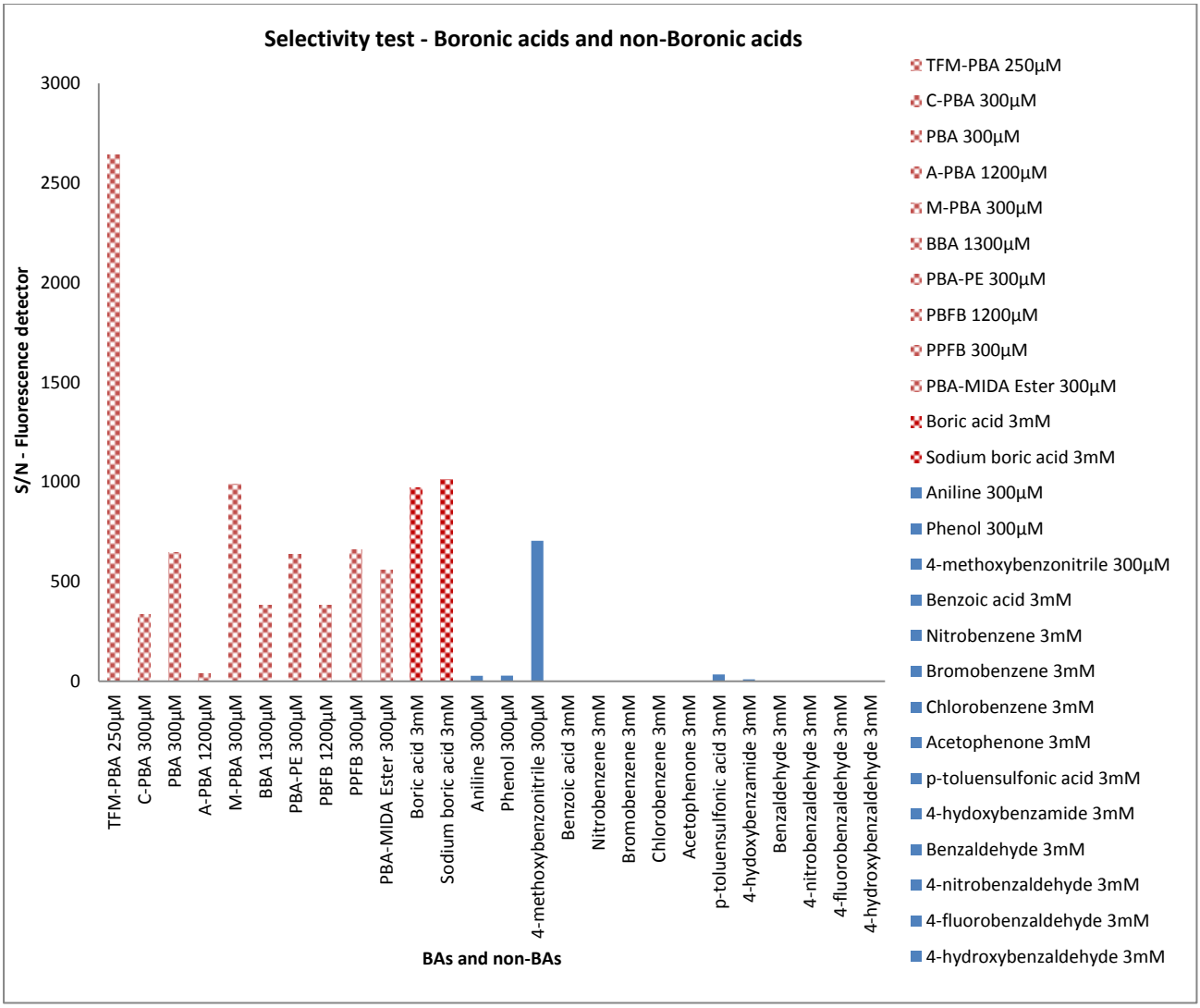


Figure 21. The signal to noise ratio of the selectivity test
*LC: mobile phase gradient program (Appendix 2), flow rate 0.4mL/min ; UV detector 270 nm; Fluorescence detector λ_{Ex} 469nm; λ_{Em} 610nm *Supeloop: Alizarin 75 µM + 0.1%TEA (v/v) in Acetonitrile, flow rate 0.6mL/min *Coil dimension: 3.50 m x 0.25 mm i.d. (T 50^{0}C). *Column**:** Alltima 5µ; ID 3.0mm x 150mm.

Table 13. The data of non boronic acids for selectivity test in the HPLC-alizarin on-line under gradient condition

Compound	Conc. (mM)	Rt (UV)	Rt (FLU)	S/N - FLU	remarks
*Aniline 300µM	0.3	8.47	8.83	27.95	*autofluorescence
*Phenol 300µM	0.3	7.80	8.14	28.55	*autofluorescence
*4-methoxybenzonitrile 300µM	0.3	12.62	12.97	704.74	*autofluorescence
Benzoic acid 3mM	3	4.55	4.90	0.13	
Nitrobenzene 3mM	3	13.32	13.67	0.13	
Bromobenzene 3mM	3	20.85	21.20	0.01	
Chlorobenzene 3mM	3	19.35	19.70	0.03	
Acetophenone 3mM	3	11.35	11.70	0.17	
p-toluensulfonic acid 3mM	3	1.88	2.07	34.63	*autofluorescence
4-hydoxybenzamide 3mM	3	2.64	2.99	9.89	*autofluorescence
Benzaldehyde 3mM	3	10.74	11.09	0.02	
4-nitrobenzaldehyde 3mM	3	11.64	11.99	0.01	
4-fluorobenzaldehyde 3mM	3	11.27	11.62	0.02	

4-hydroxybenzaldehyde 3mM	3	6.73	7.08	0.03

*LC: mobile phase gradient program (Appendix 2), flow rate 0.4mL/min ; UV detector 270 nm; Fluorescence detector λ_{Ex} 469nm; λ_{Em} 610nm *Supeloop: Alizarin 75 µM + 0.1%TEA (v/v) in Acetonitrile, flow rate 0.6mL/min *Coil dimension: 3.50 m x 0.25 mm i.d. (T 50^{0}C). *Column: Alltima 5µ; ID 3.0mm x 150mm.

3.5. Application of the HPLC-Alizarin on-line system to complex mixture

The aim of this study is to observe the applicability of the HPLC online system with alizarin to monitor the reactions involving boronic acids. During this study, two reactions were investigated, namely : the esterification of 4-carboxyphenylboronic acid [C-PBA] with ethanol and the thiol-ene reaction of (4-Allylaminocarbonyl)phenylboronic acid [AACPBA] and Boc-Cysteine.

3.5.1. Monitoring esterification of 4-carboxyphenylboronic acid (C-PBA) with ethanol

The principle of this esterification reaction is transformation into another boronic acid derivative which the detailed reaction is on Appendix 5. Thus, the HPLC on-line system with alizarin is utilized to observe the transformation of C-PBA to another boronic acid derivative.

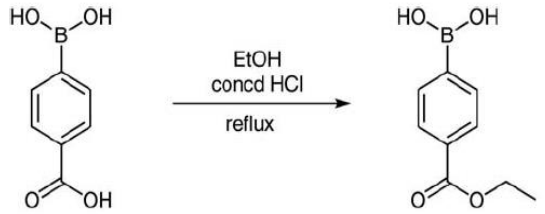
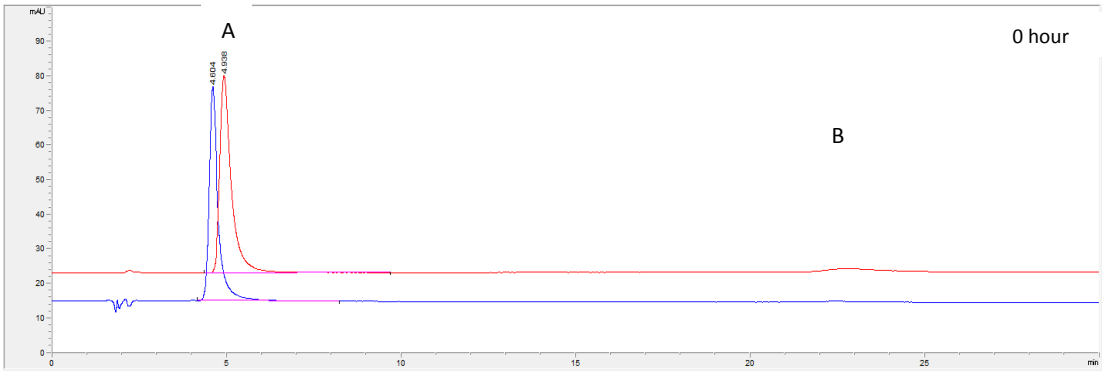


Figure 22. The scheme of the esterification of 4-carboxyphenylboronic acid [C-PBA] and ethanol.

Figure 23 and Figure 24 show the transformation process of C-PBA to another boronic acid that the peak of C-PBA decreases as the function of time. After 24 hours reaction, the starting material (A) is completely gone and the product (B) si formed, but there is new peak (C) detected on UV detector only and not detected on fluorescence detector. This peak (C) seems the impurity which yielded as the decomposition of the product. Furthermore, peak (C) is confirmed as non boronic acid compund due to un-fluorescence with alizarin. Thus, the HPLC on-line system with alizarin is able to distinguish the boronic acid and non boronic acid compound.

a)



b)

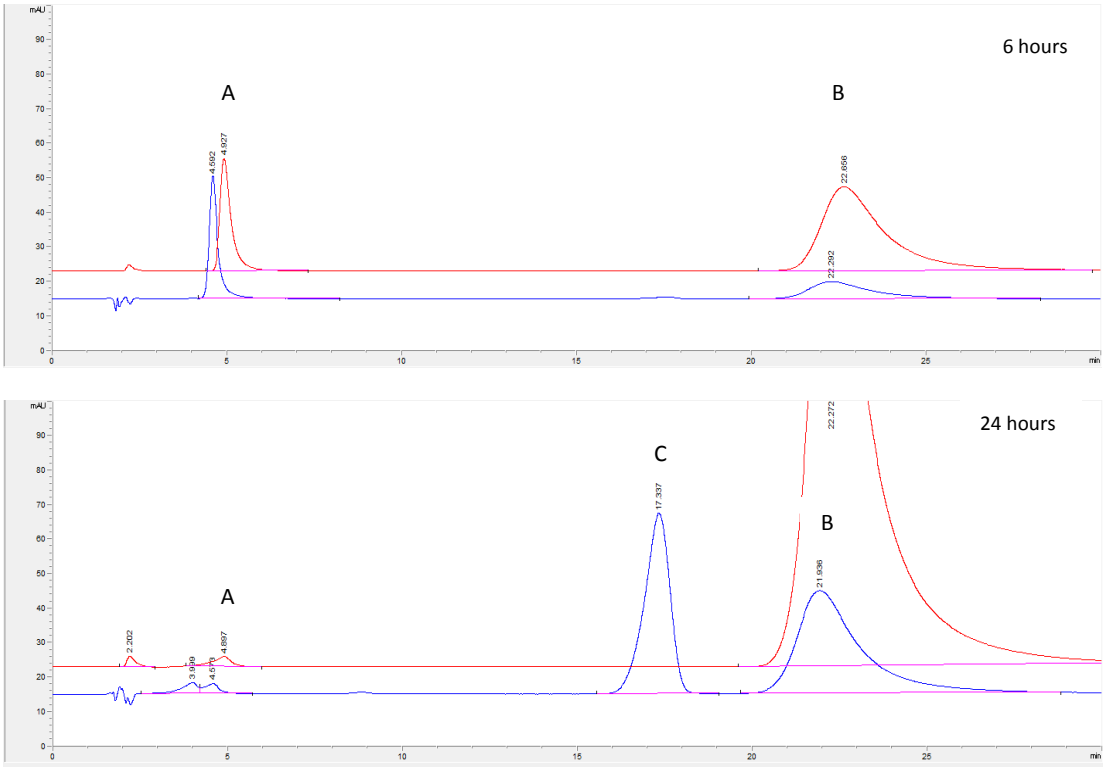


Figure 23. Chromatogram of the mixture which taken during the esterification of 4-carboxyphenylboronic acid with ethanol. (a) starting material, (b) product.
*LC: mobile phase isocratic methanol-water-acetic acid (40:60:0.1%, v:v:v); flow rate 0.4mL/min; UV detector 270 nm; Fluorescence detector λ_{Ex} 469nm; λ_{Em} 610nm *Supeloop: Alizarin 75 µM + **1%TEA** (v/v) in Acetonitrile, flow rate 0.6mL/min *Coil dimension: 3.50 m x 0.25 mm i.d. (T 50^{0}C). *Column**:** Alltima 5µ; ID 3.0mm x 150mm.
*Tested sample was taken during reaction, diluted 100x with methanol-water (40:60,v:v).

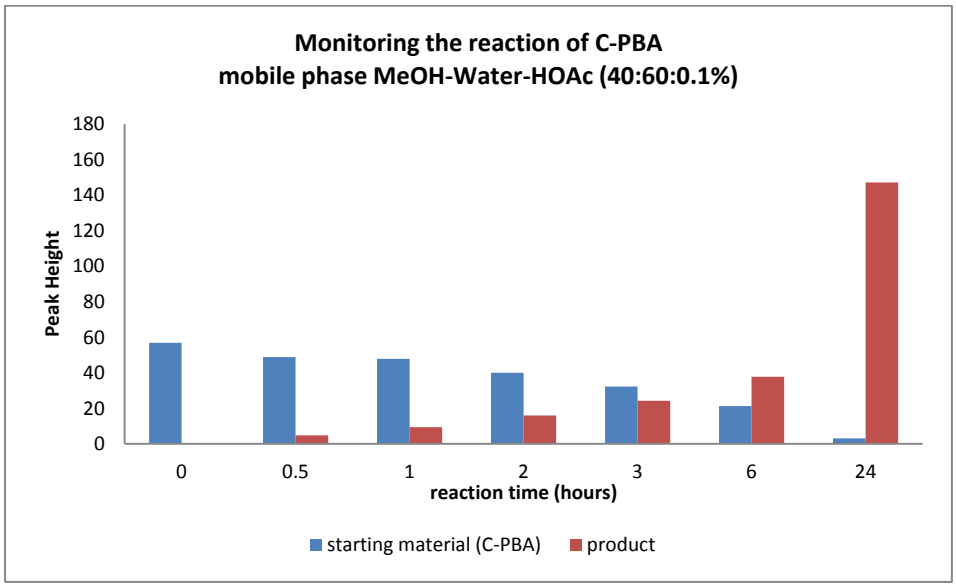


Figure 24. The transformation process of C-PBA to another boronic acid during the reaction.
*LC: mobile phase isocratic methanol-water-acetic acid (40:60:0.1%, v:v:v); flow rate 0.4mL/min; UV detector 270 nm; Fluorescence detector λ_{Ex} 469nm; λ_{Em} 610nm *Supeloop: Alizarin 75 µM + **1%TEA** (v/v) in Acetonitrile, flow rate 0.6mL/min *Coil dimension: 3.50 m x 0.25 mm i.d. (T 50^{0}C). *Column**:** Alltima C 18 5µ; ID 3.0mm x 150mm.
*Tested sample was taken during reaction, diluted 100x with methanol-water (40:60,v:v).

3.5.2. Monitoring reaction of (4-Allylaminocarbonyl)boronic acid with Boc-cystein

The principle of this reaction is also the transformation from (4-Allylaminocarbonyl)phenylboronic acid (AACPBA) to another form of boronic acid which the detailed reaction procedure is described on Appendix 6.

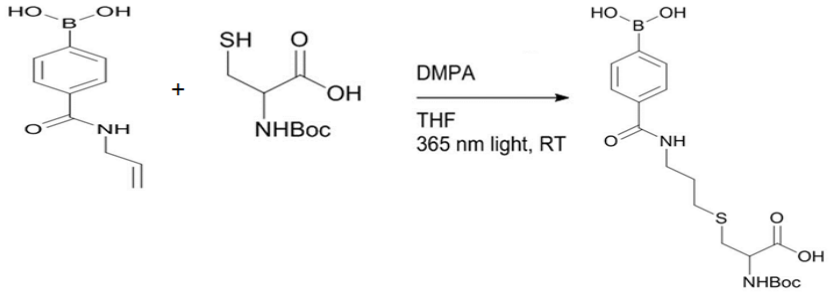
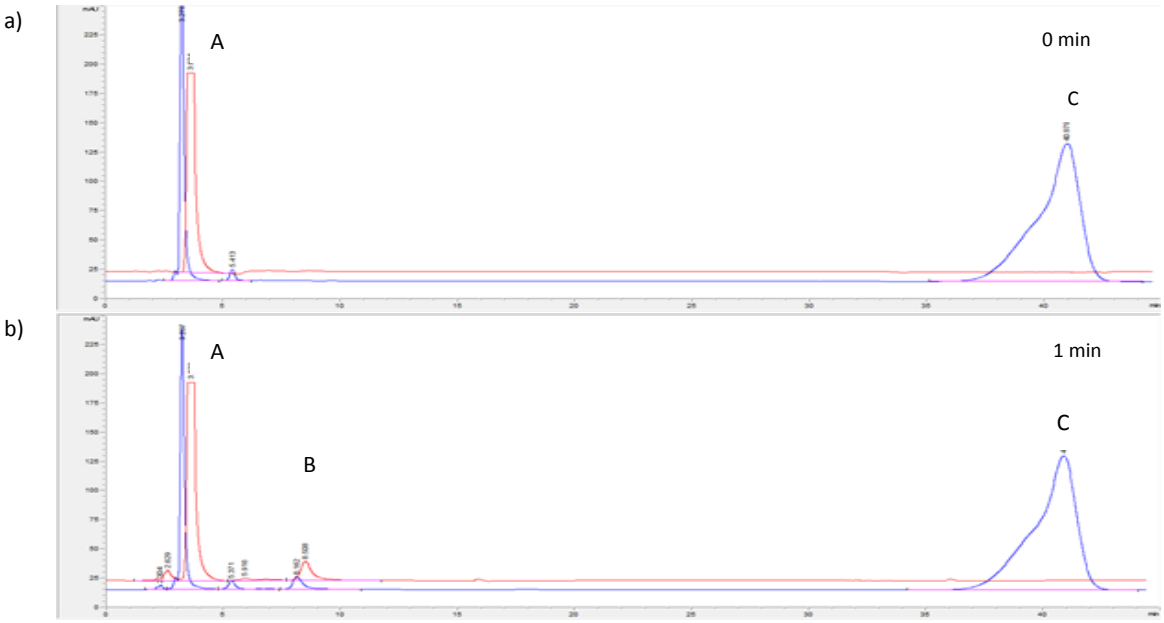
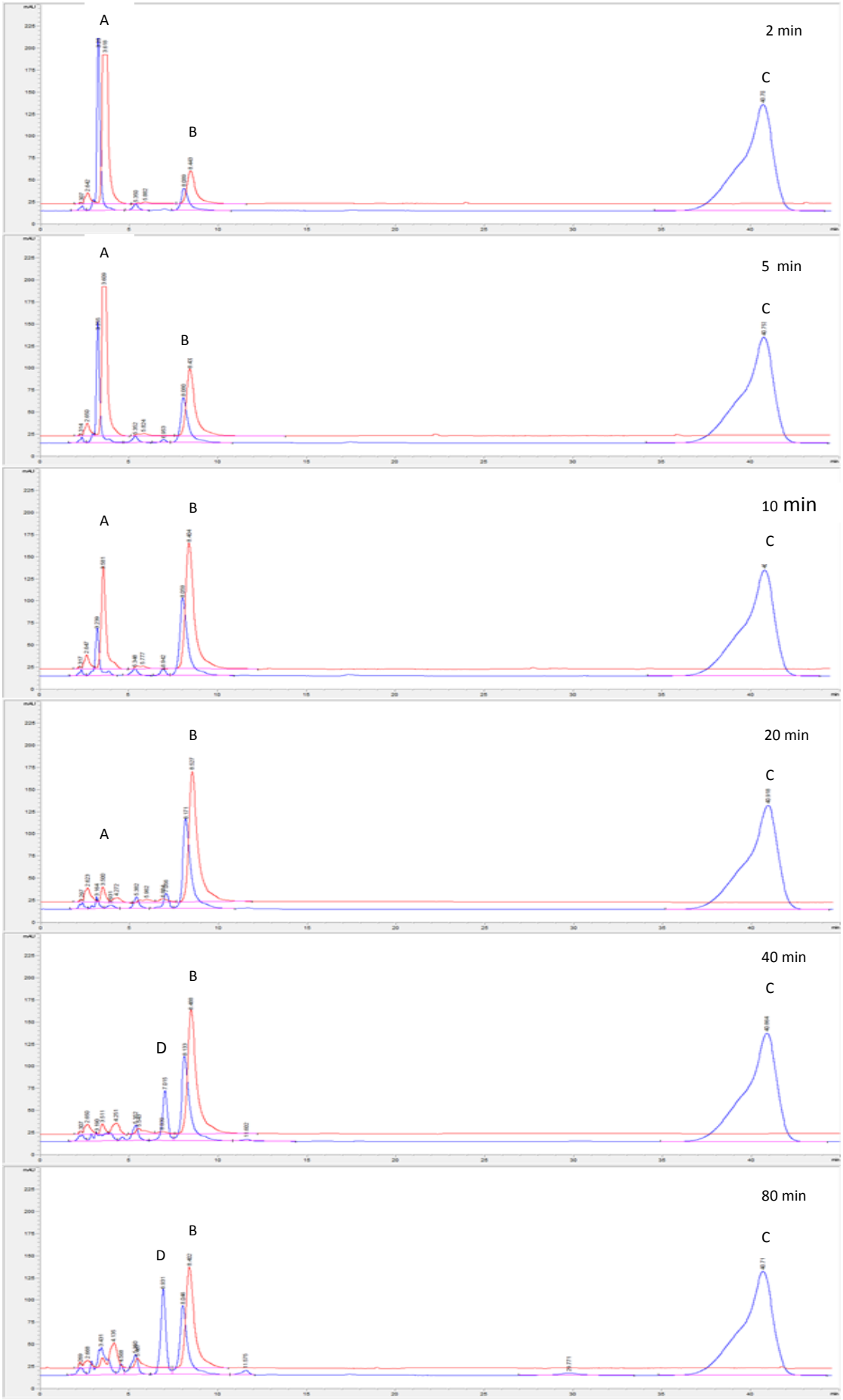


Figure 25. The scheme of thiol-ene reaction of (4-Allylaminocarbonyl)phenylboronic acid [AAC-PBA]

Figure 26 and Figure 27 show the transformation process of AAC-PBA to another form boronic acid by the function of time. This HPLC on-line system with alizarin provides information of the degradation from the product after certain time and able to define the boronic acid and non boronic acid. At the beginning, the starting material (A) is detected as a huge peak by the fluorescence detector, and after 1 min the the expected product which is another boronic acid formation starts forming, peak (B). Due to the boronic acid derivative, the expected product is detected by fluorescence detector. Unfortunately, after more than 40 min, it is clearly defined that the impurities start forming as peak (D) and (E) which not detected by fluorescence detector. Therefore, the HPLC on-line system with alizarin can be also used to define the optimum reaction time. Assuming that longer reaction time will achieve maximum yield of the product can be wrong by monitoring the reaction with this detection method. In addition, peak (E) belongs to DMPA.




A
B
C
2 min
A
B
C
5 min
A
B
C
10 min
A
B
C
20 min
A
B
D
C
40 min
B
D
C
80 min

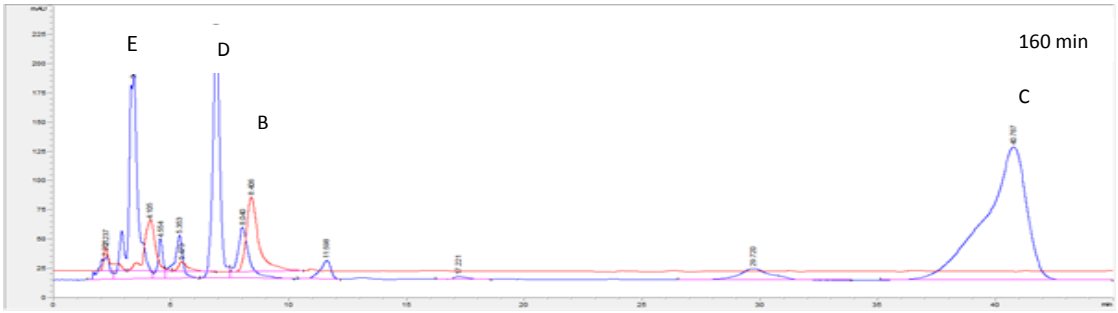


Figure 26. Chromatogram of the mixture which taken during the thiol-ene reaction of Allylaminocarbonyl)phenylboronic acid (AACPBA)

*LC: mobile phase isocratic methanol-water-acetic acid (50:50:0.1%, v:v:v); flow rate 0.4mL/min; UV detector 270 nm; Fluorescence detector λ_{Ex} 469nm; λ_{Em} 610nm *Supeloop: Alizarin 75 µM + **1%TEA** (v/v) in Acetonitrile, flow rate 0.6mL/min *Coil dimension: 3.50 m x 0.25 mm i.d. (T 50^{0}C). *Column**:** Alltima 5µ; ID 3.0mm x 150mm.
*Tested sample was taken during reaction, diluted 100x with methanol-water (50:50,v:v).

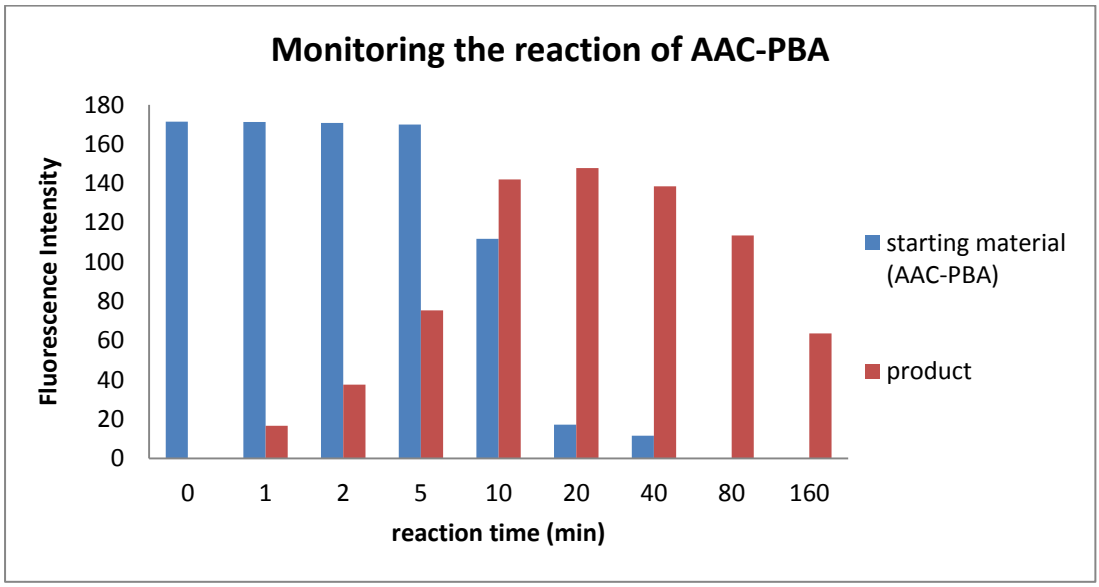


Figure 27. The transformation of AAC-PBA to another form of boronic acid via thiol-ene reaction

*LC: mobile phase isocratic methanol-water-acetic acid (50:50:0.1%, v:v:v); flow rate 0.4mL/min; UV detector 270 nm; Fluorescence detector λ_{Ex} 469nm; λ_{Em} 610nm *Supeloop: Alizarin 75 µM + **1%TEA** (v/v) in Acetonitrile, flow rate 0.6mL/min *Coil dimension: 3.50 m x 0.25 mm i.d. (T 50^{0}C). *Column**:** Alltima 5µ; ID 3.0mm x 150mm.
*Tested sample was taken during reaction, diluted 100x with methanol-water (50:50,v:v).

4. Conclusion

A rapid, sensitive and selective HPLC on-line system with alizarin for boronic acid detection in the complex mixtures was developed. This analytical method has ability to easily define between boronic acid compounds and non boronic acid compounds with high sensitivity. For further application, this method might be developed as preparative HPLC method.

However, this on-line is less sensitive for the boronic acids with the strong electron donating groups, thus it is still need to be developed.

5. References

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6. Appendix

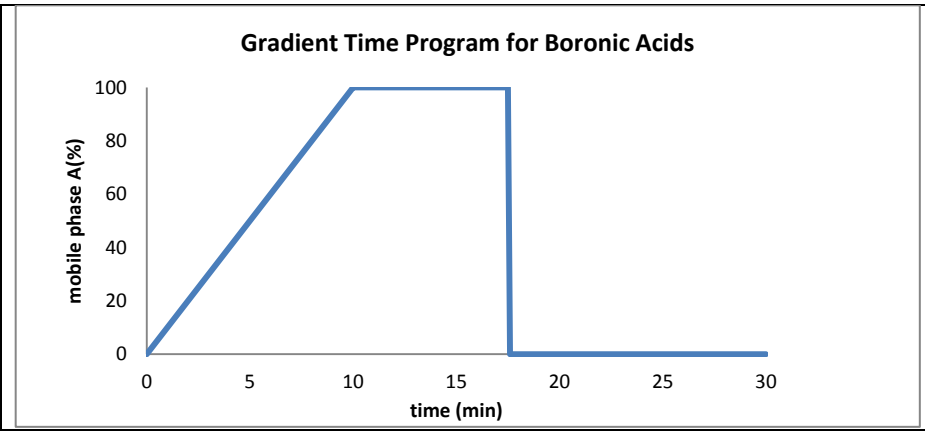
Appendix 1. Determination of signal to noise ratio

The noise was determined as three times standard deviation of the peak height of the blank, obtained from 20 data points of the peak height which taken every three seconds. Moreover, the signal was defined as the peak height in the retention time of the compound.

Appendix 2. LC-gradient time program for boronic acids

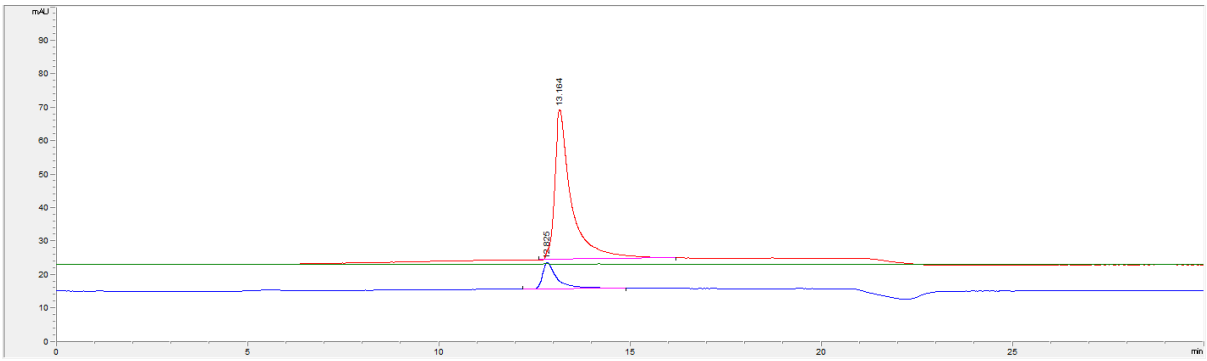
Mobile phase :
A : ACN-Water (20 : 80, v:v)
B : ACN-Water (50 : 50, v:v)

Time (min)	Solvent A (%)	Solvent B (%)
0.0	100	0
10.0	0	100
17.5	0	100
17.6	100	0
30.0	100	0
30.01	stop	

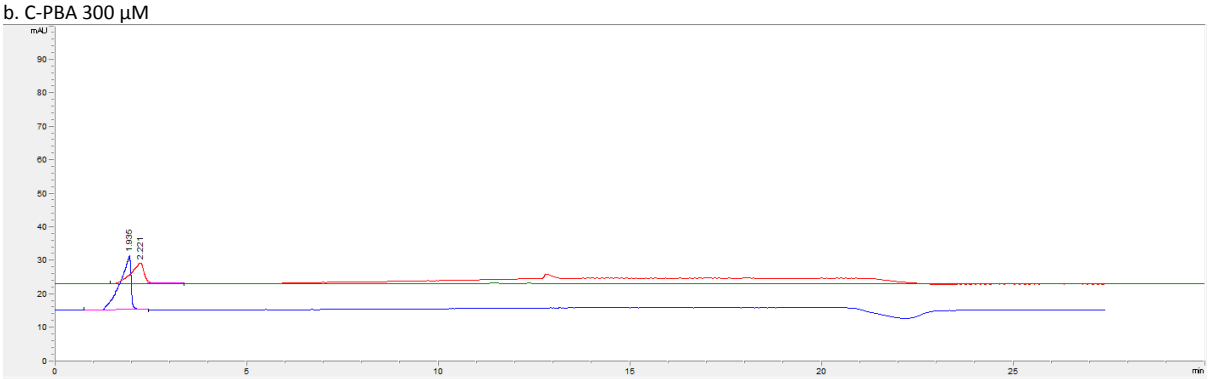


Appendix 3. Chromatogram of boronic acids under on-line HPLC system with alizarin

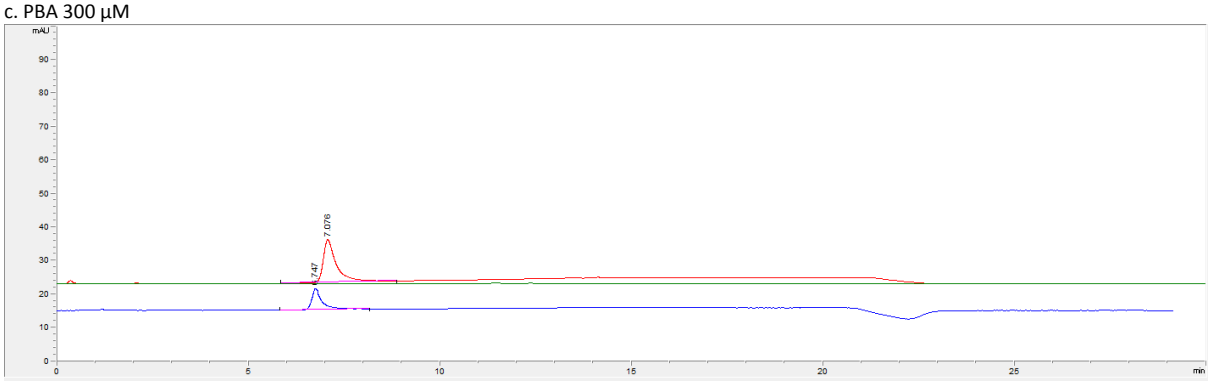
a. TFM-PBA 250µM



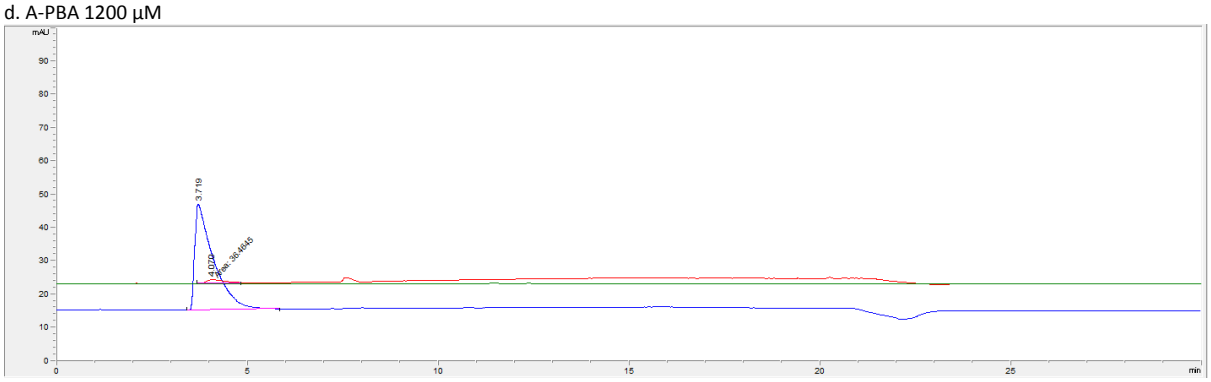
b. C-PBA 300 µM



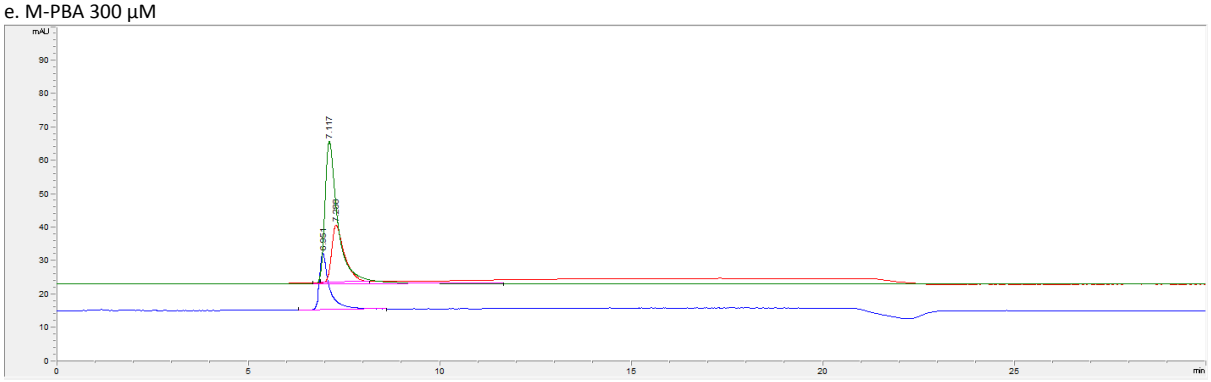
c. PBA 300 µM



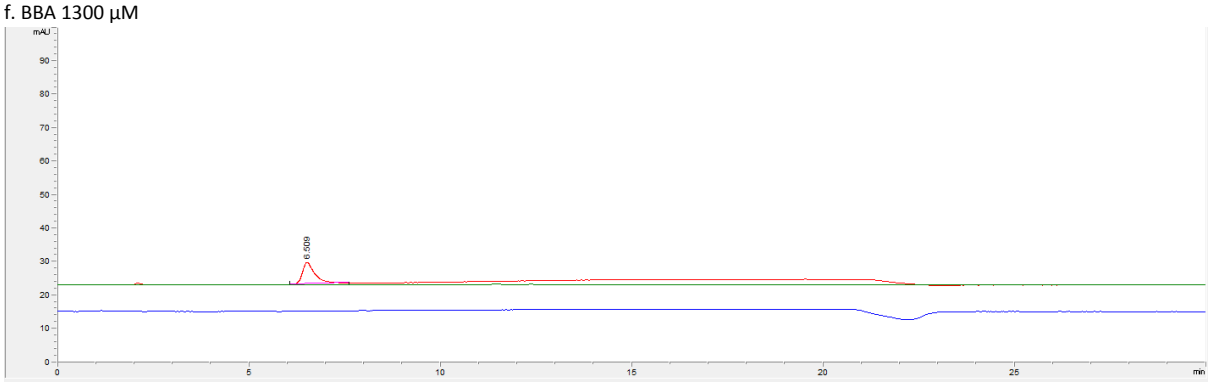
d. A-PBA 1200 µM



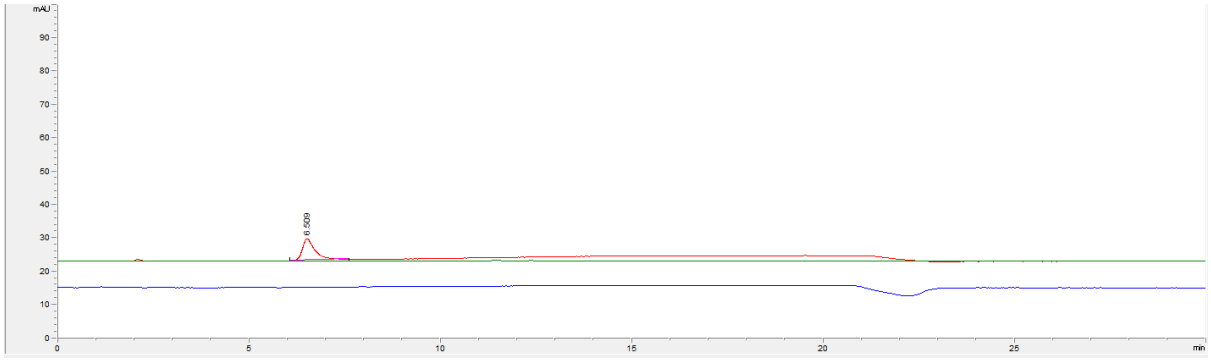
e. M-PBA 300 µM



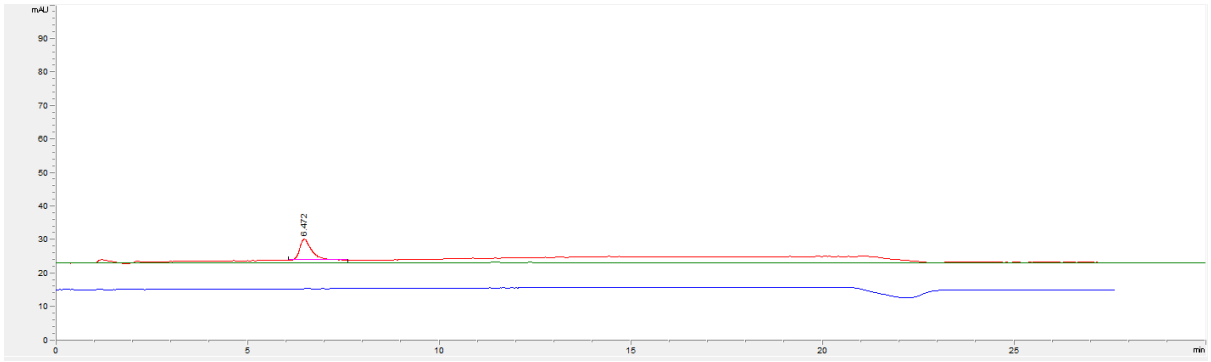
f. BBA 1300 µM



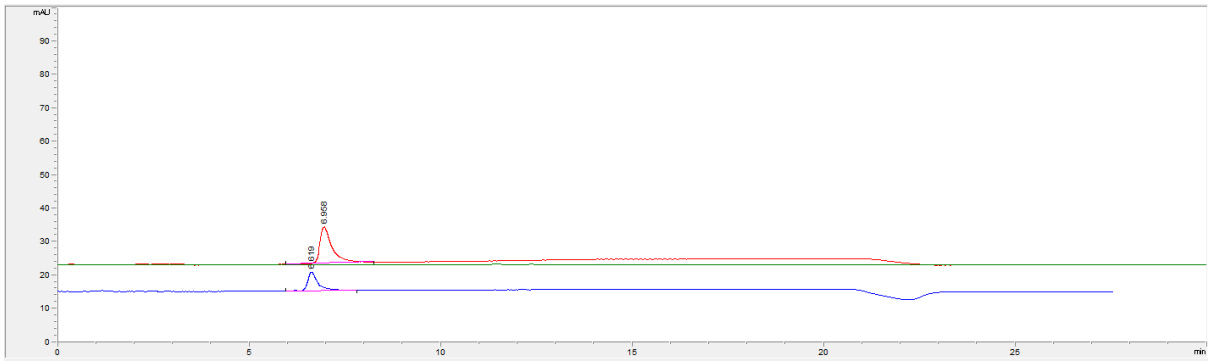
g. PBA-PE 300 µM



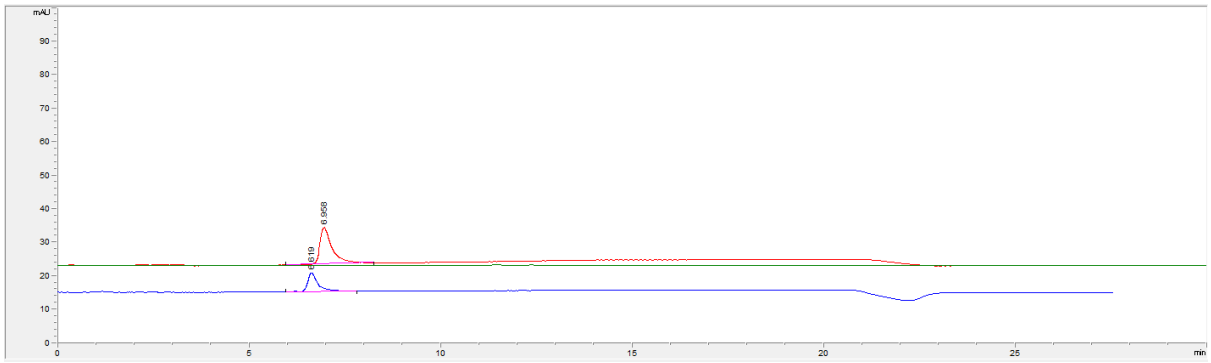
h. PBFB 1200 µM



i. PPFB 300 µM



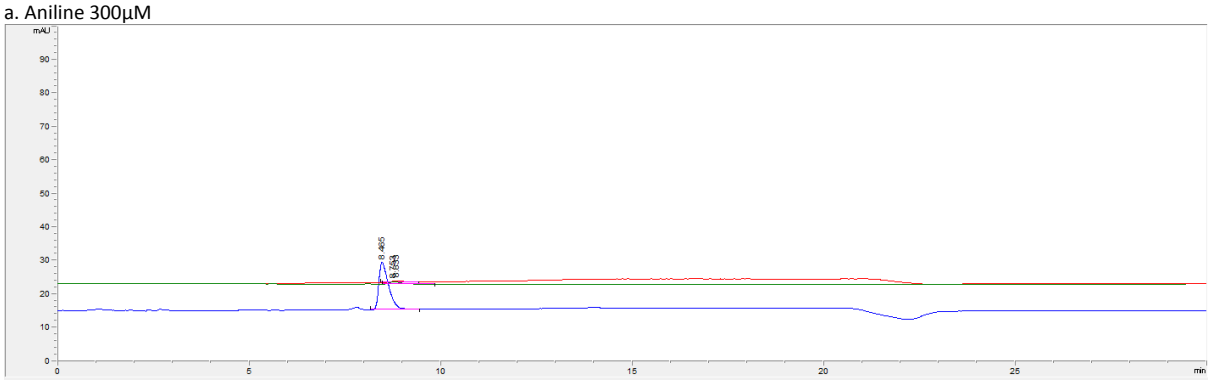
j. PBA MIDA Ester 300 µM



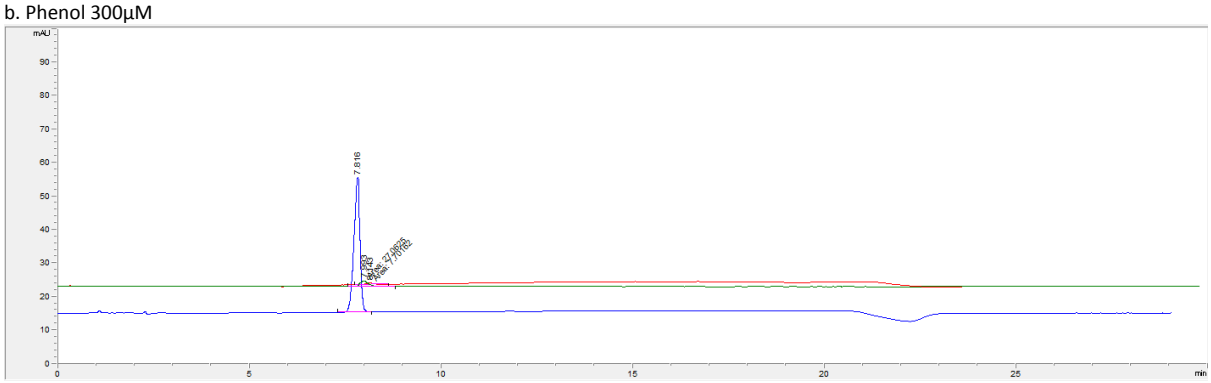
Chromatogram 1. Chromatograms of boronic acids under on-line HPLC system
(Blue: UV detector ; Red: Fluorescence detector-with alizarin ; Green: Fluorescence detector-without alizarin)

Appendix 4. Chromatograms of non boronic acids (non BA) which auto-fluorescence

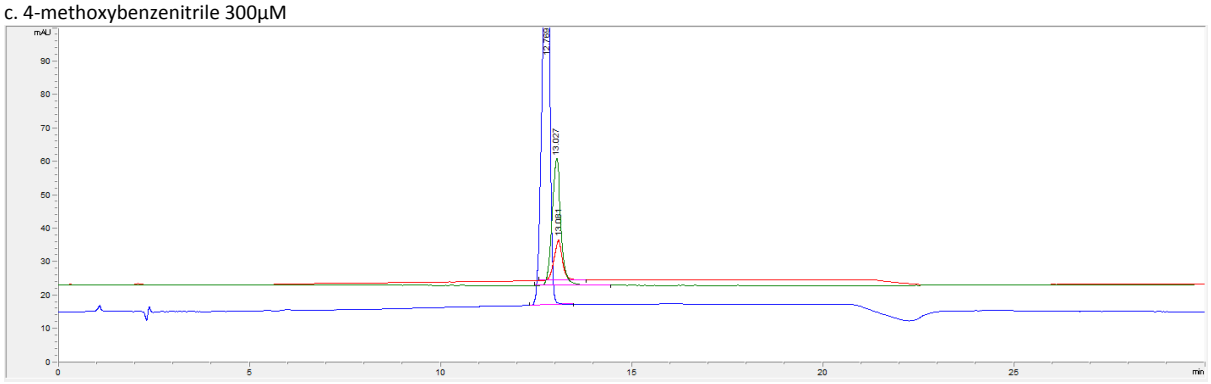
a. Aniline 300µM



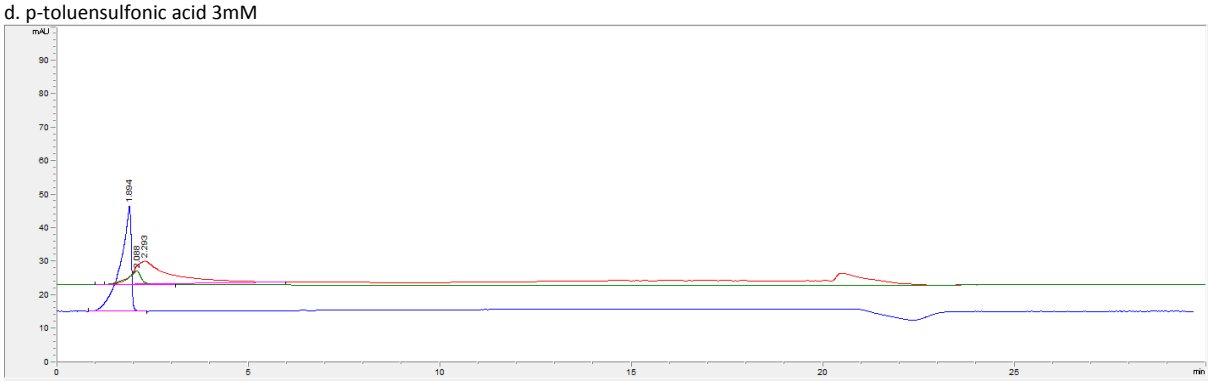
b. Phenol 300µM



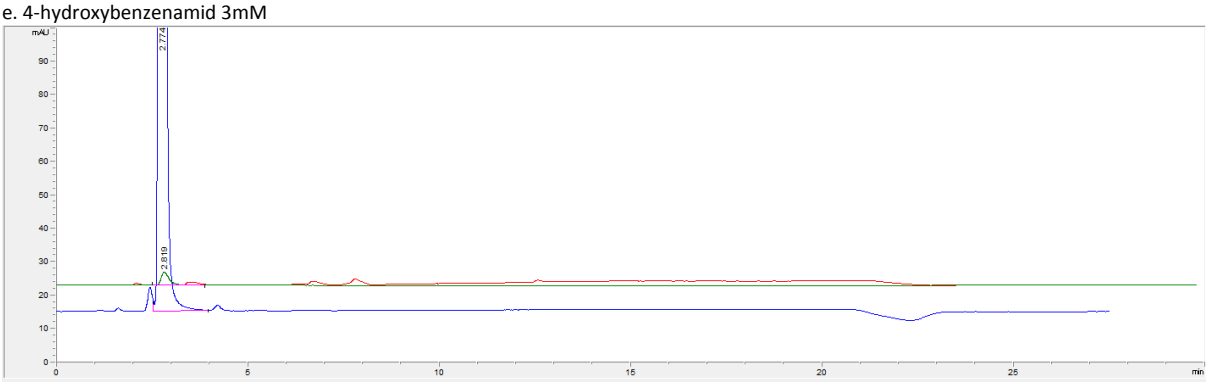
c. 4-methoxybenzenitrile 300µM



d. p-toluensulfonic acid 3mM



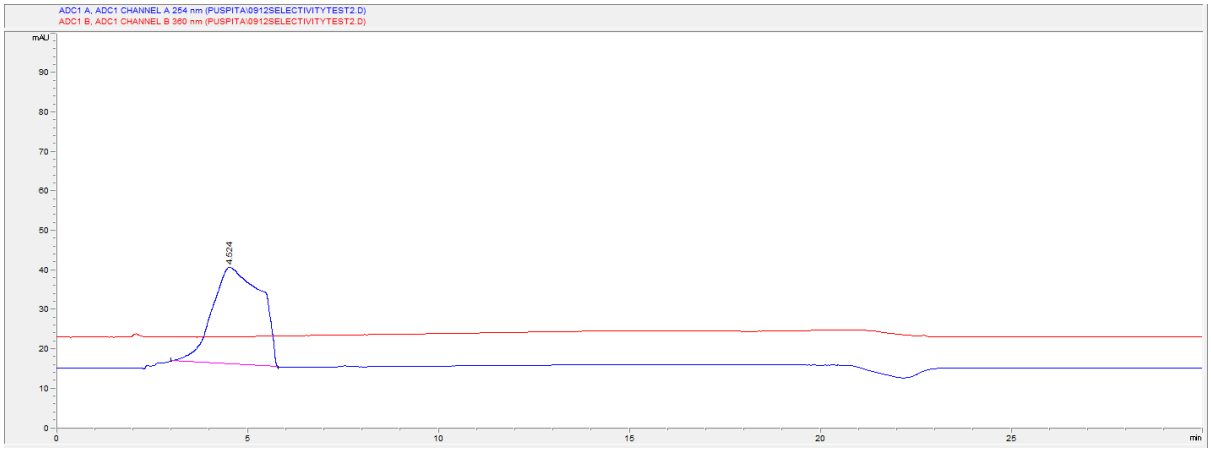
e. 4-hydroxybenzenamid 3mM



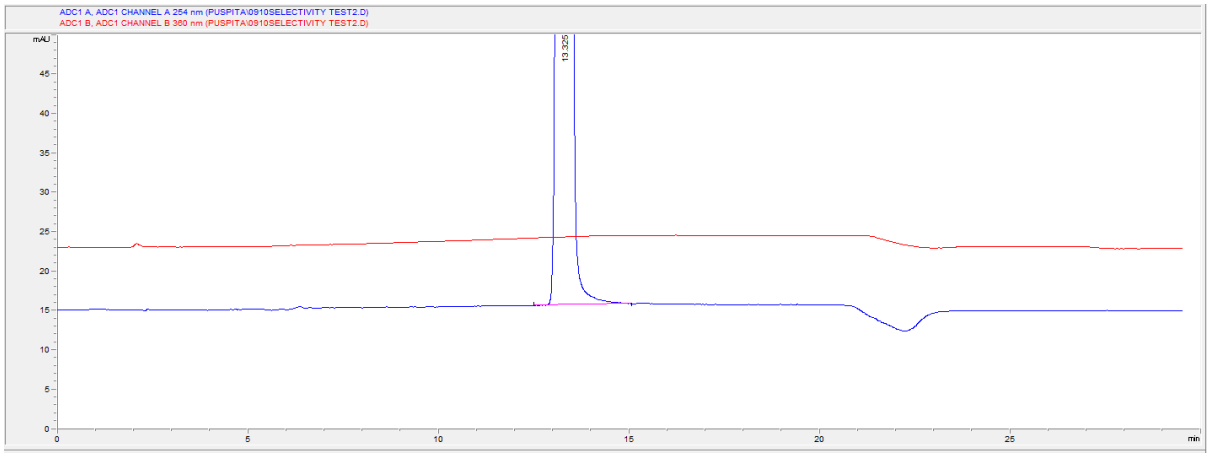
Chromatogram 2. Chromatogram of non boronic acids which auto fluorescence.
(Blue: UV detector ; Red: Fluorescence detector-with alizarin ; Green: Fluorescence detector-without alizarin)

Appendix 5. Chromatogram of non-boronic acids for selectivity test

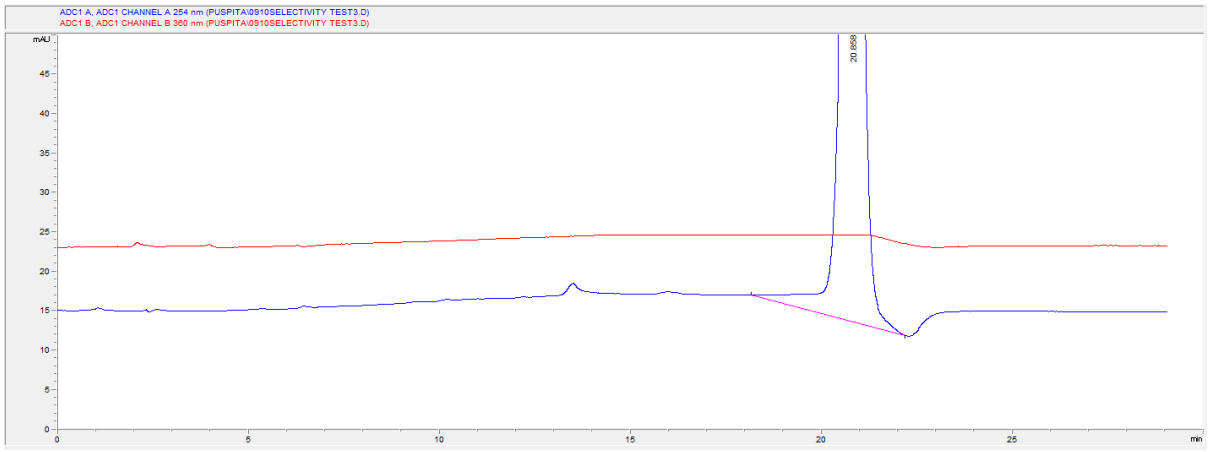
a. Benzoic Acid 3mM



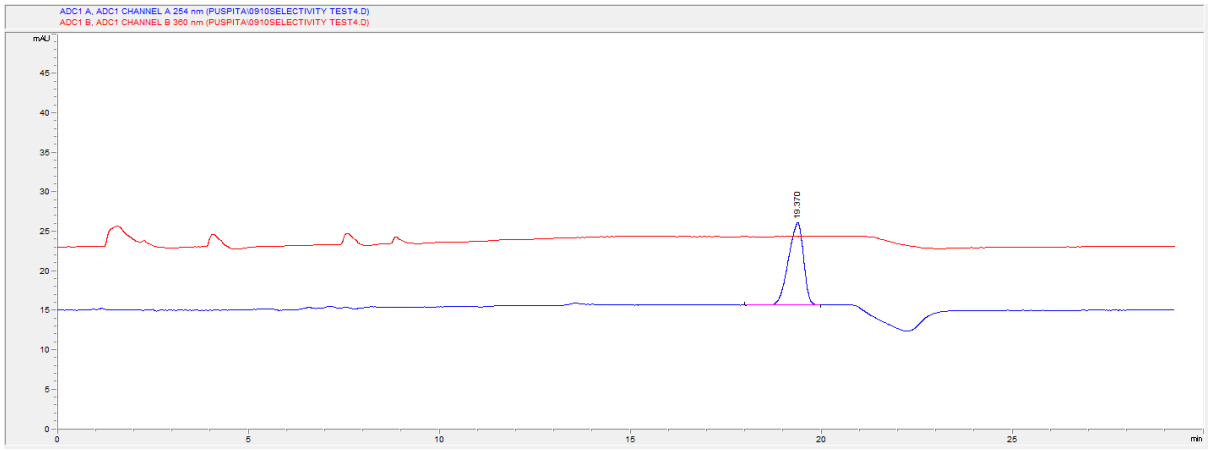
b. Nitrobenzene 3mM



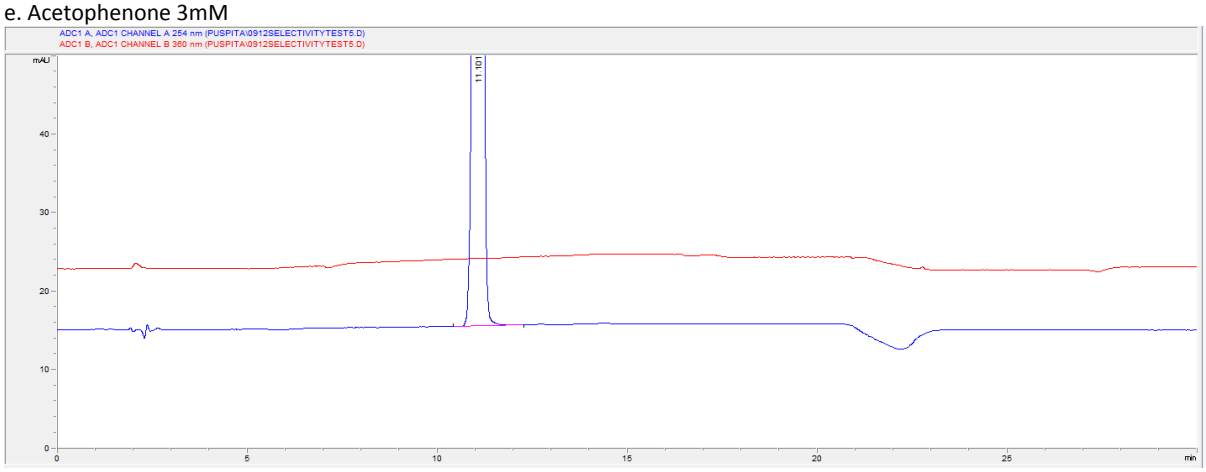
c. Bromobenzene 3mM



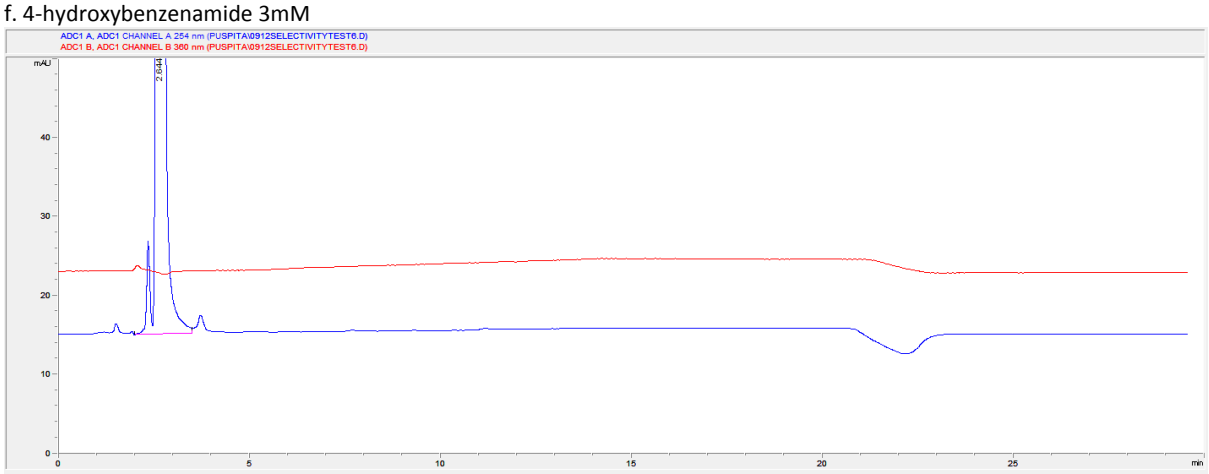
d. Chlorobenzene 3mM



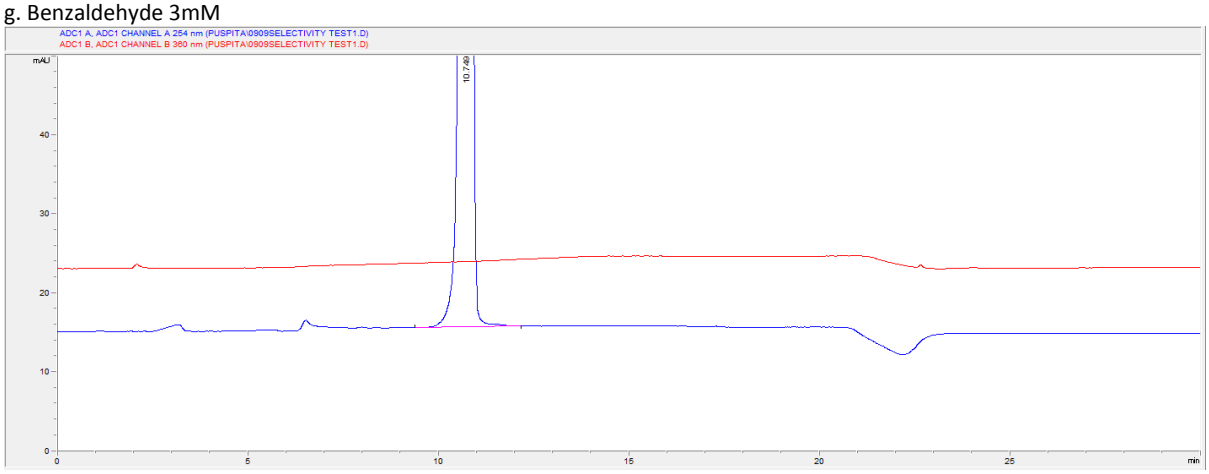
e. Acetophenone 3mM



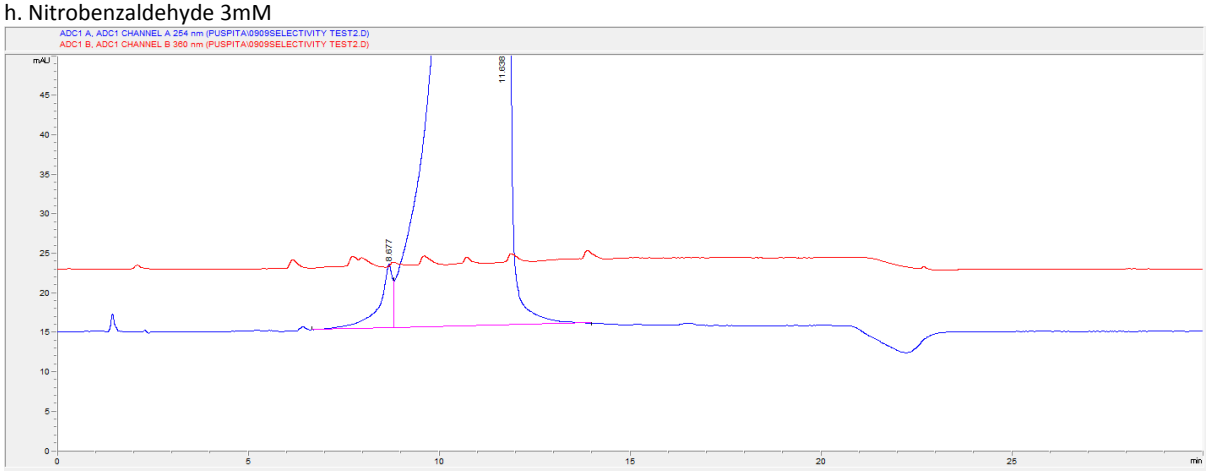
f. 4-hydroxybenzenamide 3mM



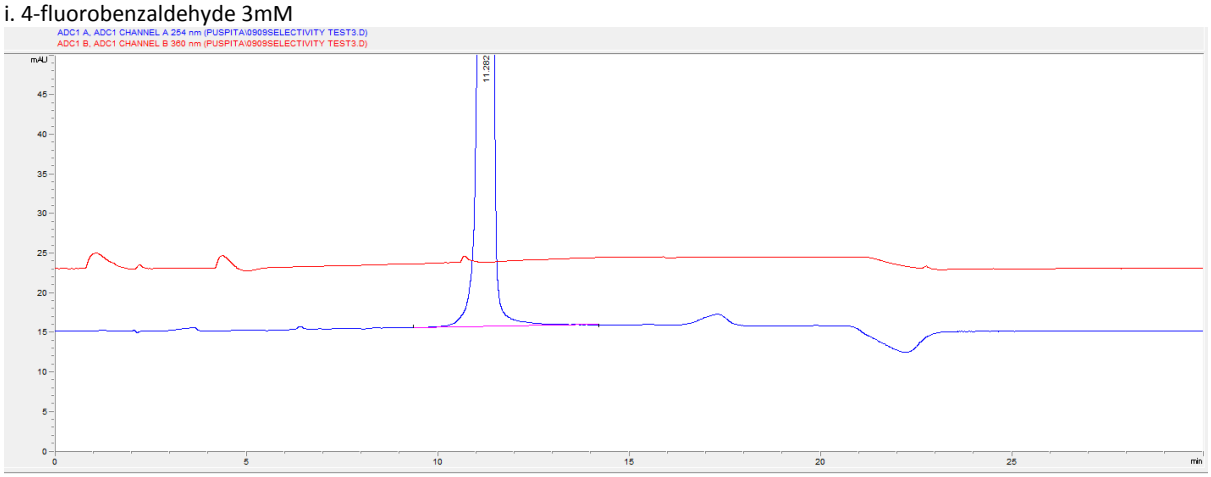
g. Benzaldehyde 3mM



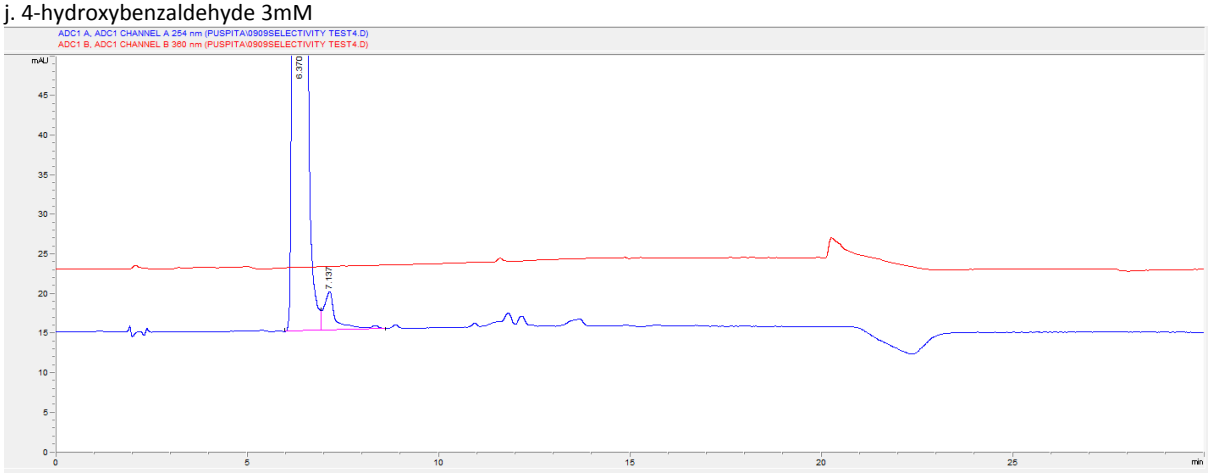
h. Nitrobenzaldehyde 3mM



i. 4-fluorobenzaldehyde 3mM



j. 4-hydroxybenzaldehyde 3mM

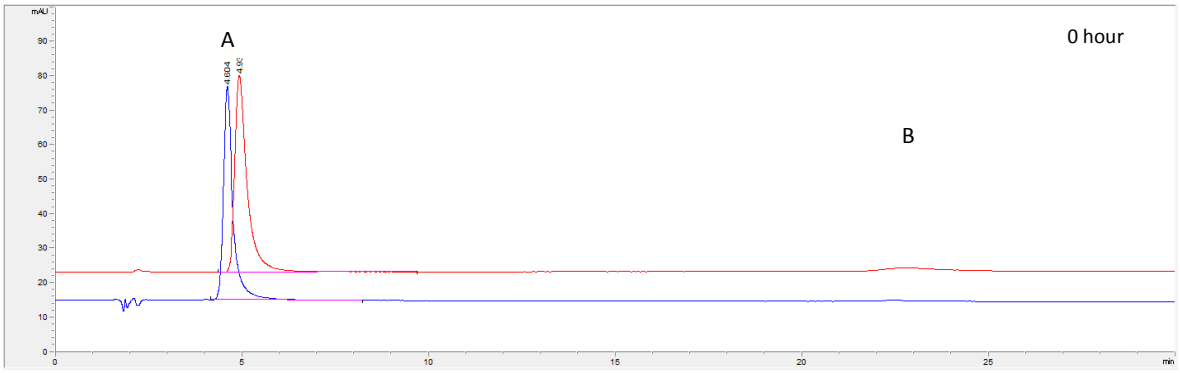


Chromatogram 3. Chromatogram of non-boronic acids for selectivity test

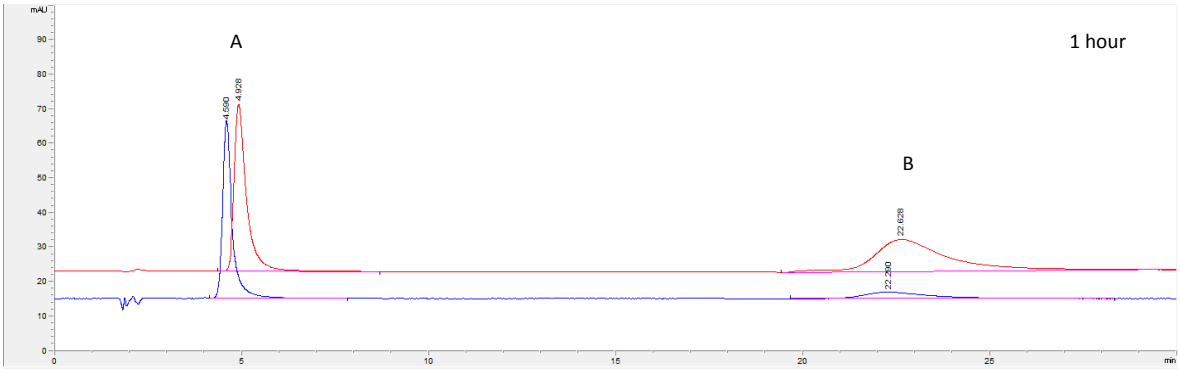
Appendix 6. Esterification of 4-carboxyphenylboronic acid (C-PBA) with ethanol

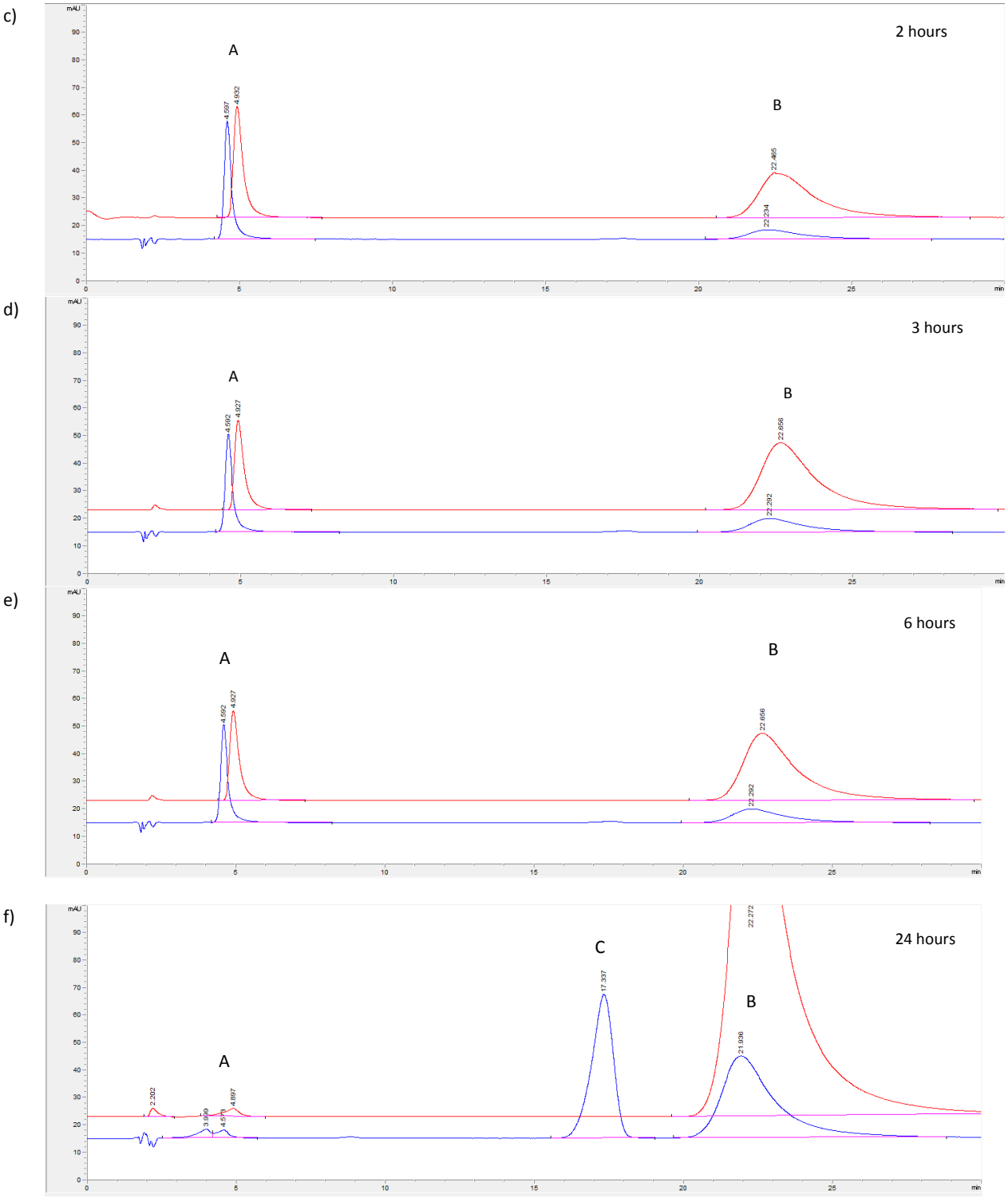
Detailed procedure : 92 mg of 4-carboxyphenylboronic acid in round-bottom flask was dissolved in 2.5 mL ethanol absolute. A mixture of absolute ethanol (2.5 mL) and HCl 37% (2 drops) was added, and the solution was heated to reflux. Tested sample was taken during reaction, diluted 100x with MeOH40%.

a)



b)

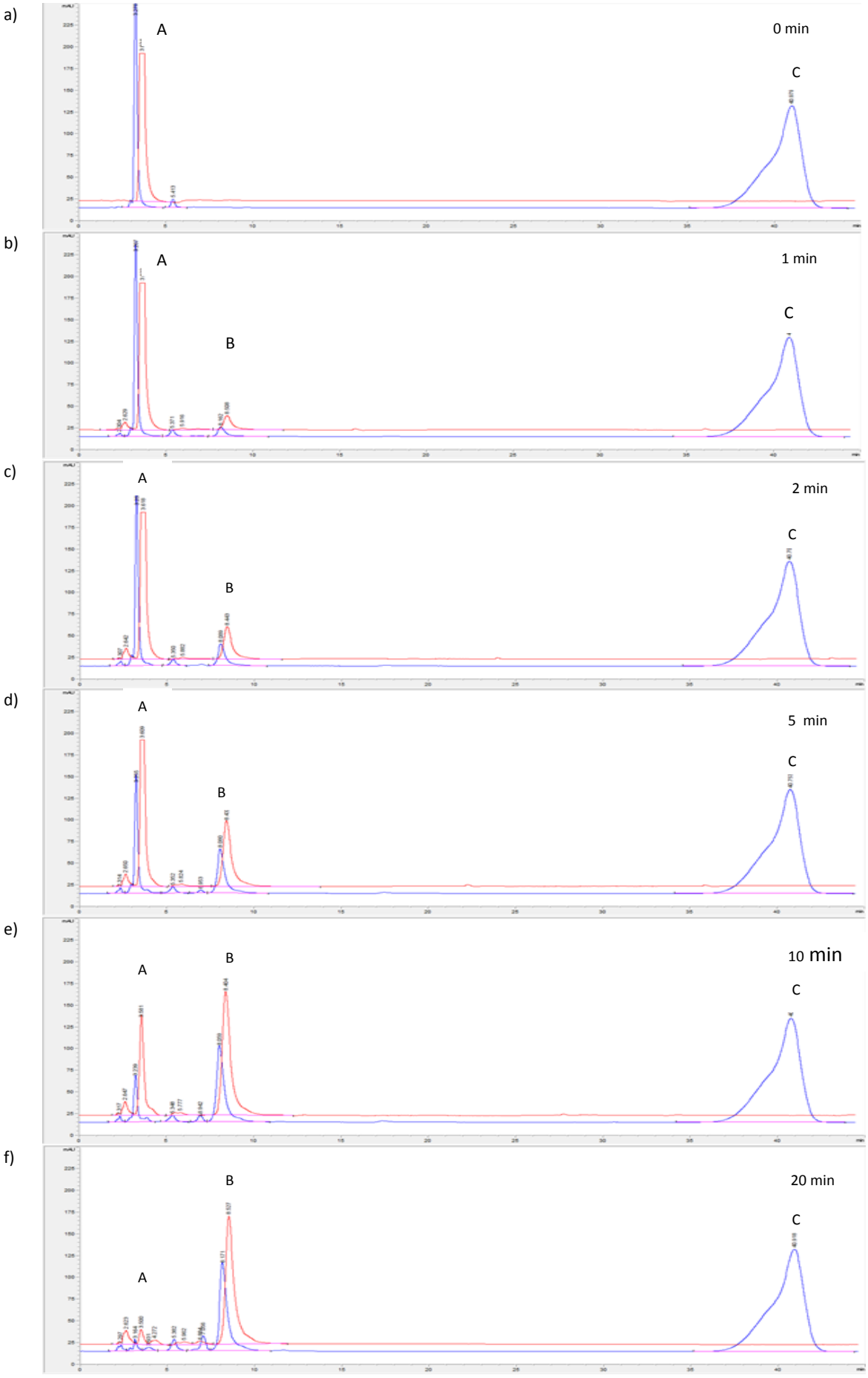




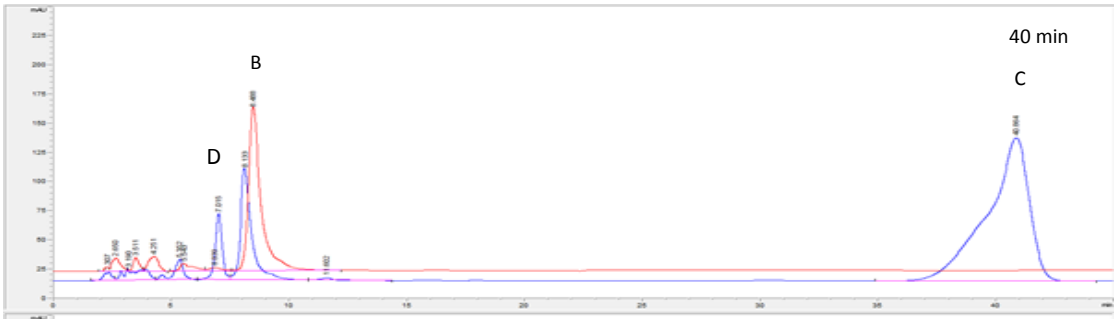
Chromatogram 4. Chromatogram of the esterification of 4-carboxyphenylboronic acid with ethanol.
(A) starting material, (B) product, (C) impurity

Appendix 7. Thiol-ene reaction between 4-Allylaminocarbonyl)phenylboronic acid (AACPBA) and Boc-Cysteine

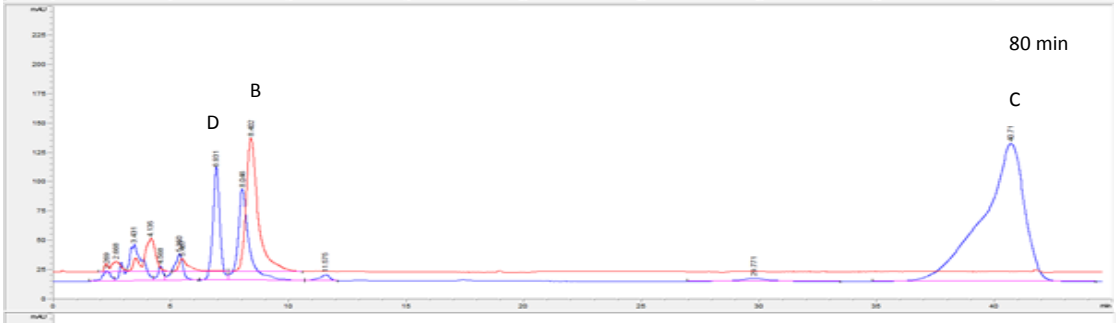
Detailed procedure : A mixture of 4-Allylaminocarbonyl)phenylboronic acid [0.125 g, 0.2mmol], Boc-cysteine [0.675 g, 1mmol] and DMPA [0.1562 g, 0.2mmol] in THF [3mL] was put together in a vial, stirred and placed in UV cabinet.


a)
A
0 min
C
b)
A
1 min
B
C
c)
A
2 min
B
C
d)
A
5 min
B
C
e)
A
B
10 min
C
f)
A
B
20 min
C

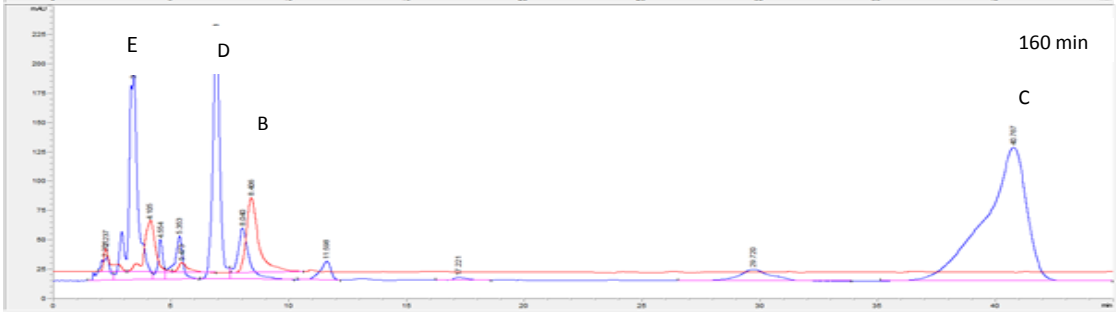
g)



h)



i)



Chromatogram 5. Chromatogram of the thiol-ene reaction of Allylaminocarbonyl)phenylboronic acid (AACPBA) wih Boc-cysteine.
Starting material; (B) product; (C) DMPA; (D) and (E) imputities