OPTIMIZATION OF BIOETHANOL PRODUCTION USING OIL PALM FROND JUICE

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OPTIMIZATION OF BIOETHANOL PRODUCTION USING OIL PALM FROND JUICE

LAWANYA A/P K.RAMU

Thesis submitted in partial fulfilment of the requirements
for the award of the degree of
Bachelor of Chemical Engineering (Biotechnology)

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JUNE 2015

SUPERVISOR'S DECLARATION

We hereby declare that we have checked this thesis and in our opinion, this thesis is adequate in terms of scope and quality for the award of the degree of Bachelor of Chemical Engineering (Biotechnology).

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STUDENT'S DECLARATION

I hereby declare that the work in this thesis is my own except for quotations and summaries which have been duly acknowledged. The thesis has not been accepted for any degree and is not concurrently submitted for award of other degree.

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Dedication

I would like to dedicate this to my parents, Mr. K.Ramu and Mrs Mala for their endless support and motivation. I would also like to dedicate to my supervisor and friends for their never ending guidance and support.

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First and foremost, I would like to express my deepest gratitude towards the Almighty Lord for giving me the strength and guidance that led to the completion of this research project.

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Thank you.

ABSTRACT

This paper presents the optimum conditions for producing bioethanol using oil palm frond juice. Bioethanol has been gaining much interest recently in terms of research and development. Since there are various factors such as rising oil price, environmental issues and high rate consumption of fossil fuel, the global demand for bioethanol has shown a remarkable increase. Oil palm frond is known to be the most generated oil palm biomass annually. OPF is obtained during pruning for harvesting fresh fruit bunch, and the juice can be produced by pressing the fresh OPF using the conventional sugarcane pressing machine. The OPF juice contains higher glucose content, which is about 70% of the total free sugar. Hence, it has a high potential to be the carbon source for producing bioethanol. The parameters investigated in this research work are temperature, pH and agitation speed. Bioethanol was produced from oil palm frond juice by fermentation with *Saccharomyces cerevisiae*. The ethanol concentration from the fermentation sample was analysed using HPLC. Using the ethanol concentration obtained from HPLC analysis, the optimum condition for the production of bioethanol was determined using the Response Surface Methodology with the aid of Design Expert software. The results revealed that, a maximum bioethanol concentration of 22.93 g/L can be obtained at the following optimum conditions : temperature of 32^{o}C, pH 6 and agitation speed of 80 rpm. The validation result revealed that at this optimum condition, a bioethanol yield of 30.1% , which is close to the predicted value, can be obtained.

Key words: oil palm frond juice, bioethanol, *Saccharomyces cerevisae,* HPLC, Design Expert

ABSTRAK

Kertas kerja ini membentangkan keadaan optimum untuk menghasilkan bioetanol menggunakan jus pelepah kelapa sawit. Bioetanol telah mendapat perhatian yang lebih baru-baru ini dari segi penyelidikan dan pembangunan. Oleh kerana terdapat pelbagai faktor seperti kenaikan harga minyak, isu-isu alam sekitar dan penggunaan kadar tinggi bahan api fosil, permintaan global untuk bioetanol telah menunjukkan peningkatan yang luar biasa. Pelepah kelapa sawit dipilih, kerana ia merupakan biojisim kelapa sawit yang paling banyak dihasilkan setiap tahun. Pelepah kelapa sawit (OPF) diperolehi semasa pemangkasan untuk penuaian buah tandan segar, dan jus yang boleh dihasilkan dengan menekan OPF segar menggunakan mesin tebu konvensional. Jus OPF mengandungi kandungan glukosa yang lebih tinggi, iaitu kira-kira 70% daripada jumlah gula bebas. Oleh itu, ia mempunyai potensi yang tinggi untuk menjadi sumber karbon untuk menghasilkan bioetanol. Kajian penyelidikan ini bertujuan untuk mengoptimumkan penghasilan bioethanol menggunakan jus pelepah kelapa sawit berasaskan kepada tiga parameter penting iaitu suhu, pH dan kelajuan kelalang goncang. Bioetanol dihasilkan dari jus pelepah kelapa sawit oleh penapaian dengan Saccharomyces cerevisiae. Kepekatan etanol daripada sampel penapaian dianalisis menggunakan HPLC. Menggunakan kepekatan etanol yang diperolehi daripada analisis HPLC, parameter optimum untuk penghasilan bioetanol ditentukan menggunakan Kaedah Respon Permukaan dengan bantuan perisian Design Expert. Hasil kajian menunjukkan, kepekatan bioetanol maksimum 22.93 g / L boleh didapati pada keadaan optimum berikut: suhu 32^{o}C, pH 6 dan kelajuan kelalang goncang 80 rpm. Keputusan pengesahan mendedahkan bahawa pada keadaan optimum ini, hasil bioetanol sebanyak 30.1%, yang berhampiran dengan nilai yang diramalkan, boleh diperolehi.

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LIST OF ABBREVIATIONS

CCD	Central Composite Design
CO_2	Carbon Dioxide
$C_6H_{12}O_6$	Glucose
C_2H_5OH	Ethanol
Elaeis guineensis Jacq.	Oil palm
EFB	Empty fruit bunch
OPF	Oil palm frond
OPT	Oil palm trunk
RSM	Response Surface Methodology

CHAPTER 1

INTRODUCTION

1.1 Background of Study

During the last few decades, the demand for alternative fuel resources has increased. Among the key factors driving a strong interest in these renewable energy sources are the ever rising fossil fuel prices, along with the growing demand for energy, and environmental concern due to excessive consumption of fossil fuels (Chin and H’ng,2013). It has been widely known that fossil fuels trigger environmental issues, such as greenhouse gas emissions. This tends to elevate the atmospheric temperature excessively (Hashem et al.,2013). Consequently, the renewable energy sources are sought after, to be utilized as biofuels. Biofuel refers to fuel energy which is derived from agricultural materials. Biofuels are divided into two groups based on the production technology. First generation biofuels are already being produced on a commercial scale, using plant materials as raw materials. However, this process may pose threat to food chain and biodiversity. Whereas, second-generation biofuels are still in R&D, pilot or lab scale, and utilize lignocellulosic biomass as raw materials. One such biofuel is bioethanol. Bioethanol is, by far, the most commercialized technology on the global market. To date, Brazil and the United States of America (USA) have already implemented mass production of bioethanol using sugarcane and corn as raw materials respectively.

1.2 Motivation and Problem Statement

Bioethanol is chemically known as ethyl alcohol with the chemical formula C_2H_5OH. It is produced from fermentation of simple sugars from plant sources using microbes. Bioethanol is biodegradable, low in toxicity and less likely to affect the environment. Among the advantageous properties of bioethanol as fuel energy include higher octane number (108), evaporation enthalpy, and flame speed and wider range of flammability. Other than that, it gives higher compression ratio (CR) with shorter burning time (Zabed et al.,2014). Bioethanol produces carbon dioxide (CO_2) and water when burned . This CO_2 is absorbed by the plant and at the same time, oxygen is released in the same volume. This proves to be advantageous over fossil fuels which emit CO_2 along with other toxic gases. Some bioprocesses have recommended possible routes to produce bioethanol in large volumes using low cost substrates (Gunasekaran and Raj, 1999). Late 1990, the concept of waste to wealth has been implemented. As for Malaysia, oil palm plantation and the palm oil industries contribute to the generation of most of the agricultural waste (Zahari et al., 2012). In 2008, Malaysia generated about 51 million tons of OPF, which accounts for 53% of the total palm biomass (Goh et al.,2010; MPOB,2009). Hence, OPF is a solid agrowaste which is available abundantly on oil palm plantations and usually disposed by directly left to decay or by burning on site, with only a small amount being composted. Bioethanol is obtained through the batch fermentation process using *Saccharomyces cerevisae.* Research by Zahari et al. (2012) suggests that OPF juice, which contains renewable sugars, could be a potential carbon source for bioethanol production using *S. cerevisea.* Hence, the optimum condition to produce bioethanol using OPF juice needs to be determined.

1.3 Objective

The objective of this research work is to determine the optimum temperature, pH and speed of agitation for bioethanol production from oil palm frond juice by yeast *S.cerevisae* with the use of Design Expert software.

1.4 Scope of the Research

The scope of this research is to produce bioethanol from oil palm frond juice and to optimize the production of bioethanol using Response Surface Methodology with the aid of Design Expert software. First of all, we need to obtain fermentation profile before measuring the final product concentration. Following that, we need to screen the effect of the parameters: temperature, pH and speed of agitation and finally, determine the optimum parameters in obtaining a high yield of bioethanol.

1.5 Main Contribution of This Work

The main contribution of this research is to determine the optimum condition for bioethanol production using OPF juice by using Response Surface Methodology with the aid of Design Expert software. The optimum condition obtained through this method may be applied in large-scale for mass bioethanol production.

1.6 Organisation of This Thesis

The structure of the reminder of the thesis is outlined as follows:

Chapter 1 provides the information on introduction of bioethanol. Findings that are related to optimum conditions to produce bioethanol using OPF juice are further discussed that gives rise to the motivation and problem statement of this research. This chapter also covers the objectives and scope of this research.

Chapter 2 gives a review on previous studies related to the research. This chapter provides the information about oil palm fronds, bioethanol and the factors affecting bioethanol production. The effects of all three factors are also discussed in this chapter.

Chapter 3 is a comprehensive description of the methodology for the research. This chapter covers explanations on the fermentation process, analysis method, and optimization method.

Chapter 4 provides the overall findings of the study with detailed description for each parameters studied and each analysis made. This chapter also covers the comparison of optimum conditions for bioethanol production using various raw materials.

CHAPTER 2

LITERATURE REVIEW

2.1 *Oil Palm Frond Juice as Renewable Energy Source*

Malaysia is the world's second largest palm oil producer, next to Indonesia. Since oil palm trees (*Elaeis guineensis* Jacq.) were introduced as a major crop from West Africa to Southeast Asian countries such as Malaysia, Indonesia and India, the increasing land area of oil palm plantation has been generating huge amounts of oil palm waste including trunks, fronds and empty fruit bunches (EFB) (Sun et al.,1999). In Malaysia alone, the quantity of oil palm waste production is estimated to be 115 million tons per annum. The oil palm fronds (OPF) represent approximately 83 million tons per year in available biomass from Malaysian plantations. This quantity, according to MPOC (2010), is 5.5 and 4.7 times larger than those of trunks and EFB in Malaysia, respectively.

OPF is a solid agricultural waste which is available abundantly on oil palm plantations (Goh et al.,2010). Currently, OPF is disposed by left to decay in the natural environment or by burning on site, with only a small amount being composted. This practice creates environmental problems, and better alternatives are needed to utilize or dispose OPF (Tan et al.,2011). Figure 2.1 shows the OPF that has been pruned.

Figure 2.1: Oil palm fronds after being pruned

OPF is currently under-utilized, since the plantation owners are having a misconception that all the OPF is necessary for nutrient recycling and soil conservation purposes (Hasan et al.,1994). Hence, the pruned fronds are just left in the plantation. However, a study by Zahari et al.(2012) proved that OPF does not contain high metal contents, but contain high carbohydrates in the form of simple sugars. Table 2.1 shows the metals composition in OPF, while Table 2.2 shows the sugars concentration in OPF juice.

Table 2.1: Nutrient and metallic elements in OPF and OPF juice

Analysis	Fresh OPF	OPF Juice
Nitrogen (%)	0.9	0.8
Carbon (%)	49	39
Organic Carbon (%)	37	29
Composition of nutrients and metal elements		
Sulphur (%)	0.2	0.4
Phosphorus (%)	0.02	0.02
Potassium (%)	0.2	2.3
Cadmium (%)	1.4	2.9
Magnesium(%)	0.2	0.5
Boron (ppm)	4	2
Manganese (ppm)	61	2
Copper (ppm)	2	2
Ferrum(ppm)	100	66

Table 2.2: Amount of sugars in OPF juice

Sugar(g/L)	
Fructose	1.68(±0.75)
Glucose	53.95(±2.86)
Sucrose	20.46(±1.56)
Total sugar	76.09(±2.85)

Production of sugars from dried OPF fibres has been reported by Fazilah et al.(2009) and Goh et al.(2010), which involves the conversion of cellulose and hemicellulose into glucose and xylose through hydrothermal treatment, followed by enzymatic hydrolysis. Other than that, research by Jung et al.(2012) suggested soaking in aqueous ammonia pretreatment. However, Zahari et al. (2012) recently found that renewable sugars can be obtained from OPF by simply pressing the fresh OPF using conventional sugarcane press machine to obtain the juice.

2.2 *Bioethanol*

The rising fossil fuel prices associated with growing demand for energy, and environmental concerns are some of the key factors driving a strong interest in utilizing renewable energy sources, particularly in biofuel. Bioethanol is one such biofuel, with the structural formula C_2H_5OH. Bioethanol is chemically known as ethyl alcohol and produced from fermentation of simple sugars from plant sources using microorganisms. Equation 2.1 shows the fermentation equation of glucose to ethanol.

$$C_6H_{12}O_6 \longrightarrow 2\ C_2H_5OH + 2\ CO_2 \qquad \text{(Equation 2.1)}$$

Bioethanol is a colourless liquid. It is biodegradable, low in toxicity and causes little environmental issues. In the 1970s, Brazil and the United States of America (USA) began their mass production of bioethanol from sugarcane and corn respectively. However, this is not applicable in long term, as the food chain could be affected due to decreasing food supply. Therefore, current interest lies in producing bioethanol from lignocellulosic materials.

The most common usage of bioethanol is to power automobiles. It can be combined with gasoline in any concentration up to pure ethanol (E100). Ethanol fuel blends are now widely available in the United States of America, Brazil, Europe and China. Today, bioethanol contributes around 3% of total road transport fuel globally on an energy basis (IEA,2010).

In Malaysia, the National Biomass Strategy 2020 was launched on year 2011 by the Malaysian government to promote the use of biofuel (AIM,2011) . Aiming to create higher value-added biomass economic activities that contribute towards Malaysia's gross national income (GNI), the Strategy outlined the production of bioethanol from lignocellulosic biomass, particularly oil palm biomass.

The cost of bioethanol depends on the raw materials that has been utilized. The cost of one litre of bioethanol produced from oil palm trunk is estimated to be at about RM 1.25/litre. The production cost from lignocellulosic materials is estimated at RM 0.26 .

2.3 *Factors Affecting Bioethanol Production*

2.3.1 *Temperature*

Temperature increases the rate of a reaction. The fermentation of ethanol at relatively high temperature is reported to be important for effective production in tropical countries, where average day-time temperatures are usually high throughout the year (Hashem et al.,2013) . The advantages of rapid fermentation at high temperatures are not only to reduce contamination, but also to reduce the cooling cost. However, yeast are greatly affected by temperature. Temperature affects yeast metabolism.

2.3.2 *pH*

The pH of a solution can have several effects of the enzymatic structure and activity. Changes in pH affects the shape of an enzyme, structure of an enzyme, and properties of the substrate, so that either the substrate cannot bind to the active site or it cannot undergo catalysis.

2.3.3 *Speed of Agitation*

Agitation speed is one of the factors which will affect the amount of dissolved oxygen in the cultivation medium along the fermentation process. Agitation rate is important for adequate mixing, mass transfer and heat transfer because it assists mass transfer between different phases present in the culture.

CHAPTER 3

METHODOLOGY

3.1 *Medium and Reagent Preparation*

3.1.1 *Medium Preparation*

Three types of mediums such as nutrient agar (NA) , nutrient broth (NB) and oil palm frond juice are used in this research work.

The nutrient agar (NA) that is used for this research work is the Yeast Extract Peptone Dextrose (YPD) agar. The agar can be prepared by using 20 g peptone, 20 g dextrose, 10 g yeast extract and 15 g agar. First of all, the agar powder is mixed with 900 mL of purified water in 1 Liter Schott bottle. Next, 5 g of glucose is mixed with 100 mL of distilled water in a 250 ml flask and covered with aluminium foil, before being autoclaved for 20 minutes at 121^{o}C. After autoclave, glucose is added to prevent Maillard reaction from occurring. After temperature has dropped to below 90^{o}C, agar is poured into Petri plates and left to solidify. All the plates are closed and sealed before stored in refrigerator at 4^{o}C.

The nutrient broth (NB) is prepared in a similar way, except that the agar powder not to be added. After autoclave, glucose is added in the broth and the solution is mixed well before being refrigerated at 4^{o}C.

3.1.2 *Preparation of OPF Juice*

The oil palm fronds are obtained from Felda Lepar Hilir, Kuantan, Pahang. The OPF juice is extracted by pressing the oil palm frond using the conventional sugarcane pressing machine. The OPF juice was then stored at -20^{o}C before use.

3.2 *Microorganism and Medium*

3.2.1 *Pure Culture Preparation*

The yeast that is used for this research work is *Saccharomyces cerevisiae* Kyokai no.7 (ATCC 26422). The yeast was dissolved in YPD broth and incubated for 1 day at 100 rpm and 30^{o}C to form yeast suspension. This suspension was used to streak on new agar plate and incubate for 2-3 days at 30^{o}C. The strain was stored at 4^{o}C.

3.2.2 *Inoculum Preparation*

About 3-4 loops were taken from the pure culture and transferred into YPD broth in shake flask. The strains were incubated for 12-18 hours at 30^{o}C and 150 rpm until reach standard initial concentration (0.2-0.4 g/L).

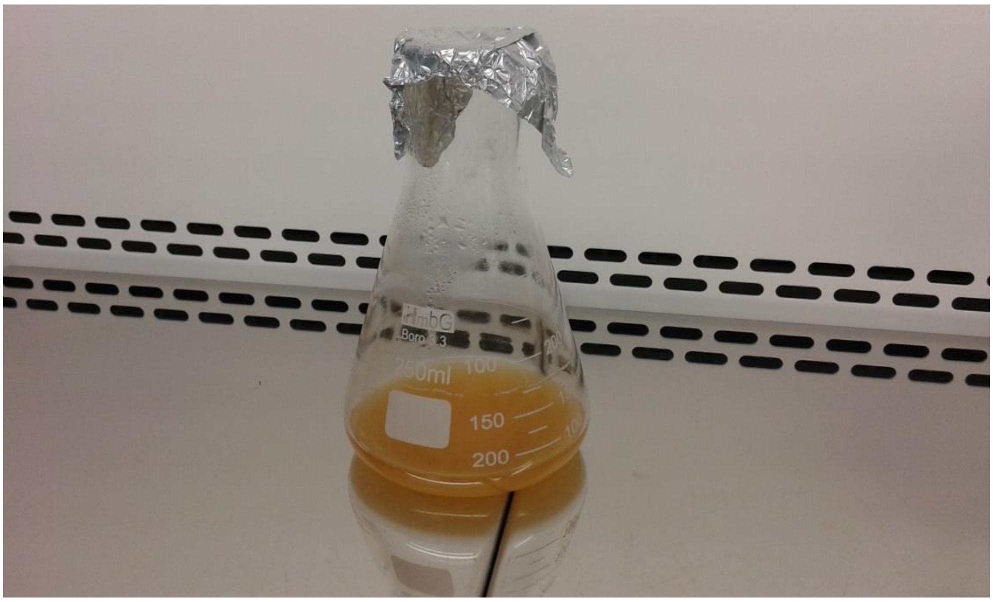

Figure 3.1 : Preparation of inoculum for fermentation

3.3 *Fermentation*

3.3.1 *Preparation of OPF Juice for Fermentation*

OPF juice was filtered and centrifuged for 30 minutes at 5000 rpm and 4^{o}C. The OPF juice was then transferred to a shake flask and pH adjustment was carried out by the addition of H_2SO_4 and NaOH using pH meter. Following that, the shake flask was covered and autoclaved for 15 minutes at 121^{o}C.

3.3.2 *Preparation of Fermentation Profile*

10% (v/v) of inoculums suspension from activated yeast flask was transferred into the sterilized OPF juice. Total working volume for each flask was kept constant at 100 mL for every run. Then, the shakeflask was purged with nitrogen gas, before being placed in incubator shaker at preferred setting. After 24 hours, 5 mL sample was collected and centrifuged at 10,000xg for 30 minutes at 4^{o}C (Thermo Fisher Scientific, NC, USA). The supernatant was then filtered using Whatman 0.22µm syringe filters into vial for HPLC analysis.

Figure 3.2 : Fermentation set-up

3.3.3 *Optimization of Parameter using Central Composite Design (CCD)*

Batch fermentation was used for the optimization of bioethanol production. The investigated parameters were temperature, pH and speed of agitation. The variable and the selected levels for the fermentation process were: pH (6-8); temperature (25-35^{o}C); agitation speed (50-100 rpm) as shown in Table 1. The range selected for the bioethanol fermentation from the OPF juice was determined according to the previous research by Nasarudin (2013).

Table 3.1: The experimental region for optimization of parameter

Independent variable	**Symbol**	**Variation levels**				
		-α	-1	0	+1	+α
Initial pH	A	5.5	6.0	7.0	8.0	8.5
Temperature (°C)	B	22	25	30	35	38
Agitation speed (rpm)	C	35	50	75	100	120

The experimental plan was generated using the Design Expert Version 7.1.6 software as shown in Table 3.2. The best combination condition suggested by the design program was validated by performing fermentation in triplicate according to the suggested parameters.

Table 3.2: Fermentation conditions for the fermentation parameter optimization

Standard Run	**Factor 1 A (Initial pH)**	**Factor 2 B (Temperature)**	**Factor 3 C (Agitation speed)**
1	6	25	50
2	8	25	50
3	6	35	50
4	8	35	50
5	6	25	100
6	8	25	100
7	6	35	100
8	8	35	100
9	5.5	30	75

10	8.5	30	75
11	7	22	75
12	7	38	75
13	7	30	35
14	7	30	120
15	7	30	75
16	7	30	75
17	7	30	75
18	7	30	75
19	7	30	75
20	7	30	75

3.4 *Methods of Analysis*

3.4.1 *High Performance Liquid Chromatography (HPLC)*

HPLC (Agilent Series 1200, USA) was used to analyse the concentration of sugar in OPF juice and ethanol content in fermentation samples. The mobile phase was acetonitrile: water (75%:25%) at a flow rate of 1.0 mL/min. The loop of injection was optimized for 10 μL injection volume. The specification for HPLC analysis for product analysis is shown in Table 3.3.

Table 3.3: Specification for HPLC for product analysis

Column	Supelcosil LC-NH2
Mobile phase	75% acetonitrile: 25% water
Standard preparation	5 g/l, 10 g/l, 15 g/l, 20 g/l and 25 g/l for each sample
Flow rate	1.0 mL/min
Injection volume	10 μL

3.4.2 *Response Surface Methodology (RSM)*

RSM consists of a set of statistical and mathematical methods which can be used to develop, improve or optimize process. RSM uses an experimental design to fit a model by least squares technique. This design must be completed by integrating experimental design with interpolation of equations in a series testing procedure (Han et al., 2011). The main advantage of RSM is the decreased amount of experimental trials required to assess various parameters and also their interactions (Karacan et al., 2007). Furthermore, even in the presence of complex interactions, RSM can be employed to analyze the relative significance of multiple affecting factors. RSM normally consists of three steps; design and experiments, followed by response surface modeling through regressions and finally optimization (Sharma et al., 2009).

Optimization of the fermentation process using RSM has been used to enhance productivity without increasing cost.Among the types of design under RSM are Central Composite Design (CCD), Box-Behnken design (B-B), One Factor design and D-optimal design. In this study, CCD was used as a method for the optimization of parameters on production of bioethanol from OPF juice.

3.4.3 *Central Composite Design (CCD)*

Central composite design (CCD) is a famous second order experimental design. The CCD is known as successful design that is used for any sequential experimentation and supply rational number of information for testing the quality of fit and not necessarily require unusually huge amount of design points thereby reducing the overall cost associated with the experiment (Korbhati et al., 2007).

CCD is an appropriate design for sequential experiments to obtain suitable information regarding lack of fit testing without a huge number of design points (Myers and Montgomery, 1996). The advantages of using CCD are easy to identify the effects of factors, optimum value determination, facilitate system modeling and offer higher precision. The important factors at different levels also can be decided by CCD design by comparing the results between predicted and experimental which can prove the accuracy and applicability of the model. This situation indicates that CCD-RSM is an effective technique to optimize the experiment (Bandaru et al., 2006).

CCD has been known by using three set of experimental runs, firstly, fractional factorial runs in which factors are studied +1, -1. Secondly, center points with all factors at their center points which help in understanding the curvature and replication helps to estimate pure error. It is followed by axial points which is similar to center points but one factor takes values above and below the median of two factorial levels typically both outside their range. The design is rotatable from its axial point (Sharma et al., 2009).

The whole fermentation process for bioethanol production can be summarized in Figure 3.3.

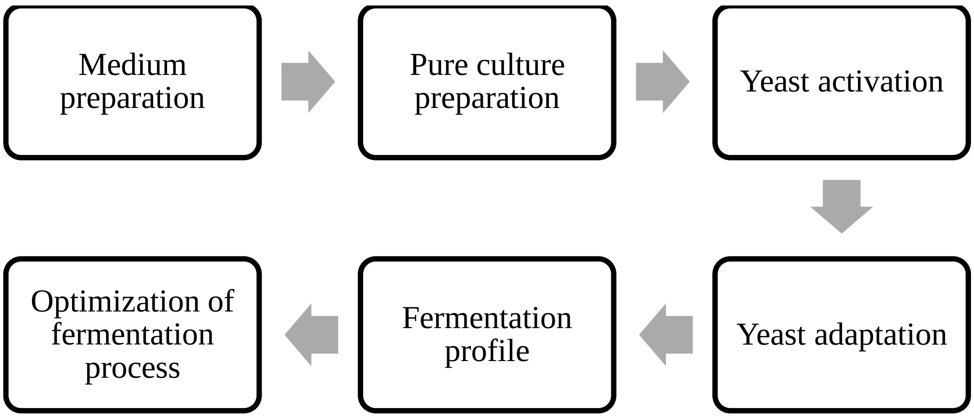

Figure 3.3: The method of fermentation process for bioethanol production

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 *Sugar Composition of OPF Juice*

Table 4.1 shows the three major types of sugars (glucose, sucrose, and fructose) found in OPF juice with their respective concentration (g/L). Glucose shows the highest percentage, followed by sucrose and fructose. These sugars are proven to be among some of the common sugars favourable for yeast consumption.

A previous study by Zahari et al. also showcased similar pattern, whereby glucose is the dominant sugar, followed by sucrose and fructose. However, the percentages of sugars composition obtained from the study were different from this study. This difference may be caused by several factors such as the way of handling OPF juice after pressing, and difference in the oil palm tree itself.

Table 4.1: Sugar composition in oil palm frond (OPF) juice

Sugar	**Amount of sugar (g/L)**	**Percentage (%)**
Glucose	99.44	66.74
Sucrose	40.70	27.32
Fructose	8.85	5.94
Total	148.99	100

4.2 *Optimization of Bioethanol Production using CCD*

4.2.1 *Statistical Analysis of Model*

Laboratory experiments were conducted in accordance with the conditions provided by Design Expert software using the optimized values of the variables as presented in Table 4.2. The concentration of bioethanol produced from each run was determined through HPLC analysis, and the results are presented in Table 4.3.

Table 4.2: Actual and coded values of the design variables

Independent variable	Symbol	Variation levels				
		-α	-1	0	+1	+α
Initial pH	A	5.5	6.0	7.0	8.0	8.5
Temperature (°C)	B	22	25	30	35	38
Agitation speed (rpm)	C	35	50	75	100	120

Table 4.3 : Bioethanol produced by *Saccharomyces cerevisae* in batch culture based on Response Surface Methodology

Std	Run	Factor 1 A: pH	Factor 2 B: Temperature (^{o}C)	Factor 3 C: Agitation speed (rpm)	Response Bioethanol (g/L)
1	5	6.0	25	50	18.76
2	17	8.0	25	50	10.67
3	2	6.0	35	50	18.98
4	6	8.0	35	50	10.98
5	18	6.0	25	100	19.05
6	8	8.0	25	100	10.66
7	3	6.0	35	100	18.66
8	15	8.0	35	100	10.78
9	12	5.5	30	75	19.9
10	19	8.5	30	75	9.14
11	7	7.0	22	75	16.76

12	10	7.0	38	75	15.48
13	9	7.0	30	35	16.43
14	11	7.0	30	120	14.78
15	1	7.0	30	75	21.35
16	14	7.0	30	75	22.45
17	13	7.0	30	75	22.79
18	20	7.0	30	75	22.08
19	4	7.0	30	75	21.87
20	16	7.0	30	75	22.54

Optimization using CCD design revealed that the resulted analysis of variance or ANOVA suggested that the quadratic model is the most appropriate model to describe the interaction between the factors. Table 4.4 shows the summary of ANOVA or analysis of variance.

Table 4.4 : Analysis of variance (ANOVA) summary

Source	Sum of Squares	Mean Square	F value	P- value Prob > F
Model	403.36	44.82	87.13	< 0.0001
A	186.41	186.41	362.42	< 0.0001
B	0.26	0.26	0.51	0.4915
C	0.67	0.67	1.29	0.2818
AB	0.045	0.045	0.087	0.7734
AC	4.050E-003	4.050E-003	7.874E-003	0.9310
BC	0.080	0.080	0.16	0.7016
A^2	108.80	108.80	211.53	< 0.0001
B^2	68.62	68.62	133.40	< 0.0001
C^2	80.54	80.54	156.59	< 0.0001
Lack of Fit	3.77	0.75	2.76	0.1451
Pure Error	1.37	0.27	-	-

According to Table 4.4, the model F-value of 87.13 implies that the model is significant. There is only a 0.01% chance that a "Model F-value" this large could occur due to noise. It has been stated by Kinley (1999) that the values of "Prob >F" less than 0.0500 indicate model terms are significant, while values greater than 0.1000 indicate the model terms are not significant. Therefore, in this research, A, A^2, B^2, and C^2 are considered as significant model terms. The "Lack of Fit F-value" of 2.76 implies that the Lack of Fit is not significant, relative to the pure error. There is a 14.51% chance that a "Lack of Fit F-value" this large could occur due to noise. A non-significant Lack of Fit F-value is considered good, since it represents the fitness of the model.

Moreover, the significant terms contributed to a quadratic model in terms of coded factor and actual factor. For coded factor,

$$\text{Bioethanol (g/L)} = 22.19\text{-}3.69A\text{-}2.75A^2\text{-}2.18B^2\text{-}2.36C^2$$

While for the actual factors,

$$\text{Bioethanol (g/L)} = -184.05 + 34.39A\text{-}2.75A^2\text{-}0.09B^2\text{-}3.78E\text{-}003C^2$$

Apart from that, the R- squared value of 0.9874 suggests that the model can precisely demonstrate the effect of the variables towards bioethanol production. The value of R-squared (R^2) gives the measure of changeability in the attained results of bioethanol that can be demonstrated by the studied parameters and the relationships between them (Jatinder et al.,2006) . The closeness of the R-squared value to one indicates that the model gives a convincing good estimate of bioethanol production (Babu and Satyanarayana, 1995).

4.2.2 *Interaction among Factors Influencing Fermentation Process and Production of Bioethanol*

The empirical predicted quadratic model for response (bioethanol production) in terms of process variables (pH, temperature, and agitation speed) are plotted in contour plots and three-dimensional diagrams to investigate the interaction among the variables and to determine the optimum condition of each factor for maximum bioethanol concentration.

Figure 4.1 shows the three-dimensional surface plot which represents the effects of varying pH and temperature on bioethanol concentration at constant agitation speed of 80 rpm. Based on the plot, the maximum bioethanol concentration is obtained at pH value 6.2 and temperature 32^{o}C.

Figure 4.2 shows the three-dimensional surface plot which represents the effects of varying pH and agitation speed on bioethanol concentration at constant temperature of 32^{o}C. Based on the plot, the maximum bioethanol concentration is obtained at pH value 6.2 and agitation speed 78 rpm.

Figure 4.3 shows the three-dimensional surface plot which represents the effects of varying temperature and agitation speed on bioethanol concentration at constant pH of 6.2. Based on the plot, the maximum bioethanol concentration is obtained at temperature 32^{o}C and agitation speed 78 rpm.

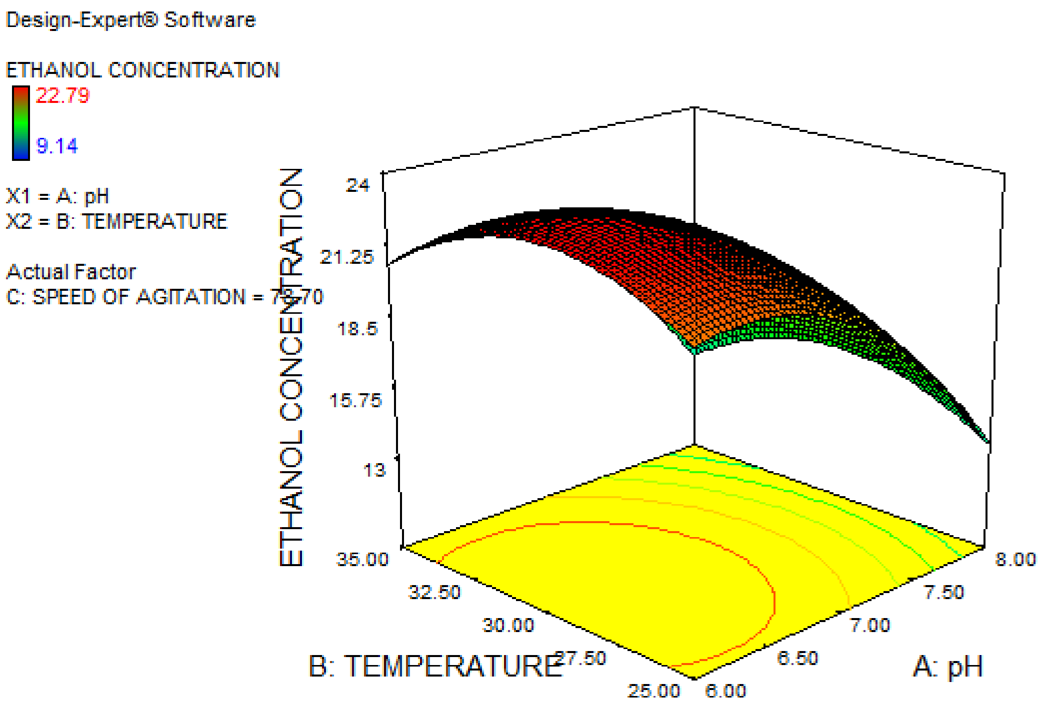

Figure 4.1 : Three-dimensional surface plot of the interaction between pH and temperature

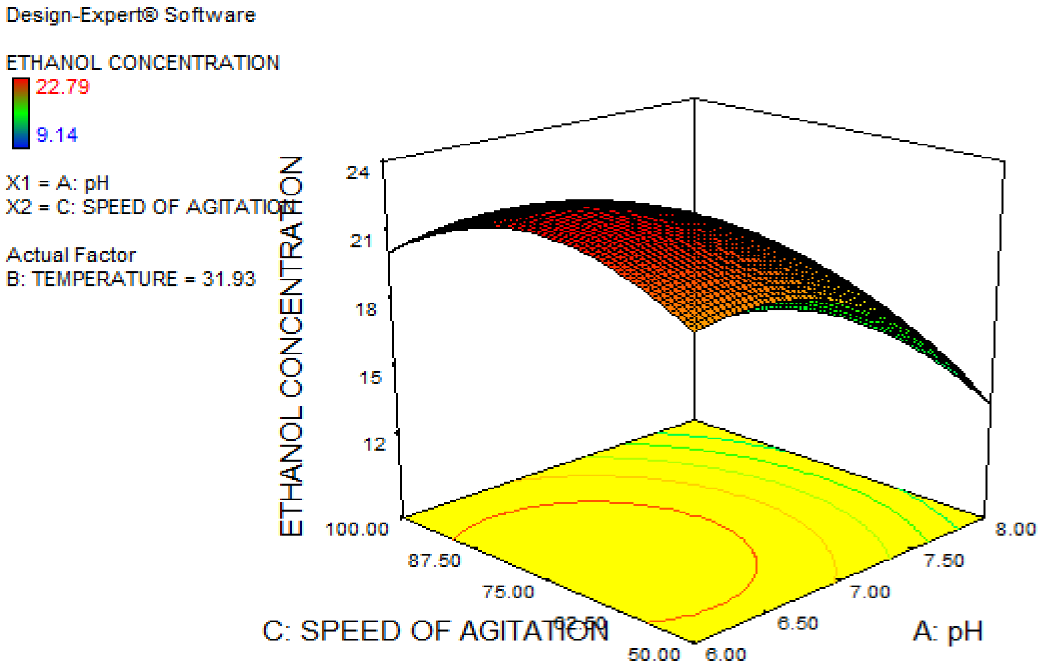

Figure 4.2 : Three-dimensional surface plot of the interaction between pH and agitation speed

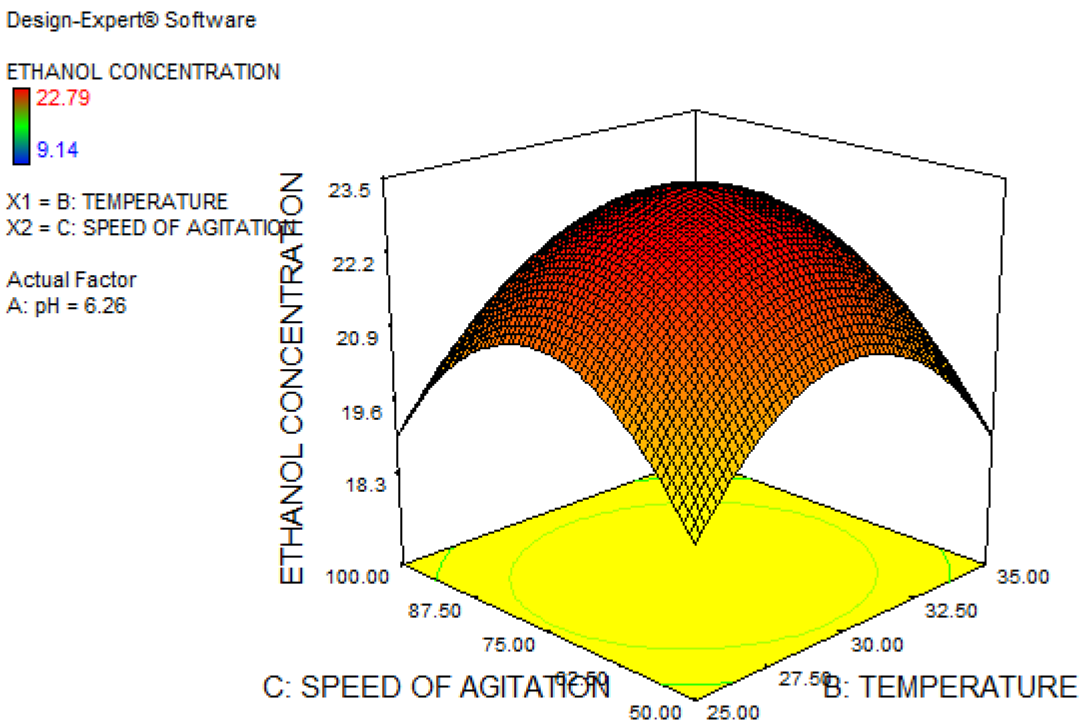

Figure 4.3 : Three-dimensional surface plot of the interaction between temperature and agitation speed

4.2.3 *Optimum Condition for Bioethanol Production*

Numerical optimization was carried out to determine the conditions which gave the maximum bioethanol concentration. Based on the results obtained, a maximum bioethanol concentration of 22.93 g/L is predicted to be obtained at pH value 6.0, temperature 32^{o}C and agitation speed 80 rpm. These optimum conditions are then used to carry out validation experiments. The bioethanol obtained are tabulated in Table 4.5.

Table 4.5: Bioethanol concentration and yield from validation experiments

Run	Bioethanol concentration (g/L)	Bioethanol yield (%)
1	23.07	30.3
2	22.89	30.1
3	22.74	29.9

The bioethanol yield was calculated as conversion using the formula as mentioned in the study by Chin et al.(2010).

$$Bioethanol\ yield\ (\%) = \frac{Bioethanol\ concentration\ (\frac{g}{L})}{Total\ sugar\ concentration\ \left(\frac{g}{L}\right) \times 0.511} \times 100$$

The reproducibility of a model is tested by performing fermentation under the suggested optimal conditions from CCD. The validation is also used as a verifying method of the accuracy of the model. The predicted bioethanol concentration under the optimized condition was 22.93 g/L. From the validation experiments, a maximum bioethanol concentration of 23.07g/L, 22.89 g/L, and 22.74 g/L were obtained respectively. Based on the results, the bioethanol yield obtained is 30.1%. These experimental findings are in close agreement with the model prediction, and hence the model developed by RSM could reliably predict bioethanol yields. The difference between experimental and predicted values of less than 10% confirm the validity of a model (Levin et al.,2008).

4.3 *Comparison of Optimum Conditions for Bioethanol Production*

4.3.1 *Optimum Conditions for Bioethanol Production using Different Raw Materials*

There are many other research works which focused on determining the optimum conditions for bioethanol production. Various raw materials are considered for the comparison. Table 4.6 shows the comparison between different raw materials.

Table 4.6 : Comparison of Optimum Conditions for Bioethanol Production for Various Raw Materials

Raw Material	Optimum Condition	Research by
Lignocellulosic hydrolysates	Temperature: 33.2^{o}C pH:5.3	Chin et al. (2010)
Sugarcane bagasse	Temperature: 35^{o}C pH:4.5	Wong and Sanggari (2014)
Nypa sap	Temperature: 30^{o}C pH:5.4 Agitation speed: 200 rpm	Natarajan and Sharmila Dewi (2012)
Cashew apple juice	Temperature: 30^{o}C pH: 4.5 Agitation speed: 150 rpm	Deenanath et al. (2013)

According to Table 4.6, the optimum temperature for bioethanol production ranges between 30^{o}C to 35^{o}C, which depends on room temperature. However, a much elevated temperature inhibits the cell growth and thus causes the fermentation process to decline. The high temperature may also result in denaturation of ribosomes and enzymes (Phisalaphong et al.,2006).

The optimum pH range for bioethanol production is between 4 to 6, which is slightly acidic. This matches with the optimum pH of this study, which is 6.0. Yeast survives in a slightly acidic environment. However, at alkaline condition, which is at a pH value of more than 7, the concentration of bioethanol produced declines, proving that a slightly acidic condition is suitable to obtain a high bioethanol yield.

CHAPTER 5

CONCLUSION AND RECOMMENDATION FOR FUTURE WORK

5.1 *Conclusion*

In conclusion, this project serves to enhance the research and development of the biofuel energy sources towards the sustainability of energy. In this context, the oil palm frond juice is gaining much significance as one of the alternative renewable energy source, in view with the worldwide depletion of petroleum and natural gas.

This project mainly focused on determining the optimum condition for bioethanol production with the aid of Design Expert software. The three parameters involved are pH, temperature and agitation speed. Based on the optimization of results using Central Composite Design, the optimum condition for bioethanol production using oil palm frond juice was successfully generated. The maximum bioethanol at 22.93 g/L is produced at pH value 6.0, temperature 32^{o}C and agitation speed 80 rpm. Laboratory experiments based on the optimum value for the variables suggested by the model was conducted and results obtained through the work proved that the model is reliable in predicting the optimum conditions for bioethanol production. The study concluded that CCD is a useful and promising technique for optimization of parameters towards the production of bioethanol.

5.2 *Recommendation for Future Work*

First and foremost, it is highly recommended to use the High Performance Liquid Chromatography or HPLC for bioethanol analysis in order to get more accurate analysis. Early booking should be made for the equipment and any failure problem may affect the accuracy of the results obtained.

Besides that, the oil palm frond should be fresh and not too dry during sugar extraction process. This is highly recommended since the total sugar content in OPF juice might decrease if the oil palm frond is not stored in a proper way.

Apart from that, in order to prevent errors, the method should be followed accordingly and all the measurements made are done correctly. Since the main parameter that needs to be optimized is related to measurement technique, a small change might affect the whole experimental result.

Lastly, the optimum condition determined in this study can be applied in large scale for bioethanol production. This can be done by scaling up in order to produce the desired amount of bioethanol.

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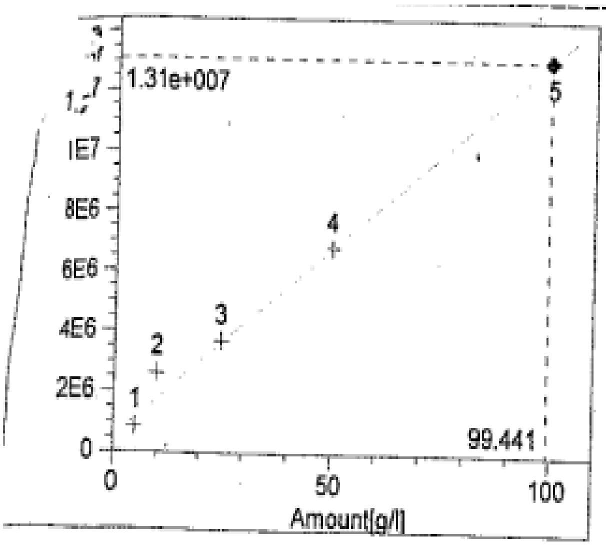
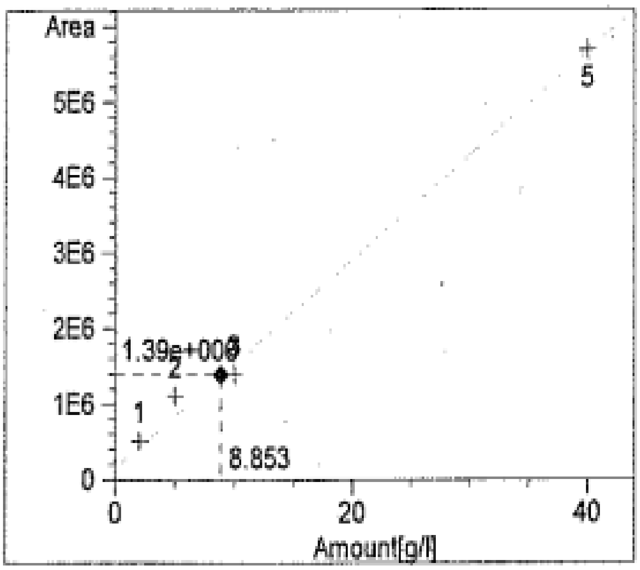
APPENDICES

Calibration curve for Glucose in OPF juice



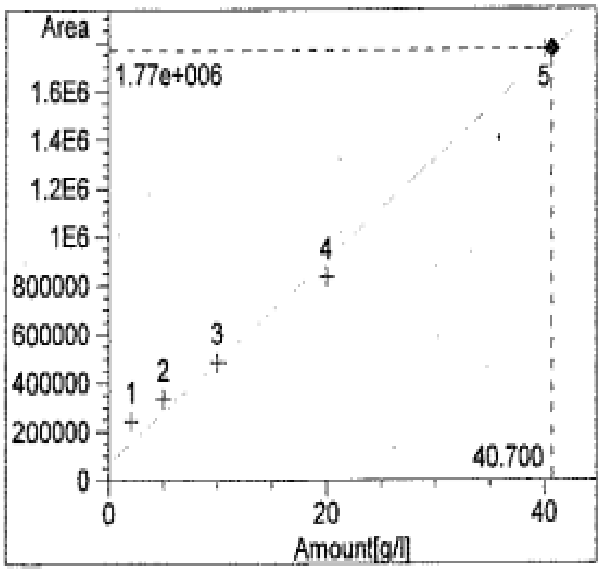
Glucose at exp. RT: 10.473
RID1 A, Refractive Index Signal
Correlation: 0.99544
Residual Std. Dev.: 514992.24247
Formula: y = mx + b
m: 126408.25709
b: 505727.30688
x: Amount
y: Area

Calibration curve for Fructose in OPF juice



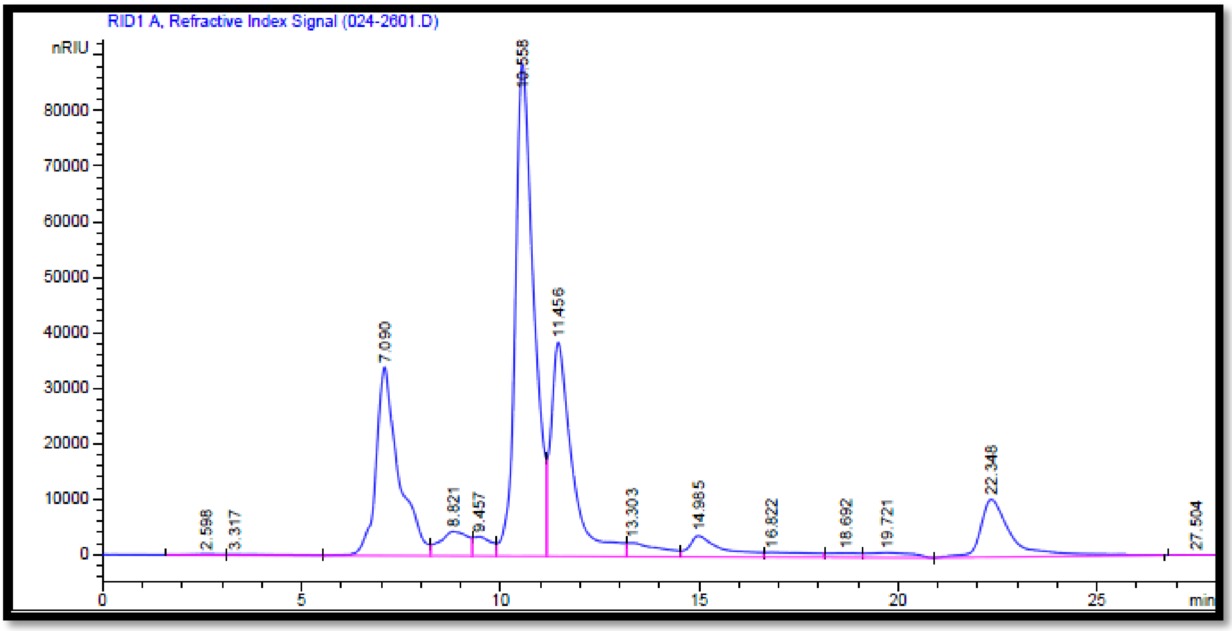
Fructose at exp. RT: 11.311
RID1 A, Refractive Index Signal
Correlation: 0.99705
Residual Std. Dev.: 200924.14421
Formula: y = mx + b
m: 137726.22900
b: 166840.97689
x: Amount
y: Area

Calibration curve for Sucrose in OPF juice



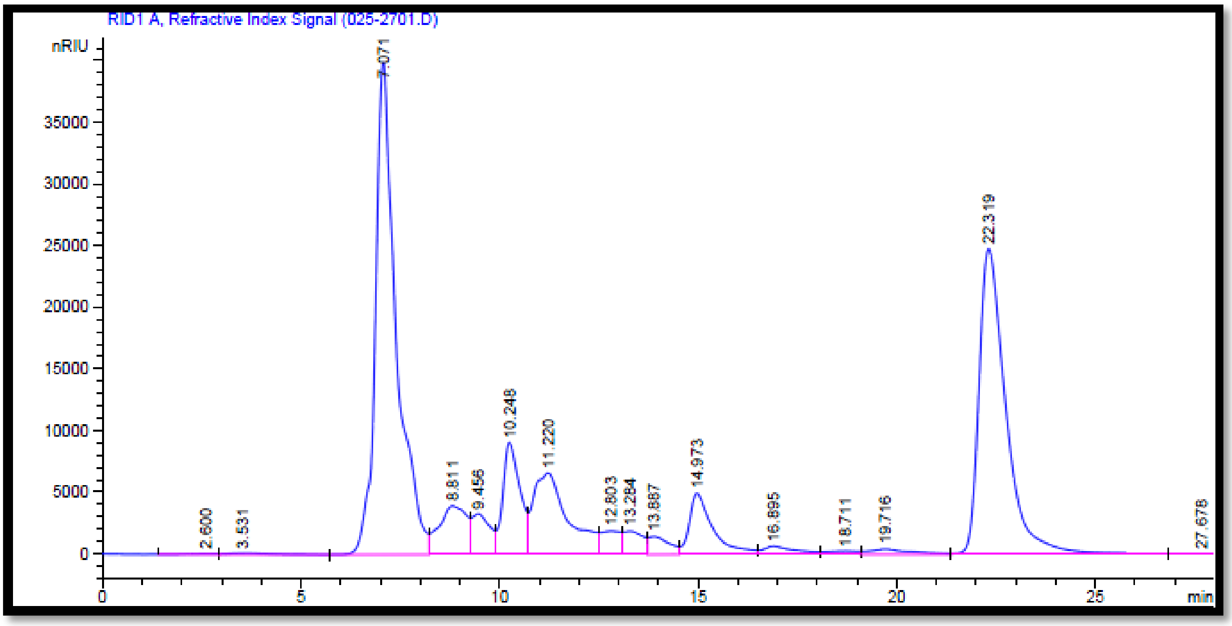
at exp. RT: 8.791
RID1 A, Refractive Index Signal
Correlation: 0.99458
Residual Std. Dev.: 73373.40993
Formula: y = mx + b
m: 41545.11743
b: 77163.13102
x: Amount
y: Area

Sample 1



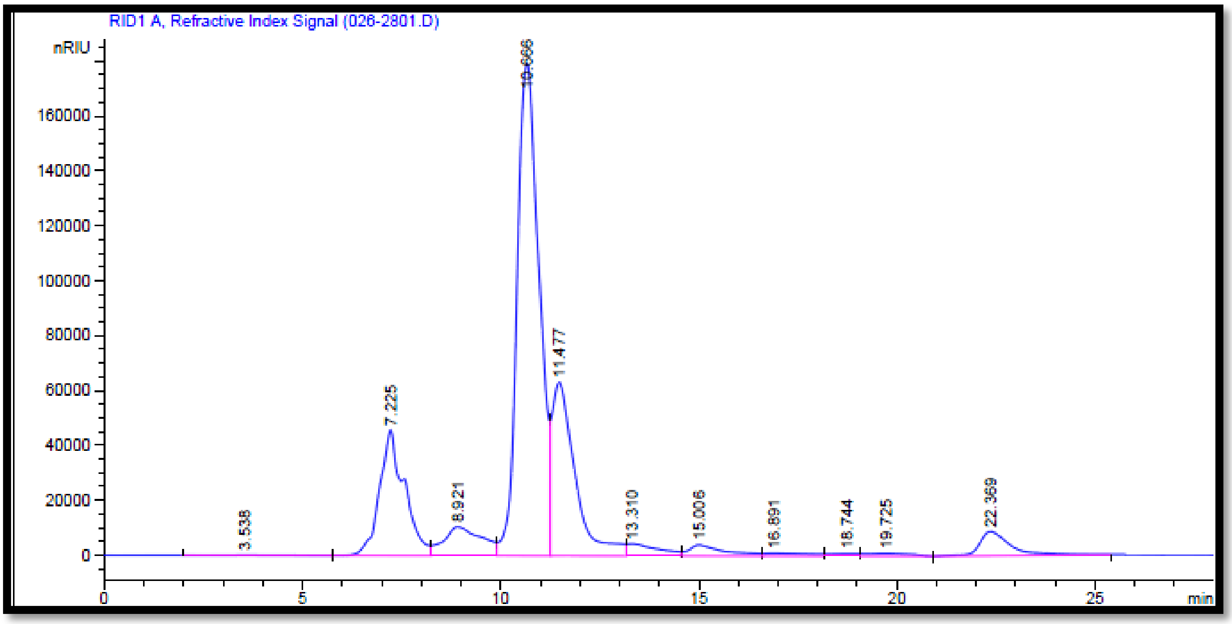
Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	2.598	BV	0.8419	7863.08154	140.73648	0.1103
2	3.317	VV	1.3027	1.40910e4	134.21007	0.1976
3	7.090	VV	0.5614	1.35495e6	3.38968e4	19.0013
4	8.821	VV	0.7217	2.16330e5	4390.49463	3.0337
5	9.457	VV	0.4437	1.08417e5	3456.64746	1.5204
6	10.558	VV	0.4740	2.87839e6	8.86253e4	40.3656
7	11.456	VV	0.5341	1.44768e6	3.84664e4	20.3017
8	13.303	VV	0.7691	1.40716e5	2435.68994	1.9734
9	14.985	VV	0.7672	2.13707e5	3757.80103	2.9969
10	16.822	VV	1.0577	6.92016e4	838.64996	0.9705
11	18.692	VV	0.8282	4.20392e4	748.34320	0.5895
12	19.721	VV	1.0494	6.61509e4	847.02539	0.9277
13	22.348	VB	0.7950	5.66261e5	1.03308e4	7.9411
14	27.504	BBA	0.9310	5010.97705	69.93696	0.0703
Totals :				7.13081e6	1.88139e5	

Sample 2



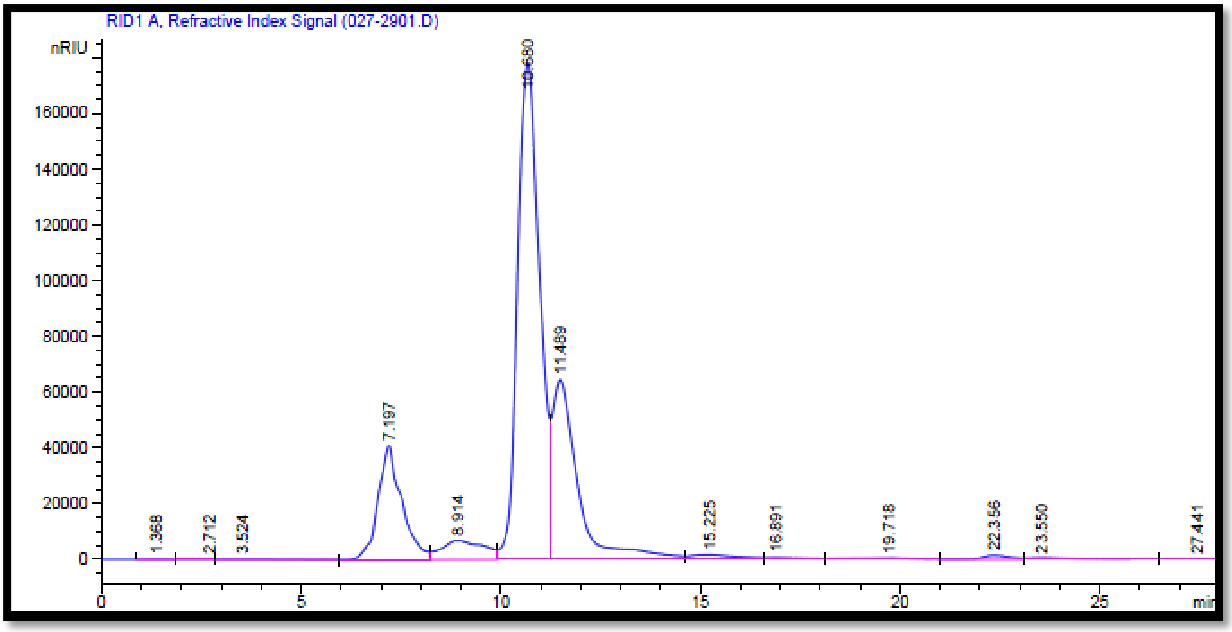
Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	2.600	BV	0.6026	2496.02734	51.26742	0.0611
2	3.531	VV	0.9381	7177.24365	96.35491	0.1758
3	7.071	VV	0.5135	1.45998e6	3.98812e4	35.7511
4	8.811	VV	0.7114	1.90417e5	3935.59082	4.6628
5	9.456	VV	0.4525	1.03340e5	3293.27661	2.5305
6	10.248	VV	0.4428	2.77407e5	9080.04297	6.7930
7	11.220	VV	0.8037	4.01351e5	6607.63574	9.8280
8	12.803	VV	0.4602	6.20469e4	1893.01758	1.5194
9	13.284	VV	0.5094	6.68009e4	1880.62109	1.6358
10	13.887	VV	0.5122	5.28544e4	1448.04797	1.2943
11	14.973	VV	0.5857	2.03805e5	4933.28711	4.9907
12	16.895	VV	0.7459	3.76645e4	675.13690	0.9223
13	18.711	VV	0.7444	1.46835e4	263.79324	0.3596
14	19.716	VV	0.9784	3.11886e4	428.66345	0.7637
15	22.319	VV	0.6974	1.16763e6	2.47462e4	28.5922
16	27.678	VBA	0.7486	4888.83496	88.43987	0.1197
Totals :				4.08373e6	9.93025e4	

Sample 3



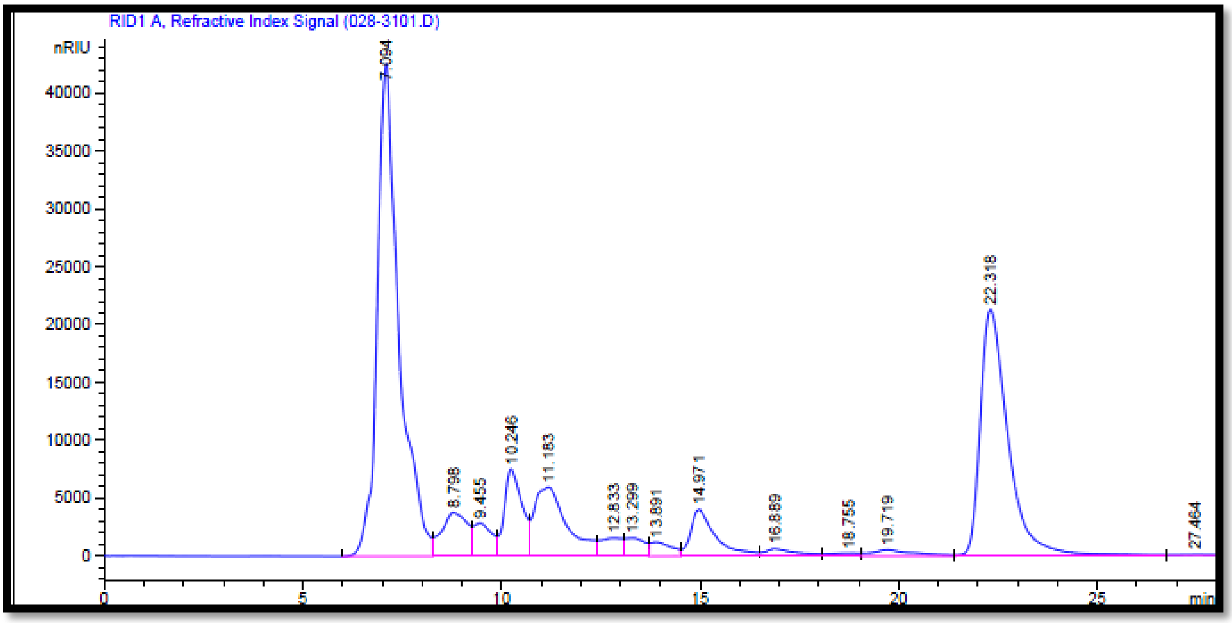
Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	3.538	BV	1.5172	1.11613e4	90.46073	0.0834
2	7.225	VV	0.5710	1.99689e6	4.56308e4	14.9288
3	8.921	VV	0.9111	6.97776e5	1.04162e4	5.2166
4	10.666	VV	0.5806	6.92120e6	1.79048e5	51.7431
5	11.477	VV	0.5731	2.58205e6	6.30166e4	19.3035
6	13.310	VV	0.7475	2.38319e5	4318.87695	1.7817
7	15.006	VV	0.8512	2.43880e5	3896.08423	1.8233
8	16.891	VV	1.0519	7.93919e4	994.97186	0.5935
9	18.744	VV	0.7453	4.22627e4	800.87518	0.3160
10	19.725	VV	1.0602	7.54555e4	963.88422	0.5641
11	22.369	VB	0.8118	4.87701e5	8786.07227	3.6461
Totals :				1.33761e7	3.17963e5	

Sample 4



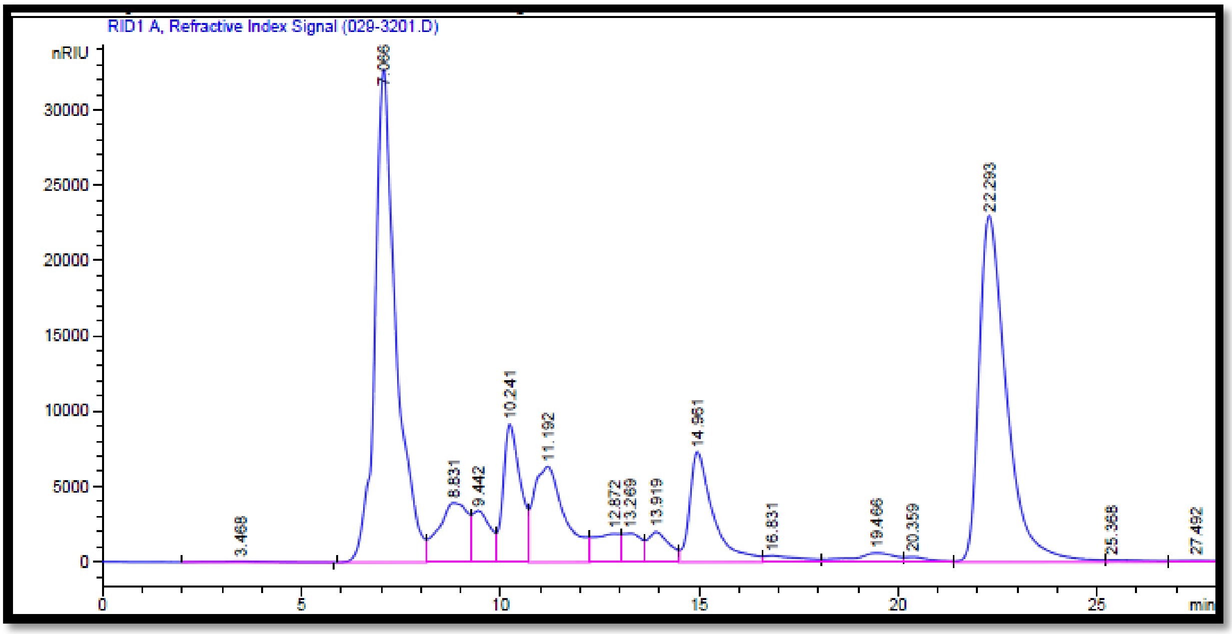
Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	1.368	BV	0.6341	1073.08008	22.50278	8.755e-3
2	2.712	VV	0.5838	1745.28503	36.49879	0.0142
3	3.524	VV	1.0259	5962.59229	70.16785	0.0486
4	7.197	VV	0.5427	1.68086e6	4.06663e4	13.7136
5	8.914	VV	1.0092	4.97245e5	6727.35254	4.0568
6	10.680	VV	0.5782	6.83137e6	1.77643e5	55.7348
7	11.489	VV	0.6242	2.87155e6	6.43595e4	23.4280
8	15.225	VV	1.0866	1.35748e5	1640.05994	1.1075
9	16.891	VV	0.9242	4.34257e4	617.41718	0.3543
10	19.718	VV	1.5014	5.66832e4	485.11728	0.4625
11	22.356	VV	0.7554	6.88547e4	1378.77905	0.5618
12	23.550	VV	1.2857	5.26726e4	547.92310	0.4297
13	27.441	VBA	1.0511	9732.93262	114.62860	0.0794
Totals :				1.22569e7	2.94309e5	

Sample 5



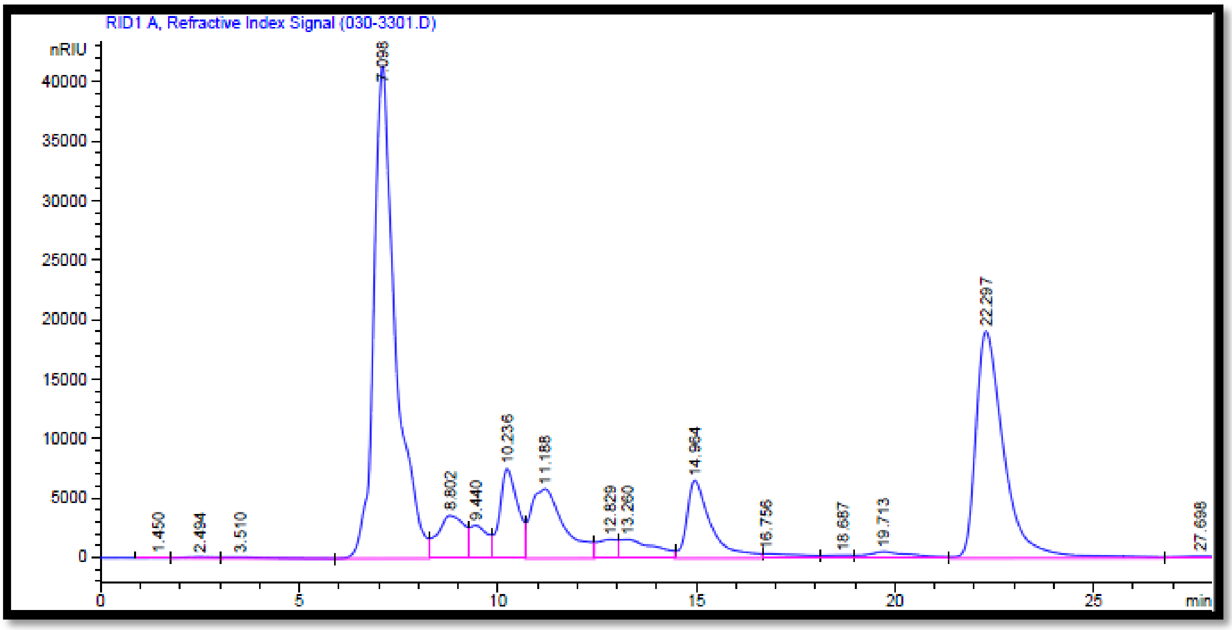
Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	7.094	BV	0.5407	1.62410e6	4.25134e4	41.1328
2	8.798	VV	0.6763	1.71723e5	3784.23096	4.3491
3	9.455	VV	0.4544	9.06559e4	2874.32788	2.2960
4	10.246	VV	0.4607	2.35993e5	7529.79541	5.9769
5	11.183	VV	0.7646	3.42502e5	5967.65430	8.6744
6	12.833	VV	0.5339	5.90220e4	1569.21289	1.4948
7	13.299	VV	0.4959	5.50889e4	1569.49426	1.3952
8	13.891	VV	0.5367	4.59596e4	1213.91125	1.1640
9	14.971	VV	0.6159	1.76191e5	4012.74756	4.4623
10	16.889	VV	0.7762	3.59815e4	640.66949	0.9113
11	18.755	VV	0.7528	1.41241e4	268.13895	0.3577
12	19.719	VV	1.0141	4.13884e4	545.91473	1.0482
13	22.318	VV	0.7378	1.04734e6	2.12995e4	26.5254
14	27.464	VBA	0.8426	8358.01855	120.66882	0.2117
Totals :				3.94842e6	9.39097e4	

Sample 6



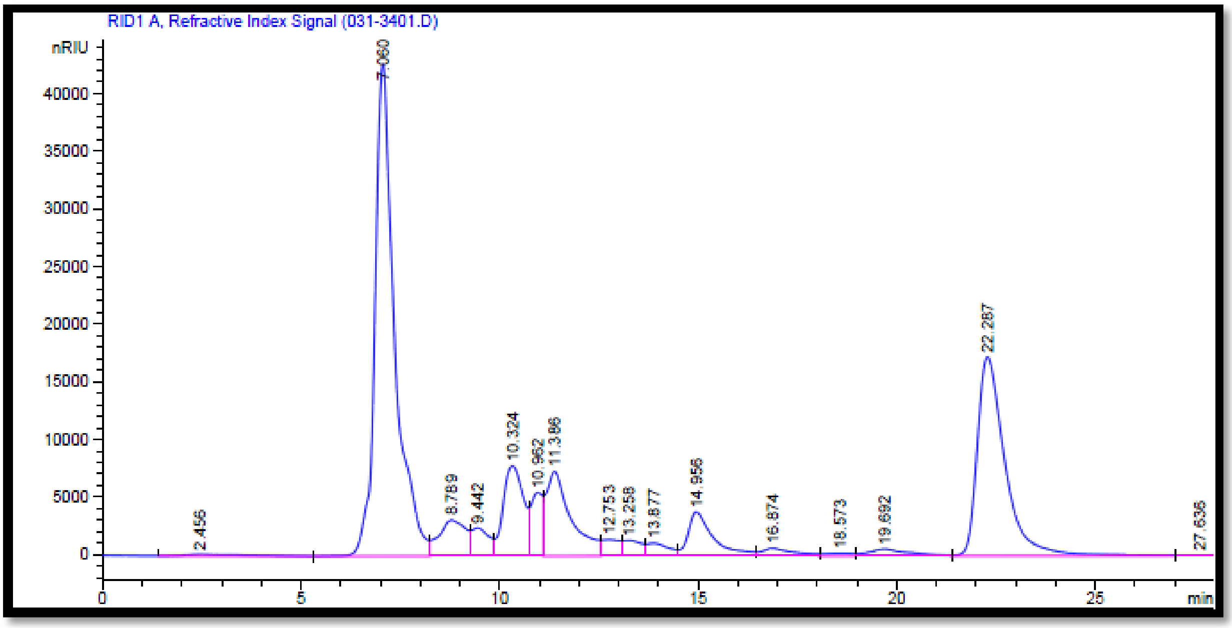
Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	3.468	BB	1.1234	5708.97461	63.69595	0.1464
2	7.066	BV	0.5280	1.21318e6	3.26936e4	31.1178
3	8.831	VV	0.7520	2.01023e5	3931.52710	5.1562
4	9.442	VV	0.4526	1.07635e5	3429.03540	2.7608
5	10.241	VV	0.4544	2.81700e5	9146.29199	7.2255
6	11.192	VV	0.7395	3.51099e5	6356.38184	9.0056
7	12.872	VV	0.6167	8.41485e4	1881.82495	2.1584
8	13.269	VV	0.4941	6.52088e4	1906.31763	1.6726
9	13.919	VV	0.5417	7.67736e4	2005.13794	1.9692
10	14.961	VV	0.5939	3.01883e5	7313.61670	7.7432
11	16.831	VV	0.8324	2.72537e4	426.24084	0.6990
12	19.466	VV	1.1097	4.56616e4	596.05481	1.1712
13	20.359	VV	0.6138	1.46733e4	335.60049	0.3764
14	22.293	VV	0.7265	1.10660e6	2.29567e4	28.3840
15	25.368	VV	0.8906	1.00355e4	135.40433	0.2574
16	27.492	VBA	0.8056	6088.81152	93.17512	0.1562
Totals :				3.89868e6	9.32706e4	

Sample 7



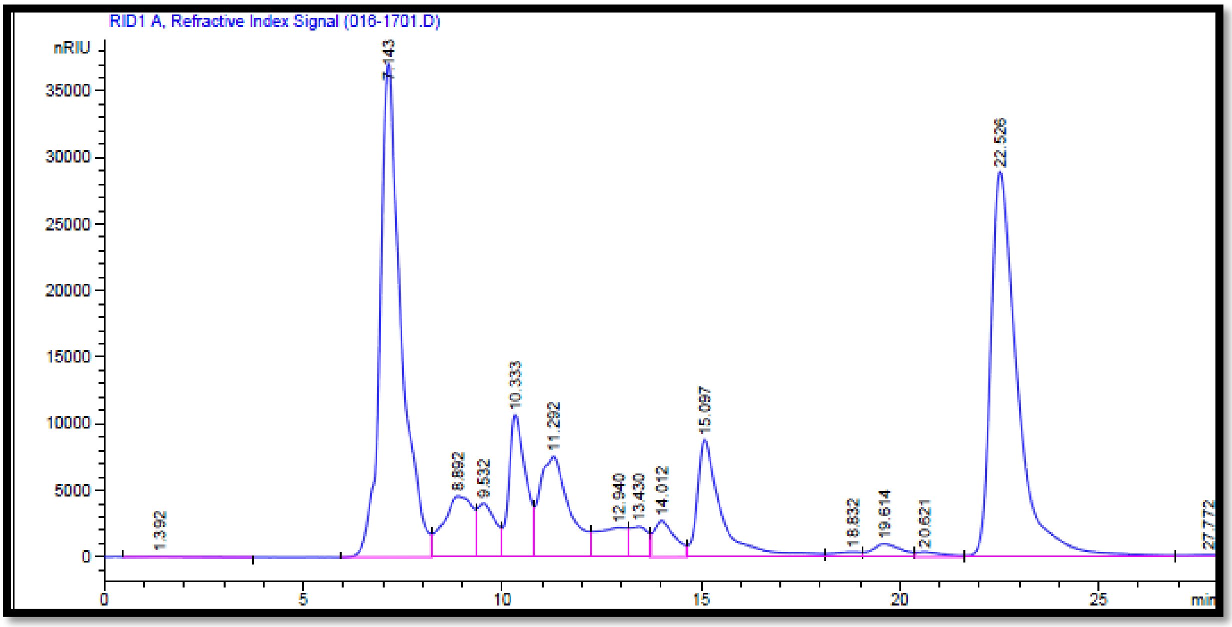
Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	1.450	BV	0.4628	942.84436	25.17681	0.0242
2	2.494	VV	0.6778	5393.11279	103.36868	0.1385
3	3.510	VV	0.9816	6149.70410	84.21094	0.1579
4	7.098	VV	0.5492	1.58054e6	4.13915e4	40.5764
5	8.802	VV	0.7041	1.69223e5	3598.57690	4.3444
6	9.440	VV	0.4283	8.30129e4	2762.56299	2.1312
7	10.236	VV	0.4713	2.42457e5	7518.47803	6.2245
8	11.188	VV	0.7942	3.45258e5	5833.06738	8.8636
9	12.829	VV	0.4913	5.61324e4	1583.48877	1.4411
10	13.260	VV	0.8269	9.69205e4	1582.32922	2.4882
11	14.964	VV	0.6060	2.77124e5	6548.57959	7.1145
12	16.756	VV	0.8658	2.57631e4	381.41116	0.6614
13	18.687	VV	0.6959	1.22084e4	244.07397	0.3134
14	19.713	VV	1.0507	4.31446e4	551.63275	1.1076
15	22.297	VV	0.7414	9.42748e5	1.90529e4	24.2028
16	27.698	VBA	0.7712	8194.49316	143.21310	0.2104
Totals :				3.89521e6	9.14045e4	

Sample 8



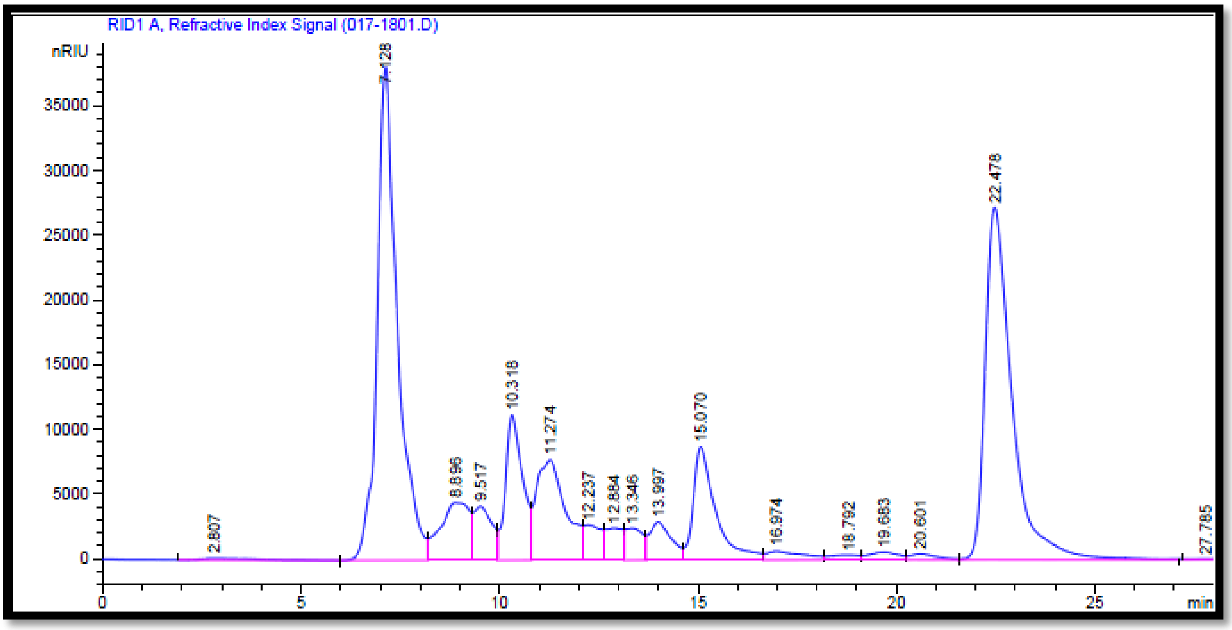
Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	2.456	BV	1.3075	1.24108e4	122.91920	0.3412
2	7.060	VV	0.4899	1.47469e6	4.26389e4	40.5460
3	8.789	VV	0.6868	1.43936e5	3110.25049	3.9575
4	9.442	VV	0.4415	7.56190e4	2425.15259	2.0791
5	10.324	VV	0.5464	2.73353e5	7812.81055	7.5157
6	10.962	VV	0.2996	1.12754e5	5501.56250	3.1001
7	11.386	VV	0.6030	3.22788e5	7289.86377	8.8749
8	12.753	VV	0.4234	4.25419e4	1400.92041	1.1697
9	13.258	VV	0.4903	4.53000e4	1337.00073	1.2455
10	13.877	VV	0.5314	4.18540e4	1097.20618	1.1508
11	14.956	VV	0.6224	1.66555e5	3809.17969	4.5794
12	16.874	VV	0.7574	3.59234e4	649.74011	0.9877
13	18.573	VV	0.6837	1.04514e4	210.21802	0.2874
14	19.692	VV	0.9746	4.01369e4	577.78888	1.1035
15	22.287	VV	0.7301	8.35397e5	1.72206e4	22.9689
16	27.636	VBA	0.6501	3365.24634	69.64492	0.0925
Totals :				3.63708e6	9.52738e4	

Sample 9



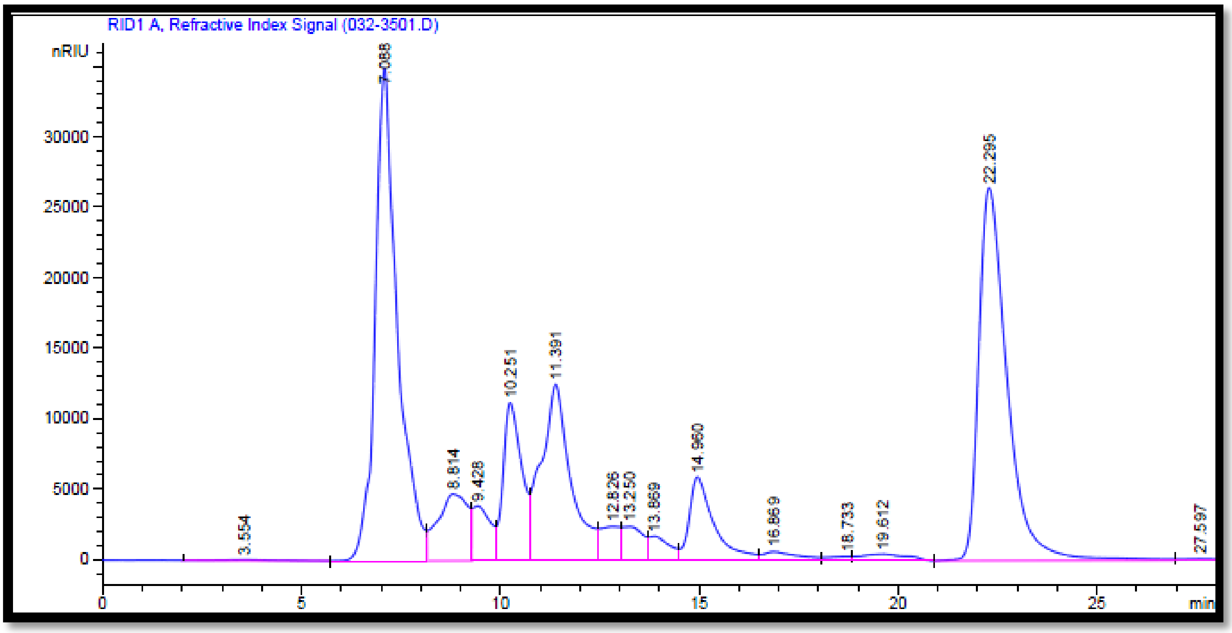
Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	1.392	VB	0.9685	1763.60266	21.82970	0.0387
2	7.143	VV	0.5190	1.37342e6	3.70394e4	30.0994
3	8.892	VV	0.7463	2.31454e5	4570.71826	5.0725
4	9.532	VV	0.4614	1.29535e5	4030.17090	2.8388
5	10.333	VV	0.4262	3.19014e5	1.06818e4	6.9914
6	11.292	VV	0.6881	3.89927e5	7560.88818	8.5455
7	12.940	VV	0.6883	1.16105e5	2218.50439	2.5445
8	13.430	VV	0.4623	7.32554e4	2273.87939	1.6054
9	14.012	VV	0.5285	1.01312e5	2726.54492	2.2203
10	15.097	VV	0.5898	3.73634e5	8811.22461	8.1884
11	18.832	VV	0.7516	1.84767e4	372.41434	0.4049
12	19.614	VV	0.7738	5.01159e4	972.81641	1.0983
13	20.621	VV	0.6619	1.76136e4	373.92432	0.3860
14	22.526	VV	0.6960	1.35965e6	2.88864e4	29.7977
15	27.772	VBA	0.7584	7669.92188	133.15659	0.1681
Totals :				4.56295e6	1.10674e5	

Sample 10



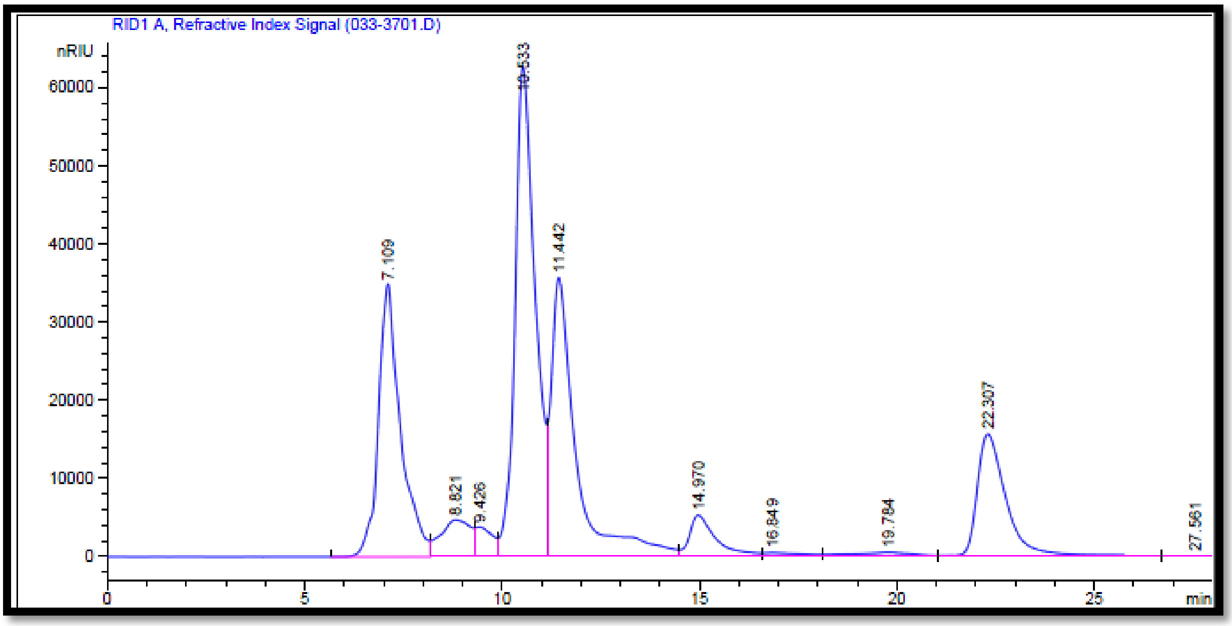
Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	2.807	BV	1.2775	1.09426e4	114.66623	0.2412
2	7.128	VV	0.5018	1.35314e6	3.80021e4	29.8271
3	8.896	VV	0.6790	2.28908e5	4442.12988	5.0458
4	9.517	VV	0.4603	1.32670e5	4140.06104	2.9244
5	10.318	VV	0.4393	3.38248e5	1.11825e4	7.4560
6	11.274	VV	0.6818	3.99979e5	7724.72168	8.8167
7	12.237	VV	0.4042	7.97861e4	2708.05933	1.7587
8	12.884	VV	0.4232	7.29055e4	2462.42041	1.6070
9	13.346	VV	0.4488	7.59698e4	2445.88135	1.6746
10	13.997	VV	0.5418	1.11765e5	2918.74048	2.4636
11	15.070	VV	0.5618	3.41440e5	8694.39941	7.5263
12	16.974	VV	0.8391	4.26677e4	668.97662	0.9405
13	18.792	VV	0.6894	1.89732e4	361.87332	0.4182
14	19.683	VV	0.7989	3.08093e4	590.40082	0.6791
15	20.601	VV	0.6794	2.11127e4	448.00717	0.4654
16	22.478	VB	0.7026	1.27529e6	2.71953e4	28.1110
17	27.785	BBA	0.7272	2003.65259	45.92318	0.0442
Totals :				4.53661e6	1.14146e5	

Sample 11



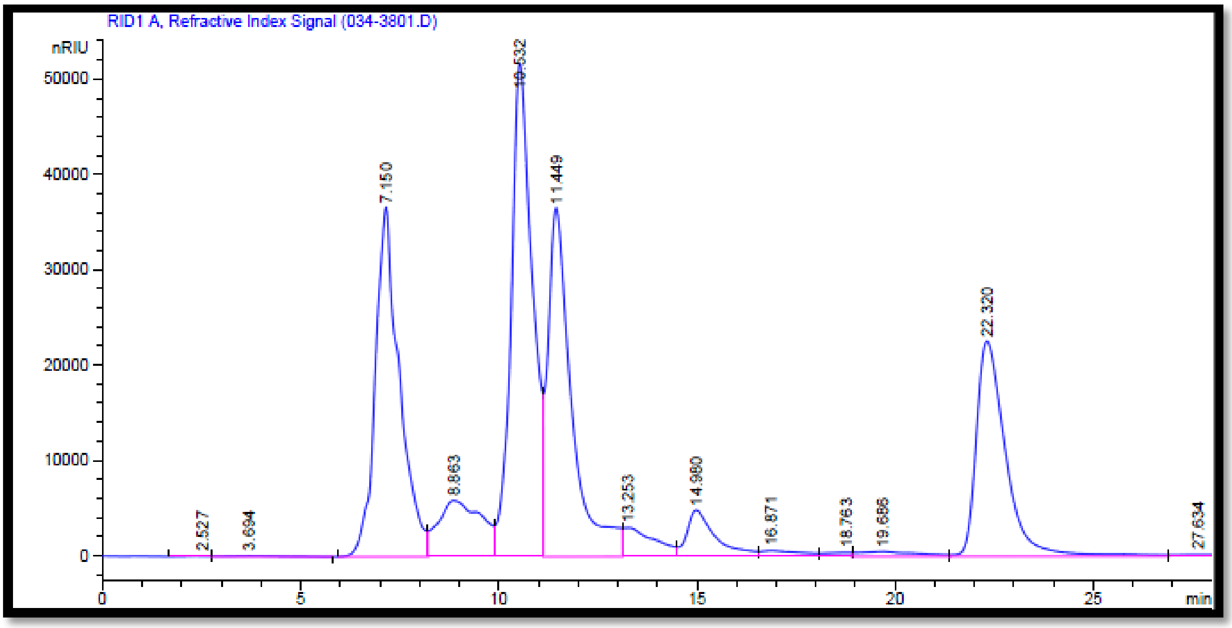
Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	3.554	VV	1.2960	6562.66797	67.64381	0.1399
2	7.088	VV	0.5618	1.37367e6	3.49821e4	29.2860
3	8.814	VV	0.7707	2.49687e5	4735.40527	5.3232
4	9.428	VV	0.4378	1.20244e5	3896.41187	2.5636
5	10.251	VV	0.4842	3.64778e5	1.11851e4	7.7769
6	11.391	VV	0.7118	6.61166e5	1.25071e4	14.0958
7	12.826	VV	0.4902	8.43738e4	2437.65698	1.7988
8	13.250	VV	0.5200	8.67697e4	2431.84082	1.8499
9	13.869	VV	0.5032	6.22125e4	1741.55762	1.3263
10	14.960	VV	0.6261	2.61215e5	5931.25049	5.5690
11	16.869	VV	0.8453	4.11430e4	647.21228	0.8772
12	18.733	VV	0.5941	1.28547e4	316.96411	0.2741
13	19.612	VV	1.2072	4.01683e4	476.94861	0.8564
14	22.295	VV	0.7462	1.31965e6	2.64508e4	28.1344
15	27.597	VBA	0.6874	6027.95850	107.71791	0.1285
Totals :				4.69052e6	1.07916e5	

Sample 12



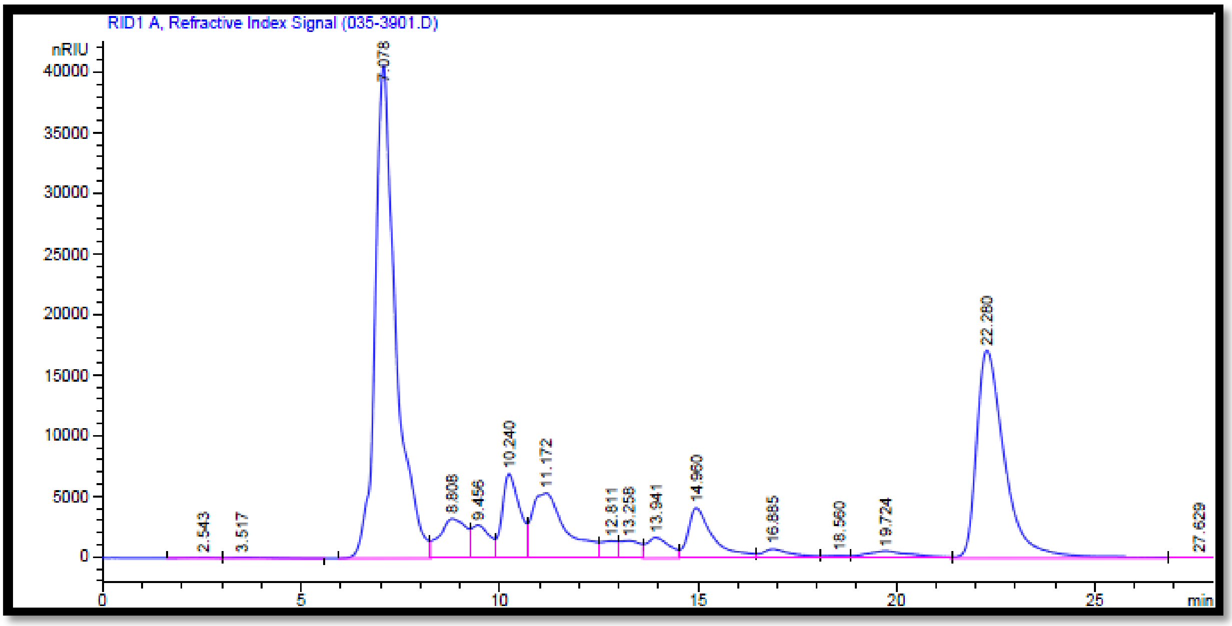
Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	7.109	VV	0.5592	1.35762e6	3.47743e4	20.3009
2	8.821	VV	0.7628	2.44975e5	4705.95508	3.6632
3	9.426	VV	0.4425	1.14943e5	3765.55127	1.7188
4	10.533	VV	0.5162	2.21308e6	6.25911e4	33.0928
5	11.442	VV	0.6207	1.57941e6	3.56393e4	23.6173
6	14.970	VV	0.6708	2.49123e5	5285.54883	3.7252
7	16.849	VV	0.9364	3.47758e4	482.24438	0.5200
8	19.784	VV	1.4432	6.22185e4	541.91425	0.9304
9	22.307	VV	0.7827	8.18257e5	1.56512e4	12.2356
10	27.561	VBA	0.9158	1.31007e4	173.44373	0.1959
Totals :				6.68751e6	1.63611e5	

Sample 13



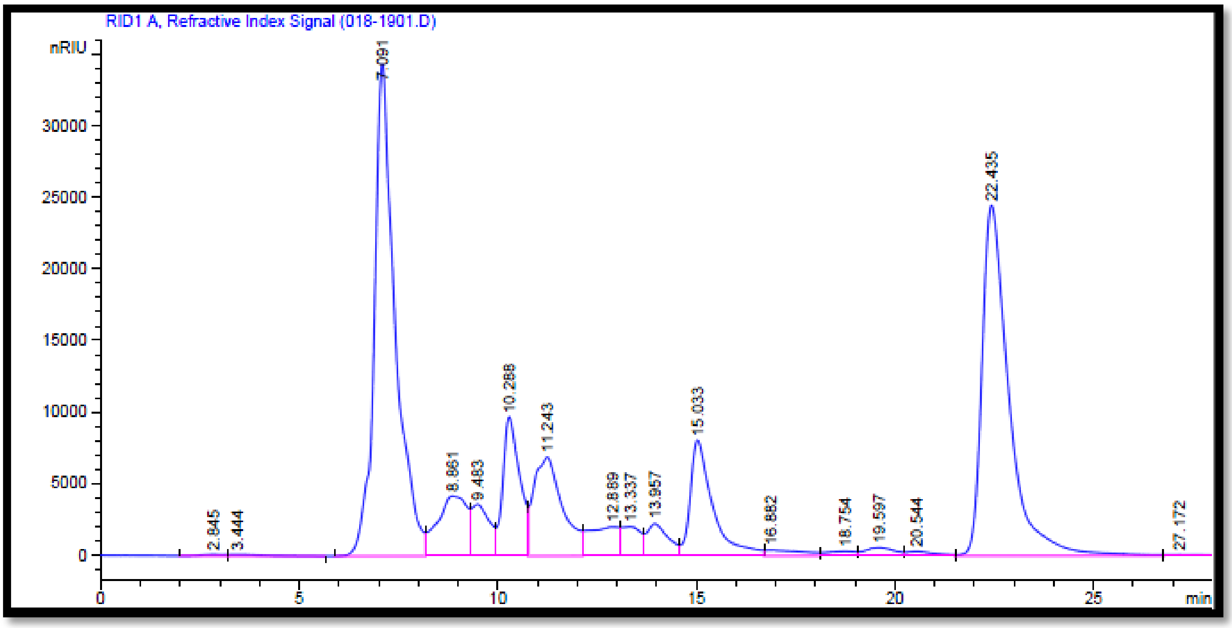
Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	2.527	VV	0.5301	1233.89197	29.05196	0.0172
2	3.694	VB	1.1172	6148.05176	68.43829	0.0858
3	7.150	BV	0.6157	1.54894e6	3.65295e4	21.6133
4	8.863	VV	1.0330	4.44714e5	5854.83008	6.2054
5	10.532	VV	0.5434	1.94615e6	5.16385e4	27.1558
6	11.449	VV	0.5945	1.53389e6	3.64629e4	21.4033
7	13.253	VV	0.7477	1.64821e5	2985.81860	2.2998
8	14.980	VV	0.6985	2.39669e5	4841.25293	3.3442
9	16.871	VV	0.9388	4.20141e4	593.07812	0.5862
10	18.763	VV	0.6421	1.76321e4	382.09103	0.2460
11	19.686	VV	1.3031	5.02596e4	499.67926	0.7013
12	22.320	VV	0.7918	1.16202e6	2.25296e4	16.2144
13	27.634	VBA	0.7330	9116.27344	150.40720	0.1272
Totals :				7.16661e6	1.62565e5	

Sample 14



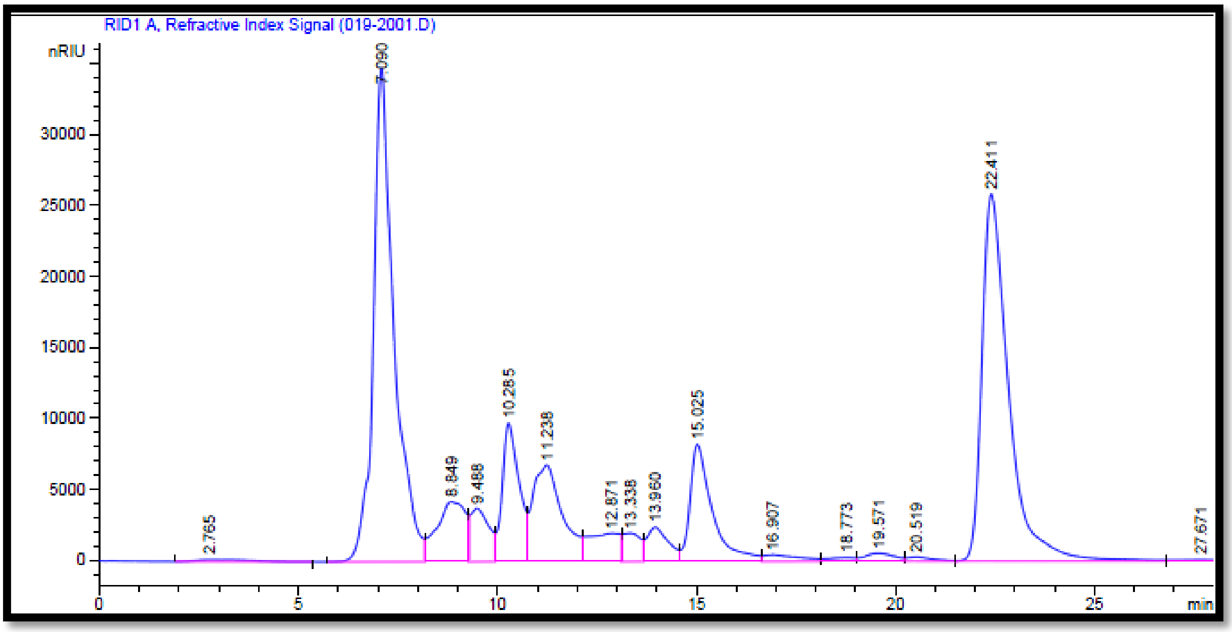
Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	2.543	BV	0.6330	2577.56567	49.53466	0.0728
2	3.517	VB	0.8858	3906.83423	57.01802	0.1104
3	7.078	BV	0.5114	1.47616e6	4.05202e4	41.7067
4	8.808	VV	0.6847	1.49244e5	3237.68335	4.2167
5	9.456	VV	0.4567	8.59729e4	2708.47778	2.4290
6	10.240	VV	0.4585	2.15464e5	6916.81641	6.0876
7	11.172	VV	0.8078	3.26731e5	5347.66504	9.2313
8	12.811	VV	0.4052	4.03562e4	1400.94873	1.1402
9	13.258	VV	0.4942	4.86161e4	1421.02466	1.3736
10	13.941	VV	0.5796	6.70893e4	1674.94666	1.8955
11	14.960	VV	0.6221	1.80062e5	4121.16406	5.0874
12	16.885	VV	0.7370	3.82751e4	724.88312	1.0814
13	18.560	VV	0.6610	8147.34326	184.90039	0.2302
14	19.724	VV	1.0589	4.57125e4	563.43469	1.2915
15	22.280	VV	0.7495	8.45852e5	1.71129e4	23.8983
16	27.629	VBA	0.7016	5216.09863	90.06010	0.1474
Totals :				3.53938e6	8.61316e4	

Sample 15



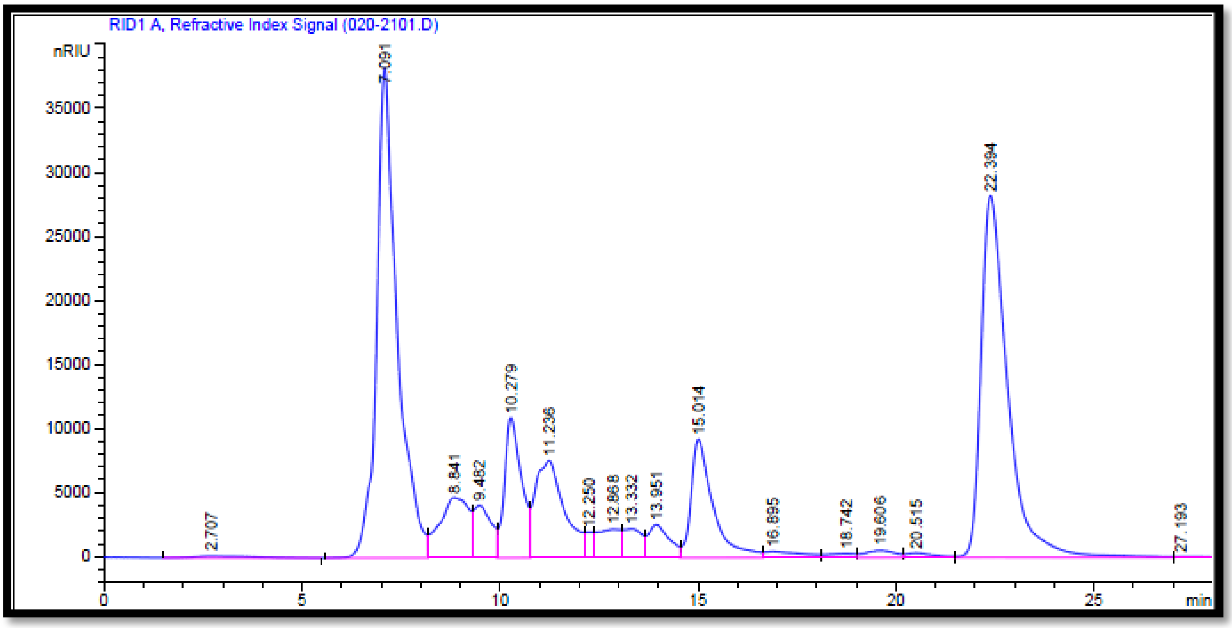
Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	2.845	BV	0.6035	3763.86987	89.40768	0.0927
2	3.444	VB	0.6860	4944.08398	90.95044	0.1218
3	7.091	BV	0.5064	1.26249e6	3.43608e4	31.0955
4	8.861	VV	0.6761	2.15026e5	4192.58105	5.2961
5	9.483	VV	0.4547	1.13907e5	3608.24121	2.8056
6	10.288	VV	0.4370	2.91136e5	9688.60352	7.1707
7	11.243	VV	0.6713	3.49722e5	6873.87158	8.6137
8	12.889	VV	0.7089	1.08954e5	2013.94666	2.6836
9	13.337	VV	0.4634	6.55162e4	2027.39111	1.6137
10	13.957	VV	0.5330	8.39475e4	2236.33960	2.0676
11	15.033	VV	0.5548	3.17841e5	8064.99951	7.8285
12	16.882	VV	0.8497	2.64784e4	400.19766	0.6522
13	18.754	VV	0.7761	1.55568e4	309.70285	0.3832
14	19.597	VV	0.7930	2.98890e4	570.16949	0.7362
15	20.544	VV	0.6740	1.46232e4	303.71890	0.3602
16	22.435	VV	0.6967	1.15262e6	2.44585e4	28.3894
17	27.172	VBA	0.7946	3630.38599	55.72308	0.0894
Totals :				4.06005e6	9.93452e4	

Sample 16



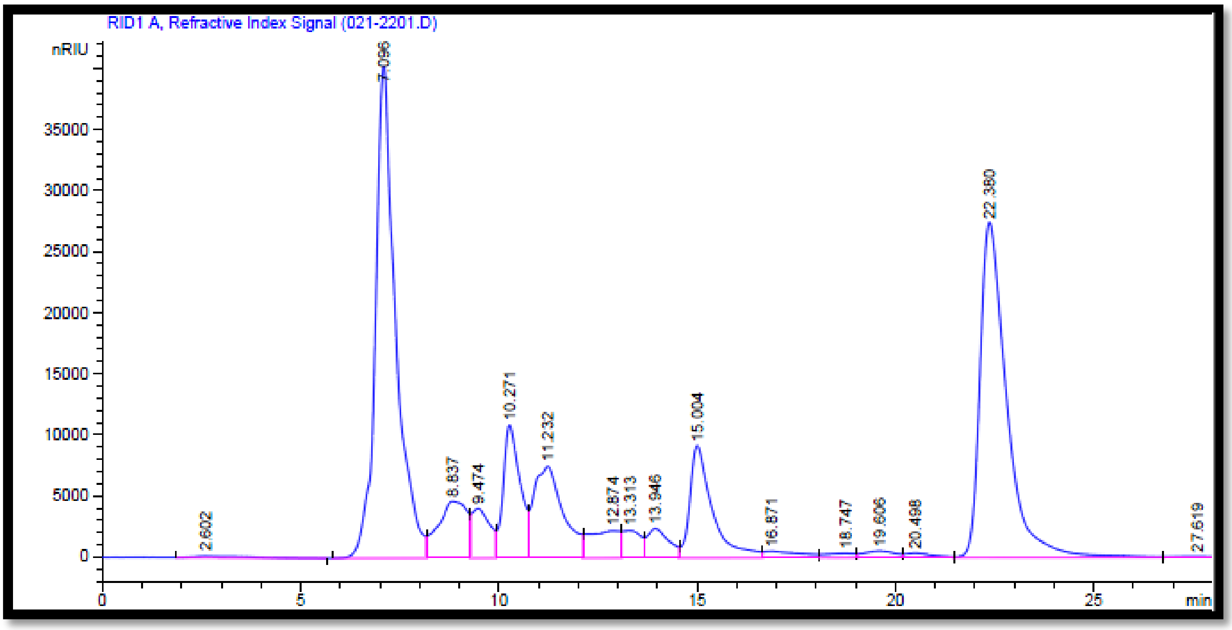
Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	2.765	BB	1.2274	1.10967e4	114.33379	0.2702
2	7.090	BV	0.5078	1.25456e6	3.47348e4	30.5504
3	8.849	VV	0.7366	2.12328e5	4200.59424	5.1705
4	9.488	VV	0.4596	1.19697e5	3741.98804	2.9148
5	10.285	VV	0.4352	2.90026e5	9701.33691	7.0626
6	11.238	VV	0.6826	3.46562e5	6781.64746	8.4393
7	12.871	VV	0.7312	1.09071e5	1973.58191	2.6560
8	13.338	VV	0.4393	6.13587e4	1979.74133	1.4942
9	13.960	VV	0.5350	9.06824e4	2404.71338	2.2083
10	15.025	VV	0.5480	3.18639e5	8207.43457	7.7593
11	16.907	VV	0.8465	2.89530e4	460.09061	0.7050
12	18.773	VV	0.6821	1.21538e4	260.71277	0.2960
13	19.571	VV	0.7998	2.91047e4	556.86975	0.7087
14	20.519	VV	0.6443	1.34935e4	300.82401	0.3286
15	22.411	VV	0.6914	1.20401e6	2.57962e4	29.3194
16	27.671	VBA	0.8477	4791.55859	77.85007	0.1167
Totals :				4.10652e6	1.01293e5	

Sample 17



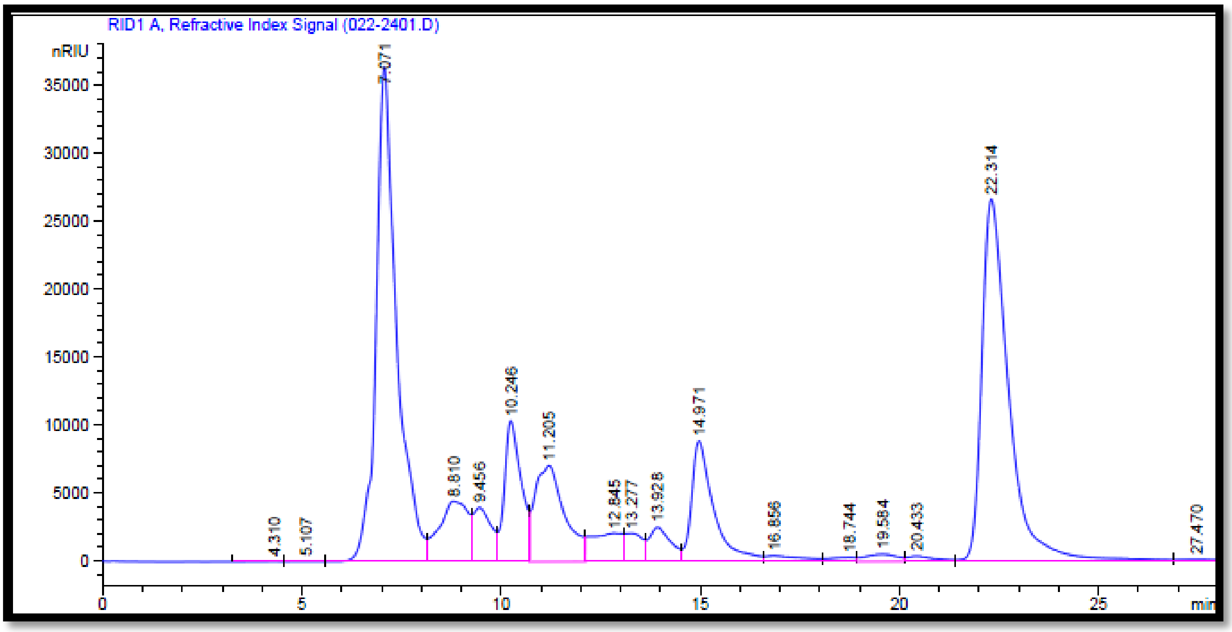
Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	2.707	BB	1.2914	1.39447e4	138.99693	0.3063
2	7.091	BV	0.5020	1.39175e6	3.82751e4	30.5747
3	8.841	VV	0.6797	2.38418e5	4688.52197	5.2377
4	9.482	VV	0.4477	1.29674e5	4089.66748	2.8487
5	10.279	VV	0.4369	3.27367e5	1.08962e4	7.1918
6	11.236	VV	0.6856	3.89016e5	7573.87256	8.5461
7	12.250	VV	0.1880	2.63984e4	2006.59875	0.5799
8	12.868	VV	0.5669	9.31376e4	2221.54883	2.0461
9	13.332	VV	0.4690	7.39181e4	2253.83569	1.6239
10	13.951	VV	0.5221	9.61735e4	2575.02271	2.1128
11	15.014	VV	0.5460	3.56530e5	9224.11816	7.8324
12	16.895	VV	0.8496	3.24965e4	485.75467	0.7139
13	18.742	VV	0.6472	1.52188e4	311.81604	0.3343
14	19.606	VV	0.8219	2.92748e4	548.03693	0.6431
15	20.515	VV	0.6838	1.76745e4	360.83914	0.3883
16	22.394	VB	0.6916	1.31783e6	2.82260e4	28.9509
17	27.193	BBA	0.6399	3141.04639	60.53253	0.0690
Totals :				4.55196e6	1.13936e5	

Sample 18



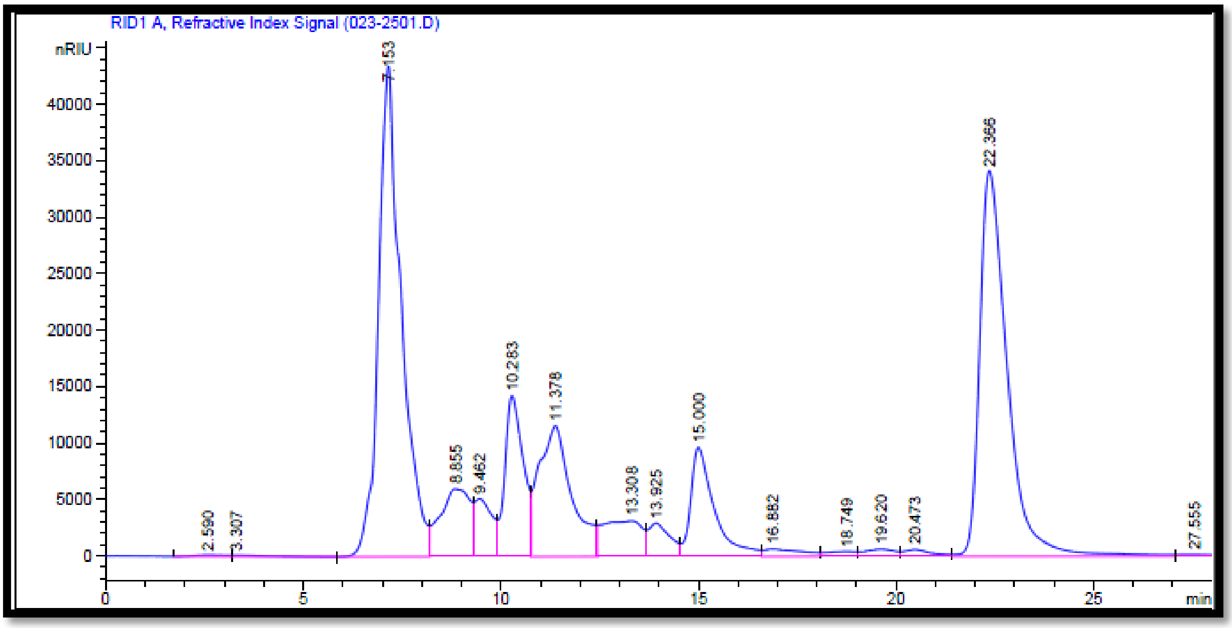
Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	2.602	BB	1.2746	1.13189e4	115.33575	0.2495
2	7.096	BV	0.5020	1.43418e6	4.02583e4	31.6139
3	8.837	VV	0.6821	2.36764e5	4637.22119	5.2190
4	9.474	VV	0.4443	1.26658e5	4030.98291	2.7919
5	10.271	VV	0.4284	3.26006e5	1.08484e4	7.1862
6	11.232	VV	0.6872	3.84485e5	7465.55664	8.4753
7	12.874	VV	0.7196	1.19193e5	2196.28662	2.6274
8	13.313	VV	0.4634	7.16084e4	2215.85425	1.5785
9	13.946	VV	0.5343	9.01161e4	2393.53491	1.9864
10	15.004	VV	0.5536	3.59204e5	9138.47168	7.9180
11	16.871	VV	0.8397	3.33218e4	516.07385	0.7345
12	18.747	VV	0.6906	1.74261e4	336.46182	0.3841
13	19.606	VV	0.8169	2.82085e4	525.03040	0.6218
14	20.498	VV	0.6987	1.67884e4	354.96646	0.3701
15	22.380	VV	0.6911	1.27722e6	2.73802e4	28.1538
16	27.619	VBA	0.8242	4060.84619	63.47285	0.0895
Totals :				4.53656e6	1.12476e5	

Sample 19



Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	4.310	VV	0.6513	1006.14917	19.58102	0.0233
2	5.107	VV	0.6302	1215.19812	23.46338	0.0282
3	7.071	VV	0.5087	1.31366e6	3.62951e4	30.4666
4	8.810	VV	0.6636	2.22762e5	4436.25781	5.1663
5	9.456	VV	0.4568	1.26071e5	3970.69751	2.9239
6	10.246	VV	0.4257	3.08650e5	1.03494e4	7.1583
7	11.205	VV	0.6920	3.66121e5	7052.97852	8.4912
8	12.845	VV	0.7223	1.13056e5	2074.34839	2.6220
9	13.277	VV	0.4481	6.42370e4	2071.93359	1.4898
10	13.928	VV	0.5347	9.44795e4	2507.03711	2.1912
11	14.971	VV	0.5466	3.41976e5	8836.36230	7.9312
12	16.856	VV	0.8289	2.72996e4	424.00632	0.6331
13	18.744	VV	0.7174	1.41459e4	303.06708	0.3281
14	19.584	VV	0.8374	2.94257e4	523.37677	0.6824
15	20.433	VV	0.7018	1.93872e4	383.71765	0.4496
16	22.314	VV	0.6987	1.25984e6	2.66380e4	29.2186
17	27.470	VBA	0.7667	8453.80957	133.14261	0.1961
Totals :				4.31179e6	1.06043e5	

Sample 20



Peak #	RetTime [min]	Type	Width [min]	Area [nRIU*s]	Height [nRIU]	Area %
1	2.590	BV	0.7939	6411.23975	117.16441	0.1087
2	3.307	VV	0.7253	5806.61230	98.07634	0.0985
3	7.153	VV	0.5752	1.75488e6	4.34204e4	29.7622
4	8.855	VV	0.7656	3.12936e5	5984.20313	5.3073
5	9.462	VV	0.4440	1.60424e5	5110.71191	2.7207
6	10.283	VV	0.4738	4.61266e5	1.42088e4	7.8229
7	11.378	VV	0.7808	6.70067e5	1.15453e4	11.3641
8	13.308	VV	0.9072	2.19237e5	3149.26465	3.7182
9	13.925	VV	0.5418	1.12508e5	2938.02905	1.9081
10	15.000	VV	0.5863	3.98965e5	9643.44238	6.7663
11	16.882	VV	0.8885	4.45382e4	662.04547	0.7554
12	18.749	VV	0.7704	2.22544e4	428.25638	0.3774
13	19.620	VV	0.7907	3.41246e4	644.29175	0.5787
14	20.473	VV	0.6792	2.89325e4	604.65240	0.4907
15	22.366	VB	0.7308	1.65722e6	3.41202e4	28.1058
16	27.555	BBA	0.6822	6773.64209	125.36317	0.1149
Totals :				5.89634e6	1.32800e5	