EXTRACTION OF GLYCOLIC ACID FROM NATURAL SOURCES

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SUPERVISOR'S DECLARATION

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

Signature
Name of Supervisor:
Position:
Date:

STUDENT'S DECLARATION

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

Signature
Name: Faznurfariza binti Firdaus @ Nicholas
ID Number: KE08029
Date:

"Specially dedicated to my beloved mother, father and sisters"

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ABSTRACT

Glycolic acid (GA) is a type of alpha hydroxyacetic acid (AHA). It is a small molecule of AHA that is colorless, odorless and hygroscopic. Since GA is capable of penetrate the skin, it is suitable for exfoliate and anti ageing application. It is widely used especially in dermatology, medical and pharmaceutical applications. Since GA is very useful in cosmetic and pharmaceutical field, it yields a high market demand. By using rotten fruits obtained from the market as the sources to produce GA, the waste management in the market could be overcome. Thus, the production cost of GA could be reduced. The purpose of this study is to study the highest production of GA from different type natural sources such as sugarcane juice and banana in term of fresh and rotten state. The study is ought to investigate the effect of concentration of ethylene glycol (0.2 M to 1 M), temperature (40 °C to 80 °C) and time (30 min to 60 min) on extraction of GA compounds from natural sources and to optimize the production of glycolic acid from the sample after screening using response surface methodology (RSM). The ultrasonic homogenizer was used for the extraction of product with different independent variables. HPLC is used to analyze the concentration of GA in the samples. From the present research, fresh banana peels contain the highest GA concentration at 0.914 M. From RSM the most optimum combination variables are temperature at 70 °C, solvent concentration at 1 M and extraction time at 50 min with desirability 1.000. The optimization of GA can be further investigated by using different types of solvent, extraction method and ultrasonic frequency.

ABSTRAK

Asid glikolik (GA) adalah sejenis asid alfa hidroksil asetik (AHA). Ia adalah molekul kecil AHA yang tidak mempunyai warna, bau dan bersifat higroskopik. Memandangkan GA mampu menembusi lapisan kulit, ia sesuai digunakan untuk pengelupasan dan anti penuaan. Ia digunakan secara meluas dalam bidang dermatologi, perubatan dan farmaseutikal. Disebabkan GA sangat berguna dalam bidang kosmetik dan farmaseutikal, ia mempunyai permintaan yang sangat tinggi. Dengan menggunakan buah-buahan yang rosak dari pasar sebagai sumber untuk menghasilkan GA, pengurusan bahan sisa di pasar dapat diatasi. Di samping itu kos penghasilan GA dapat dikurangkan. Tujuan kajian ini dijalankan adalah untuk mengkaji penghasilan GA yang paling tinggi daripada sumber semula jadi yang berbeza-beza, iaitu daripada air tebu dan pisang (*sebatu*) sama ada dalam keadaan segar atau rosak. Kajian ini dijalankan untuk mengkaji kesan kepekatan ethylene glikol (0.2 M hingga 1 M), suhu (40 °C hingga 80 °C) dan masa (30 min hingga 60 min) untuk pengekstrakan GA daripada sebatian sumber semula jadi dan untuk mengoptimumkan pengeluaran GA daripada sampel selepas menggunakan kaedah sambutan permukaan (RSM). Homogenizer ultrasonik digunakan untuk mengekstrak produk dengan pembolehubah bebas yang berbeza. Kromatografi cecair berprestasi tinggi (HPLC) digunakan untuk menganalisa kepekatan GA dalam setiap sampel. Keputusan kajian ini telah menunjukkan kulit pisang segar mengandungi kepekatan GA yang paling tinggi iaitu 0.914 M. Berdasarkan kombinasi pembolehubah yang ditentukan dari RSM, keadaan yang paling optimum adalah pada suhu 70 °C, kepekatan pelarut pada 1 M dan masa pengestrakkan 50 min dengan kecenderungan 1.000. Kajian pengoptimuman GA yang lebih lanjut boleh dilakukan dengan menggunakan jenis pelarut, kaedah pengekstrakan dan frekuensi utrasonik yang berbeza.

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LIST OF SYMBOLS

A_1	Fresh banana peel
A_2	Rotten banana peel
B_1	Fresh banana pulp
B_2	Rotten banana pulp
b_0	Constant term
b_1, b_2, b_3	Linear effects
b_{11}, b_{22}, b_{33}	Quadratic effects
b_{12}, b_{13}, b_{23}	Interaction effects
C_1	Fresh sugarcane
C_2	Rotten sugarcane
X_1	Temperature
X_2	Solvent concentration
X_3	Time
y	Response function

LIST OF ABBREVIATIONS

AHA	Alpha hydroxyl acid
ANOVA	Analysis of variance
CCD	Central composite design
EG	Ethylene glycol
GA	Glycolic acid
GC	Gas chromatography
HPLC	High performance liquid chromatography
IC	Ion chromatography
LC	Liquid chromatography
RSM	Response surface methodology
US$	American dollar
₩	Won (South Korea money name)
¥	Renminbi (China money name)

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF STUDY

Cosmetic industry has a high potential due to increasing consumers around the world. It was reported, in 2010 cosmetic industry in South Korea was expected to grow to ₩8 trillion (US$6.7 billion) by Amorepacific, Korea's biggest cosmetics company. According to Analysys International, China's online retailing market is aspected to increase to ¥713 billion (US$104.4 billion) from 3.56% of the nation's total social commodity retail sale in 2012.

Nowadays, environmental pollution generated from economics activities such as chemical, petrochemical, agricultural and food industries are common problems faced by the world. There is a potential for solid waste from fruits to be used as raw material, or for conversion into useful and higher value added products. The fruit waste can be used to produced protein, ethanol, methane, pectins, extracts and enzymes.

Glycolic acid (GA) is considered as a very important chemical compound with significant application in pharmaceutical, chemical industry and has been well

known for being used as a cosmetic ingredient and a superficial peeling agent in dermatology (Kataoka et al., 2001). Recently, the use of glycolic acid containing cosmetics has received increasing public interest owing to their supposed ability to improve acne as well as premature aging of the skin (Clark et al. 1996 and Murad et al. 1995) to reduce wrinkles, roughness, age spots and other skin damage (Males and Herring, 1999). It can be prepared by chemical synthesis and be produced from fermentation broth or from glycolonitrile hydrolysis by mineral acid such as sulfuric acid (Grether and Vall, 1936; Shi, et al., 2005).

In plants, GA is an important intermediate in the photorespiratory carbon oxidation cycle (Jolivet et al., 1985). Experiment conducted by Jolivet et al. (1985), in order to examine the rate and sequence of photorespiratory metabolism following ^{18}O incorporation in the glycolate synthesized by leaves exposed to $^{18}O_2$. Under these conditions, the glycolate analyzed by mass spectrometry was labeled in ^{13}C and ^{18}O. The GC-MS analytical method which has been developed is suitable for the quantitative determination of GA especially from plant extracts. However in this research, HPLC been applied for GA analysis due to accuracy and better quantification.

1.2 PROBLEM STATEMENT

Usually banana can be found in the local market. Normally, bananas do not stay fresh for long. They are always being sold in a bunch at local markets and the flesh is commonly sold as banana fritters while the peel will be discarded and become a solid waste. However, if waste can be transformed into a valuable product such as organic acid, this would heighten the profits and competitiveness of the industry. For instance, the banana waste collected from the local market can be used as a substrate for organic acid production such as glycolic acid (GA). Therefore, the use of banana waste for glycolic acid production may be an option for utilizing low value waste material in producing a commercial product while solving environmental problems. Previous research use enzymatic methods to produce GA, however those method suffer from instability and high

cost of the commercially purified enzyme glycolate oxidase (Oungpipat and Alexander, 1994). Hence reduce the price of glycolic acid.

In this study, glycolic acid been produced because it has a high market demand especially in the cosmetic and pharmaceutical field (Clark et al. 1996 and Murad et al. 1995). Based on Bergfeld et al. (1997) results illustrated that GA treatment is superior to mechanical exfoliation in improving the cosmetic appearance of photo aged skin. Zhu et al. (2004) and Chauhan et al. (2002) mentioned that it is also used in numerous areas of technology such as adhesive, metal cleaning and electroplating.

1.3 OBJECTIVE

The main objective of this research is to optimize the production of glycolic acid (GA) from natural source. The measureable objectives are:

a) To screen natural sources (sugarcane juice and banana) that contain high yield of glycolic acid.
b) To determine the effect concentration of solvent (ethylene glycol).
c) To determine the effect of temperature.
d) To determine the effect of extraction time.

1.4 SCOPE

Glycolic acid (GA) can be produced from plants extraction or chemical synthesis. In this study, GA will be produced from two natural sources from rotten and fresh fruits, which will be collected from the local market. Six types of samples that will be used are fresh banana peel, fresh banana pulp, fresh sugarcane, rotten banana peel, rotten banana pulp and rotten sugarcane. Sample that produces the highest production of glycolic acid will be chosen for optimization using response surface methodology (RSM). Variable parameters used are temperature in the range 40 °C to 80 °C, solvent concentration from 0.2 M to 1 M of ethylene glycol and extraction time in range 30 min to 60 min. Ultrasonic

homogenizer be used for extraction of glycolic acid. The analysis of the glycolic acid will be done by using high performance liquid chromatography (HPLC).

2.4 RATIONALE AND SIGNIFICANT

This research, high production of glycolic acid isolated from natural sources has potential cosmetic and pharmaceutical benefits especially as a therapeutic skin corrector. It is a chemical exfoliant that can regenerate the skin (Kataoka et al., 2001). In fact, it can act as a useful adjuvant for the treatment of acne because of its unique properties (Chauhan et al. 2002 and Katherine, 2000). The significant of this research is to help waste management in the market by reducing solid waste from fruits. This could also help fruit's seller in Malaysia by buying solid waste from fruits rather than throwing them away. Economic effective because can produce GA by our own technology instead of importing from other country.

CHAPTER 2

LITERATURE REVIEW

2.1 ALPHA HYDROXY ACIDS

Alpha5hydroxyl acids (AHA) are water-soluble and are often added to skin care products. AHA mostly prepare by chemical synthesis or fermentation. AHA are also referred to as 'fruit acids.' It is usually found in citrus fruits, apricots, apples, grapes and sugarcane juice. AHA are meant to improve skin problems such as pimples, blackheads, whiteheads, reduce acne scars, improve skin's texture and bring out radiance, treat fine lines, wrinkles and lighten freckles. AHA promotes the shedding of the dead cells and instigates the renewal scene to take place in order to make skin smooth-looking and radiant. AHA is usually used for medical and pharmaceutical application (Van Scott and Yu, 1974).

2.2 GLYCOLIC ACID

2.2.1 Glycolic Acid

For many year glycolic acids (GA) have been used in cosmetic products to remove undesirable signs of skin ageing (Kurtzweil, 1998). GA or defined by IUPAC as hydroxyethanoic acid is a type of fruit acids or alpha hydroxyl acid (AHA). Other names for GA are hydroxyacetic, glucohydroxyacid and kyselina glykolova.

According to Chauhan et al. (2002), GA is crystalline, colorless, odorless and hydroscopic. GA penetrates easily into the skin as compared to other types of alpha hydroxyl acid because it is the smallest molecule within the homologous series of AHA with two carbon atoms. It has high acidity but easily soluble in water and proved to be an effective dermatologic and cosmetic ingredient as it can be used as natural skin exfoliant and moisturizer (Katoaka et al., 2001). It is also easy soluble in methanol, ethanol, acetone, ethyl acetate, ether and acetic acid (Budavari, 1996 and Miltenberger, 1989)

GA is a low mole weight compound (76.05 g/mol) but has unique properties and dual functionality of alcohol and acid. In acidic condition, it forms cyclic and linear polymers known as glycolides. In aqueous environments GA dissociates into glycolate and hydrogen ion. Make GA ideal for a broad spectrum of consumer and industrial applications such as rust removal and degreasing (Chauhan et al., 2002).

Katoaka et al. (2001) stated that it is prepared by chemical synthesis or extraction from plants. According to Chauhan et al. (2002) GA can be produced from fermentation broth or from glycolonitrile hydrolysis by mineral acid such as sulfuric acid (Grether and Vall, 1936 and Shi et al. 2005).

2.2.2 Benefits of Glycolic Acid

There are a lot of benefits from GA such as stimulated the synthesis of new collagen (Van Scott and Yu, 1989) and decreasing keratinocytes cohesion (Katherine, 2000).

Researchers found that GA in low concentrations decreases corneocyte cohesion by promoting exfoliation of the outer layers of the stratum comeum (Katz, 1995 and Van Scott and Yu, 1984). This is important because most pigmentation alterations associated with photo damage can be attributed to be thickening of the stratum comeum (Bergfeld et al., 1997).

Ditre et al. (1996) noted that a significant increase in overall epidermal thickness that appeared to be secondary to augmented synthesis of glycosaminoglycans and collagen. Besides that they also found significant reversal of basal cell atypia, dispersal of melanin pigmentation, and a return to a normal pattern (Ditre et al., 1996).

GA can act as a useful adjuvant for the treatment of acne. The combination with topical retinoid makes it more effective in preventing comedonal acne (Katherine, 2000).

Stagnone (1989) reported that, repeated and regular applications of GA to the face have been shown to diminish fine facial wrinkles significantly.

2.2.3 Application of Glycolic Acid

Glycolic acid, perhaps the best-known AHA, is used in various fields. It is widely used especially in dermatology (Katherine, 2000), medical and pharmaceutical applications (Guzel, 1996; Moy et al. 1993; Van Scott and Yu, 1984 and Van Scott and Yu, 1989).

William (1978) reported that the concentration of glycolic acid in biological fluids has been used as an index for differential diagnosis of the hyperoxaluria. It is also used as inhibitors for harmful oxidation biochemical processes (Moy et al., 1993).

GA is one of the most important fine chemicals. It is used in numerous areas of technology such as in adhesive, metal cleaning, textiles, leather processing (Krochta et al., 1988), biodegradable polymers (Hayes and Lauren, 1994), electroplating, dairy cleaning, water-well cleaning, masonry, detergents (Kirk-Othmer, 1981), and as a component in personal care product (Yamamoto et al., 2006). Besides that, GA is also used as automotive oil additives, oil and water well flow enhancers, pH controlling, cosmetics, and chemical intermediate manufacture (Guzel, 1996 and Van Scott and Yu, 1984).

2.2.4 Glycolic Acid Structure

The structures of AHA family group are illustrated in Figure 2.1. GA permeates at slightly slower rate because it is more hydrophilic than acetic acid (Leo et al., 1971).

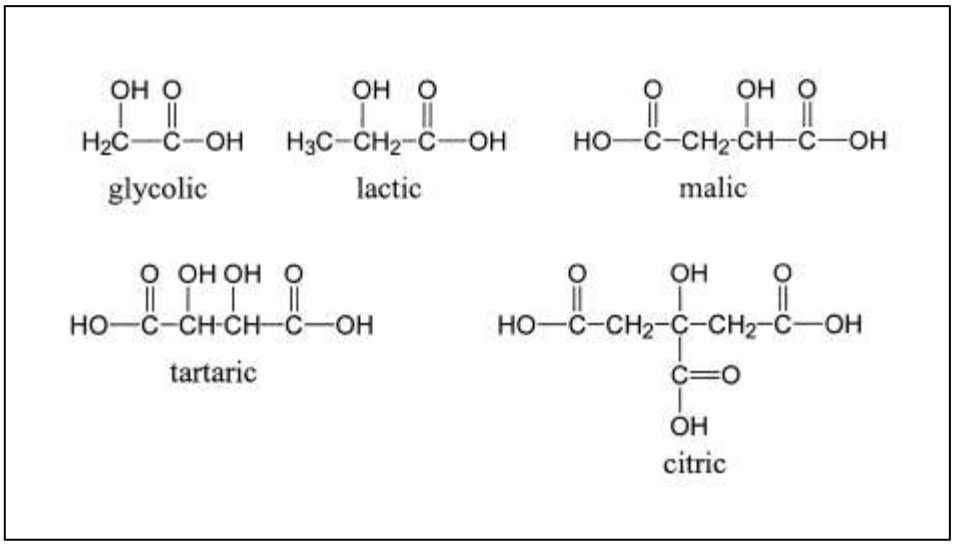

Figure 2.1: The alpha hydroxyl acid family

Adapted from: Males, R.G. and Herring, F.G. 1999. A H-NMR study of the permeation of glycolic acid through phospholipid membranes. *Biochimica et Biophysica Acta*. **1416**(1-2): 333-338

GA can diffuse easily throughout the intercellular phase in plasma and skin because it is hydrophilic and small compound of AHA. Smith (1994) proved that GA has a pH-dependent ability to stimulate cell renewal and it was observed at pH 3.

2.3 ANALYSIS

Numerous methodologies have been applied for the measurement of GA. These include calorimetric (Niederwieser et al. 1978 and Viccaro et al. 1972), isotope dilution (Hockaday et al., 1965), chromatographic (Petrarulo et al. 1989; Petrarulo et al. 1990 and Wandzilak et al. 1991) and enzymatic methods (Bais et al. 1985 and Kasidas and Rose, 1979).

Oungpipat et al. (1994) claimed that colorimetric, isotope dilution, gas chromatographic (GC), liquid chromatographic (LC) and enzymatic methods have inherent problems in analysis GA. The colorimetric methods are non-specific. The isotope dilution methods are used in combination with calorimetric methods, which are unreliable. GC and LC methods require complex isolation and derivatisation steps as well as involve the use of an expensive apparatus. Enzymatic methods suffer from instability and high cost of the commercially purified enzyme glycolate oxidase (Oungpipat et al., 1994).

GA are examined using chromatographic methods, including gas chromatography (GC) (Asano, 2002) after derivatization, ion chromatography (IC) (Chen et al., 2005) and liquid chromatography (HPLC) (Scalia et al., 1998). De Bruijn et al. (1984) concluded that even though GC provided superior resolution, HPLC had advantages of shorter sample preparation and analysis times.

Other researchers proved that HPLC is the best method for the analysis of GA and other acid compounds in sugar (Abeydeera, 1983; Morel du Boil et al. 1990; Thompson et al. 1990; Tsang et al., 1990 and Schäffler et al. 1990).

2.4 SUBSTRATE

GA is mainly extracted from sugarcane, sugar beets and various fruits (Chauhan et al., 2002). Van der Poel et al. (1998) proved that cane juice contains GA and other organic acid such as citric, malic, succinic, fumaric, lactic, oxalic, acetic, formic, itaconic and aconitic acid (1-propene-1,2,3-tri-carboxylic acid).

Table 2.1: Glycolic acid from various sources

Source	Authors
Tomato (*Lycopersicum esculentum* Mill.) Maize leaves (Zea mays L.)	Jolivet et al. (1985)
Algae	Tolbert and Zill (1956) Fogg (1966) Watt (1969) Jolivet et al. (1985)
Glycolonitrile hydrolysate	Yunhai et al., (2006)
An aqueous solution of glyoxal in the presence of a catalytic	Kiyoura and Kogure (1997)
Ethylene glycol	Wei et al. (2009)
DGA-utilizing *Rhodococcus* sp. No. 432	Yamanaka et al. (1991)
Paenibacillus sp. AUI 311	Isobe et al. (2007)
Gluconobacter oxydans DSM 2003	Wei et al. (2009)
Microorganisms	Kataoka et al. (2001)

2.4.1 Banana

Bananas are a tropical fruit from *Musaceae* family and produced in large quantities. In Malaysia, it was estimated to be 33,704.2 hectares total planted area of bananas in 2001 (MAO, 2006). Banana peel contains 30% to 40% of total banana weight (Oberoi et al., 2011). Nowadays, there is a large quantity of banana peel was wasted and consumed as animal feed. This activity is unfriendly to the environment and non-economical. Based on the reasons, banana-processing industries have been searching for applications of these by-products which proved to be a source of important natural compounds, such as protein, ethanol, methane and pectin (Cordenunsi et al. 2008 and Willats et al. 2001). Banana peel also contains highly valued antioxidant compounds and can be used as food livestock and biosorbent for heavy metals, dyes and the removal of phenolic compounds (Achak et al. 2009; Annadurai et al. 2002; Anwar et al. 2010 and Onwuka et al. 1997).

According to Juliana et al. (1989), raw banana juices are turbid, gray in colour, very viscous and tend to settle during storage and contain polysaccharides such as pectin and starch. Research doing by reported that green banana flour contained high total starch,

resistance starch and dietary fibre content about 73.4%, 17.5% and ~14.5% and high-sugar content in ripe banana.

2.4.2 Sugarcane

Brazil and Australia are the biggest county planted sugarcane (*Saccharum Officinarum*). In sugarcane, organic acid constitutes a significant proportion of the ionic organic non-sugar. Sugarcane contains organic acid such as citric, malic, succinic, fumaric, glycolic, lactic, oxalic, acetic and aconitic acic (Walford, 2002). Solid waste from sugarcane like bagasses can be applied for animal feed, paper, pulp and board (Banerjee and Pandey, 2002).

2.5 SOLVENT

Acid can be used as catalysts for fruit's hydrolysis because according to Aguilar et. al. (2002), acid can break down heterocyclic ether bonds between sugar monomers in the polymeric chain, which are formed by hemicellulose and cellulose.

Ethylene glycol is one of the cheap starting materials, can be use for the production of glycolic acid through an oxidation reaction (Katoaka et al., 2001). It was expected that microbial conversion of ethylene glycol to glycolic acid was an attractive alternative method for the value-added production of glycolic acid with no by-production. (Wei et al., 2009).

CHAPTER 3

METHODOLOGY

3.1 RESEARCH DESIGN

Figure 3.1 simplified the process methodology of GA production.

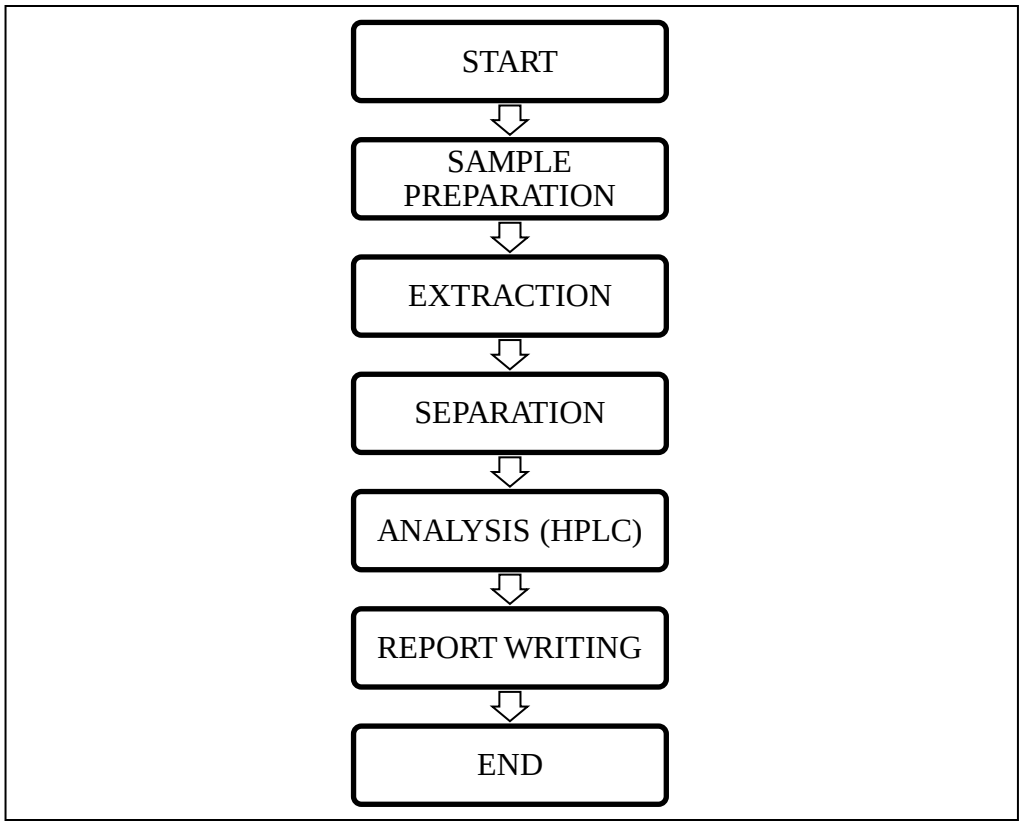

Figure 3.1: Process methodology of GA production

3.2 PROCEDURE

3.2.1 Reagents

All the chemicals used were of analytical grade and purchased from various suppliers.

3.2.2 Natural Sources

Banana (*pisang sebatu*) and sugarcane juice (Figure 3.2) are obtained from local market. Half of the fresh banana and sugarcane juice were kept for a few days to prepare rotten fruit samples.

Figure 3.2: Fresh banana (*pisang sebatu*) and sugarcane juice

3.2.3 Sample Preparation

The fresh and rotten fruits were washed and separated into pulp and peel (Figure 3.3). Banana pulp and peel were cut into small pieces and dry into the oven (MEMMERT, Model CELSIUS 2000, Malaysia) at 60 °C for several days.

Figure 3.3: Separation of banana peel and pulp

The dried peel and pulp were blended with blender (SHARP, Model EM-11, Malaysia). The fresh and rotten sugarcane juices were freeze dried (BioTron, Model Clean vac 8) for several days.

3.2.4 Extraction

For the experimental setup, a 100 W and 30 kHz frequency LABSONIC®M ultrasonic homogenizer (SARTORIUS, Germany) with amplitude of 100 % was used. Each six freeze-dried sample (2 g) was placed into centrifuge tube (50 ml, conical bottom) containing 20 ml of ethylene glycol and directly sonicated. The temperature and time were adjusted to the desired level using an ultrasonic homogenizer. The independent variables for the production of glycolic acid were the concentration of solvent, ethylene glycol (0.2–1.0 M), temperature (40-80 °C), and time (30–60 min) for extraction.

3.2.5 Separation

The treated juices were centrifuged at 3000 rpm for 10 min using a centrifuge (EPPENDORF, Model Centrifuge 5810 R) and the supernatant was collected (Lee et al., 2006). The supernatant was filtered through a filter paper (Whatman No. 1, Whatman International Ltd. Maidstone, England).

3.2.6 HPLC Analysis

The concentration of glycolic acid in the supernatant was measured by high-performance liquid chromatography (Agilent 1200, Model G1311A, Switzerland) using a ZORBAX ECLIPSE XDB-C18 column (4.6 x 250 mm) (Agilent Technologies, USA) at 70 °C. The eluent was 5 mM sulphuric acids. It was filtered through a 0.45 µm membrane, degassed under vacuum before use. Flow-rate was 0.5 ml/min and injection volume was 10 µl. The system was calibrated using the external standard method. Five injections of each eluate were then made onto the HPLC column to determine linearity and precision. When the absorbance of the eluate was monitored at 210 nm, glycolic acid was observed at retention time of 3.826 min. GA (0.2 to 1.0 M) from Sigma-Aldrich were used as standard.

3.2.7 Experimental Design and Statistical Analysis

RSM was used in designing this experiment using Design Expert 6.06 software (Stat-Ease Inc., Minneapolis, MN, USA) to generate the experimental designs, statistical analysis and regression model.

Table 3.1: The central composite experimental design (CCD) (in coded level of three variables) employed for extraction of glycolic acid in natural sources.

No.	Temperature (^{0}C)	Solvent concentration (M)	Time (min)
1	60	0.6	45
2	60	0.6	45
3	60	0.6	45
4	60	0.6	45
5	60	0.6	45
6	60	0.6	45
7	60	0.6	60
8	40	0.6	45
9	60	0.2	45
10	60	1.0	45
11	60	0.6	30
12	80	0.6	45
13	80	1.0	30
14	40	1.0	60
15	80	0.2	60
16	40	0.2	30

The central composite design (CCD) with a quadratic model (Box & Draper, 1987) was used. In this study, the effects of temperature(x_1), solvent concentration, ethylene glycol(x_2) and times (x_3) were investigated on the response of GA yield on six different natural sources (fresh banana peel (A_1), fresh banana pulp (B_1), fresh sugarcane (C_1), rotten banana peel (A_2), rotten banana pulp (B_2) and rotten sugarcane (C_2) using RSM.

Each independent variable had 3 levels, which were -1, 0 and +1. Table 3.1 show, a total of 16 different combinations were chosen in random order according to a CCD configuration for three factors. The response function (y) measured was concentration of glycolic acid. These values were interrelated to the coded variables (xi, i = 1, 2 and 3) by a quadratic polynomial using the Equation 3.1.

$$y = b_0 + b_1 x_1 + b_2 x_2 + b_3 x_3 + b_{12} x_1 x_2 + b_{13} x_1 x_3 + b_{23} x_2 x_3 + b_{11} x_1^2 + b_{22} x_2^2 + b_{33} x_3^2 \quad (3.1)$$

The coefficients of the polynomial were symbolized by b_0 (constant term), b_1, b_2 and b_3 (linear effects), b_{11}, b_{22} and b_{33} (quadratic effects), and b_{12}, b_{13} and b_{23} (interaction effects). The analysis of variance (ANOVA) tables were created and the effect and regression coefficients of individual linear, quadratic and interaction terms were found out. The significances of all terms in the polynomial were evaluated statistically by calculating the F-value at a probability (p) of 0.001, 0.01 or 0.05. The regression coefficients were applied to make statistical calculation to generate contour maps from the regression models.

CHAPTER 4

RESULT AND DISCUSSION

4.1 SCREENING

4.1.1 Distribution of Glycolic Acid-Producing Ability in Natural Sources

Sixteen experiments were carried out according to the conditions indicated in Table 3.1 (Section 3.2.7). Response values (glycolic acid concentration) were reported in the last column of this table. Six natural sources that been used were fresh banana peel (A_1), fresh banana pulp (B_1), fresh sugarcane juice (C_1), rotten banana peel (A_2), rotten banana pulp (B_2) and rotten sugarcane juice (C_2).

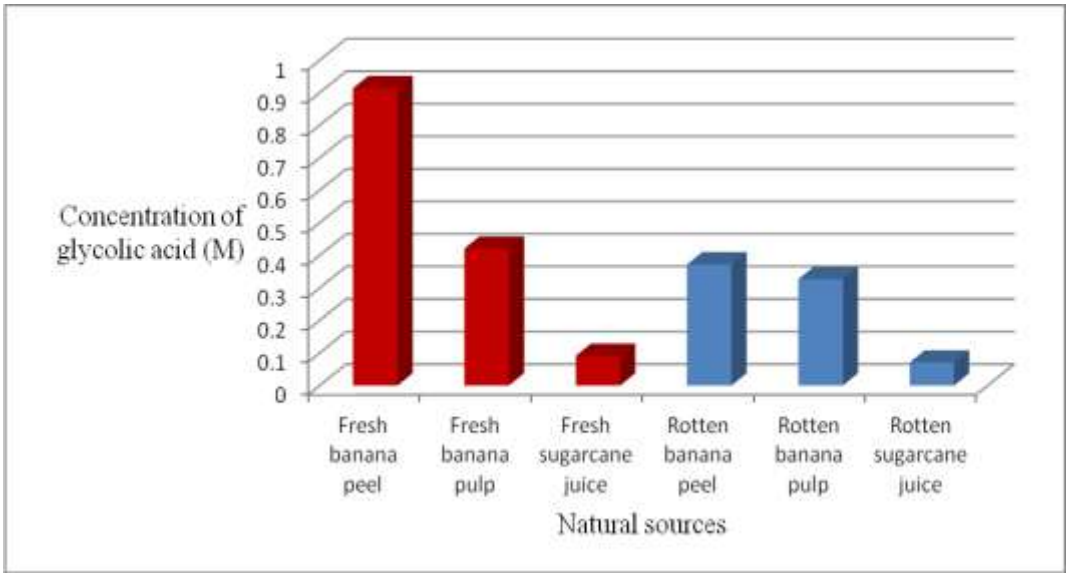

Figure 4.1: Concentration of glycolic acid (GA) in each sample after RSM

In agreement with all previously published data, glycolic acid (GA) is usually found in sugarcane juice. However, after extraction with ethylene glycol, as shown in Figure 4.1, fresh banana peel produced high yield of glycolic acid at 0.914 M compared to other sources. Other sources like fresh banana pulp produced 0.4211 M, fresh sugarcane 0.0911 M, rotten banana peel 0.3723 M, rotten banana pulp 0.3270 M and rotten sugarcane produced 0.0710 M of glycolic acid.

According to Blake and Clarke (1987), glycolic acid might co-elute with other acids and complicated by the presence of non acidic components. In their research, they used ion chromatography to separate organic acid from the neutral and basic compound. This method also can overcome the qualification of poorly resolved peek and identification problems. Although ion chromatography do not been applied in this research, the production of glycolic acid still can be obtained with the highest amount of GA compared with other studies. Thus the elimination of ion chromatography in this study seems to be more appropriate where it can decrease the cost of extraction of glycolic acid.

However, although ion chromatography was applied, aconitic acid is the major constituent in fresh sugarcane (Blake and Clarke, 1987). Another research that had been conducted by Van der Poel et al. (1998) reported that, aconitic acid is responsible for the most of the titratable acidity and resultant buffering capacity of the expressed juice. It has a high molar absorptivity within the wavelength range 200-220 nm most commonly used for detection where it could interfere resultant time.

4.1.2 Statistical Analysis

The experimental values for concentration of glycolic acid (GA) from six samples under different extraction conditions are presented in Table 4.1.

Table 4.1: Effect of temperature, solvent concentration and extraction time on six samples

No	X_1 (^{0}C)	X_2 (M)	X_3 (min)	Concentration of glycolic acid (M)					
				A_1	B_1	C_1	A_2	B_2	C_2
1	60	0.6	45	0.3926	0.1633	0.0441	0.0695	0.1829	0.0524
2	60	0.6	45	0.3926	0.1633	0.0441	0.0695	0.1829	0.0524
3	60	0.6	45	0.3926	0.1633	0.0441	0.0695	0.1829	0.0524
4	60	0.6	45	0.3926	0.1633	0.0441	0.0695	0.1829	0.0524
5	60	0.6	45	0.3926	0.1633	0.0441	0.0695	0.1829	0.0524
6	60	0.6	45	0.3926	0.1633	0.0441	0.0695	0.1829	0.0524
7	60	0.6	60	0.9149	0.2559	0.0911	0.0679	0.2163	0.0579
8	40	0.6	45	0.2128	0.3884	0.0289	0.0677	0.2848	0.0190
9	60	0.2	45	0.0946	0.2232	0.0317	0.0171	0.2092	0.0340
10	60	1.0	45	0.8110	0.1234	0.0441	0.0199	0.1094	0.0371
11	60	0.6	30	0.0911	0.3288	0.0708	0.3723	0.2288	0.0710
12	80	0.6	45	0.3515	0.2119	0.0351	0.0720	0.2041	0.0540
13	80	1.0	30	0.7530	0.4189	0.0461	0.0221	0.3151	0.0230
14	40	1.0	60	0.2902	0.4211	0.0493	0.0201	0.3270	0.0270
15	80	0.2	60	0.2587	0.4033	0.0671	0.0287	0.3053	0.0480
16	40	0.2	30	0.2041	0.4132	0.0689	0.0239	0.3110	0.0460

Subscripts: X_1 = temperature, X_2 = solvent concentration, X_3 = time

Based on the result in Table 4.1, it is clearly shown that sample A_1 and C_1 produced high yield of glycolic acid at 60 °C in 60 min using 0.6 M ethylene glycol. Sample A_2 and C_2 produced high yield of glycolic acid at same temperature and solvent concentration with sample A_1 and C_1 but the extration time is at 30 min. Sample B_1 and B_2 produced high yield of glycolic acid at temperature 40 °C, 1 M of ethylene glycol and 60 min extration time. Since fresh banana peel appeared to be superior GA production compared to the other sources, it was selected for further production of GA.

4.2 REGRESSION ANALYSIS OF FRESH BANANA PEEL

The regression coefficients for the second order polynomial equation and results for the linear, quadratic and interaction terms are presented in Table 4.2.

Table 4.2: Analysis of variance (ANOVA) for model of relationship between response variables (glycolic acid extraction) and independent variables (X_1, X_2, X_3) and variance analysis of items of regression equation of glycolic acid yield from fresh banana peel

Item	Quadratic Sum	DF	Mean Square	*F* Value	Prob > *F*
X_1	0.0096	1	0.0096	42.05	0.0013
X_2	0.2566	1	0.2566	1121.81	< 0.0001
X_3	0.3393	1	0.3393	1483.38	< 0.0001
X_1^2	0.0452	1	0.0452	197.77	< 0.0001
X_2^2	0.0036	1	0.0036	15.90	0.0104
X_3^2	0.0198	1	0.0198	86.45	0.0002
X_1X_2	0.3522	1	0.3522	1539.64	< 0.0001
X_1X_3	0.0605	1	0.0605	264.69	< 0.0001
X_2X_3	0.0048	1	0.0048	20.98	0.0059
Model	0.8521	9	0.0947	413.88	< 0.0001
Residual	0.0011	5	0.0002		
Lack of Fit	0.0011	1	0.0011		
Pure Error	0	4	0		
Cor Total	0.8552	15			

Subscripts: X_1 = temperature, X_2 = solvent concentration, X_3 = time

Regression analysis in Table 4.2 was made to the experimental data intend at an optimal region for the responses studied. The significance of each coefficient was verified using the *F*-test and *p*-value in Table 4.2. The corresponding parameter variables would be more significant if the absolute *F*-value becomes greater as the *p*-value becomes smaller (Atkinson & Donev, 1992). The statistical analysis shows that the proposed model was adequate, possessing insignificant lack-of-fit and with a very satisfactory value of the R^2 at 0.9987. It showed that the variables with the largest effect were linear terms of solvent concentration (X_2) and extraction time (X_3), the quadratic term of temperature (X_1^2) and the interaction effects of temperature with solvent concentration (X_1X_2) and temperature with time (X_1X_3). From Table 4.2 the model is significant when Prob>*F* or *p*-value less than 0.05 and not significant when the value greater than 0.1. The Model *F*-value of 413.88 implies the model is significant and only 0.01 % to achieve this large could occur due noise. From the Table 4.2, it comes out with the following second order polynomial Equation 4.1 based on coded values obtained from the analysis:

$$Y = 0.4 + 0.069X_1 + 0.36X_2 + 0.41X_3 - 0.13X_1^2 + 0.038X_2^2 + 0.088X_3^2 + 0.51X_1X_2 + 0.21X_1X_3 - 0.06X_2X_3 \quad (4.1)$$

4.3 ANALYSIS OF ONE FACTOR PLOT

4.3.1 Effect of Temperature When Solvent Concentration and Time Constant

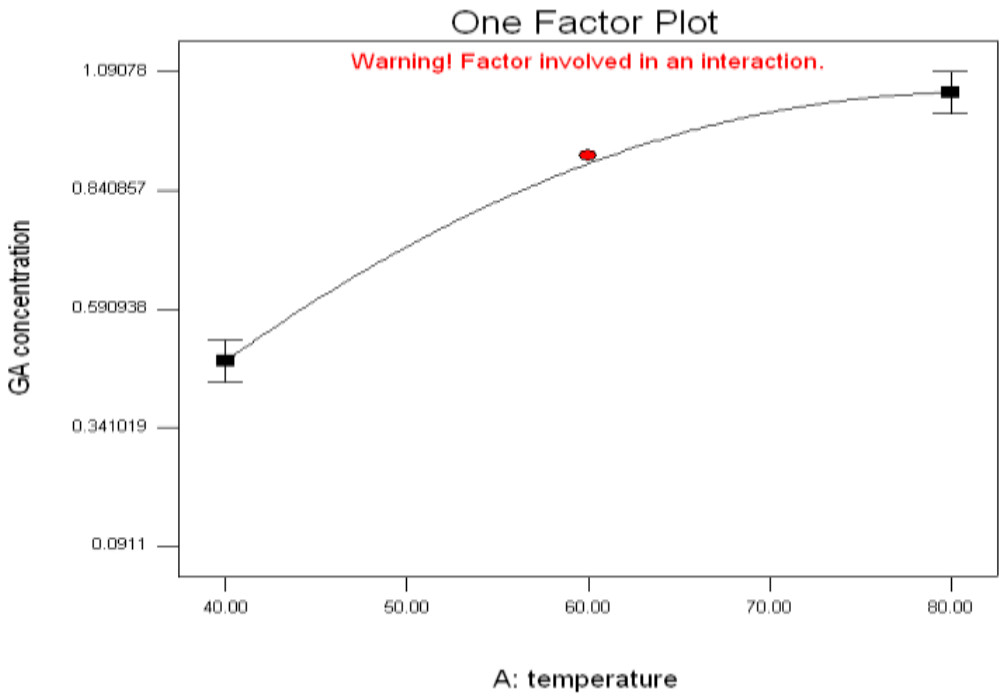

Figure 4.2: Response surface of concentration of glycolic acid as a function of temperature when solvent concentration and time constant at 0.6 M and 45 min

Figure 4.2 illustrates the linear effect of temperature on the concentration of glycolic acid (GA) from fresh banana peel extract. It can be seen in Figure 4.2, as the temperature increase the production of glycolic acid also increase when solvent concentration at 0.6 M and time at 60 min. Previous research done by Coulson and Richardson (1979) and Qui et al. (2010) indicated that this may be because of the improvement of solubility of the extracted glycolic acid by increasing temperature in certain range and the increasing of diffusion coefficient also improve the extraction rate.

4.3.2 Effect of Solvent Concentration When Temperature and Time Constant

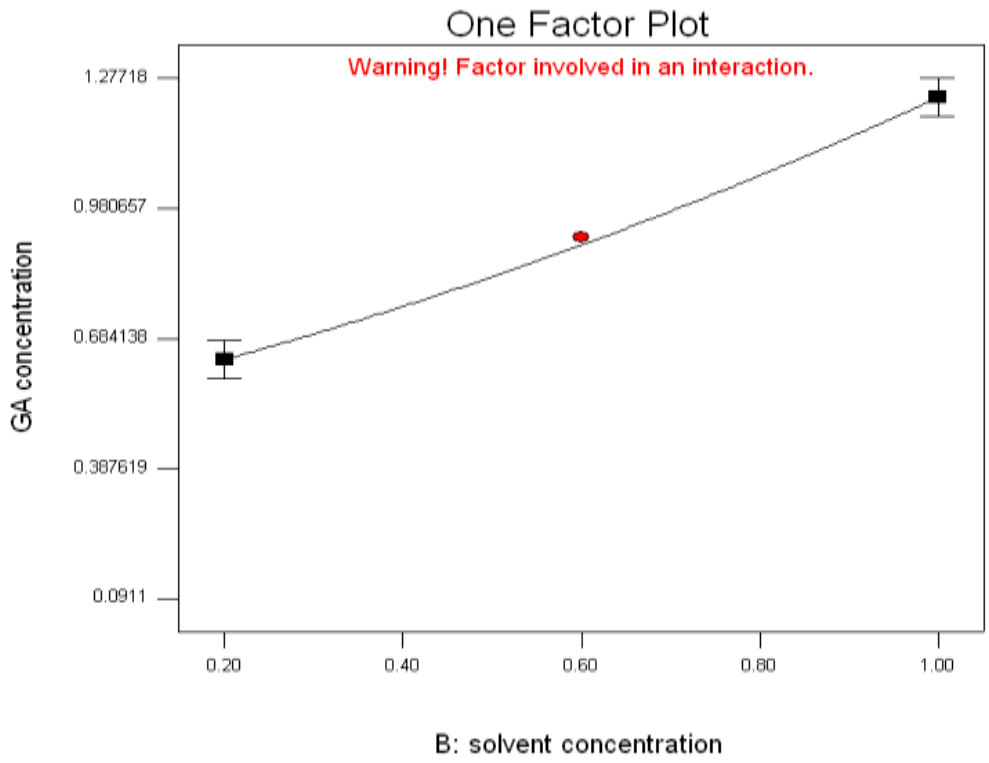

Figure 4.3: Response surface of concentration of glycolic acid as a function of solvent concentration when temperature and time constant at 0.6 °C and 45 min

Figure 4.3 explains the linear effect of solvent concentration on the concentration of glycolic acid (GA) from fresh banana peel extract. It can be seen in Figure 4.3, as the temperature increase the production of glycolic acid also increase when temperature at 60 °C and time at 60 min. According to Aguilar et. al. (2002), acid can break down heterocyclic ether bonds between sugar monomers in the polymeric chain, which are formed by hemicellulose and cellulose. Previous study says that suitable acidic conditions favored the hydrolysis of the insoluble pectin, thus raising the pectin recovery (El Nawawi and Shehata, 1988)

4.3.3 Effect of Time When Solvent Concentration and Temperature Constant

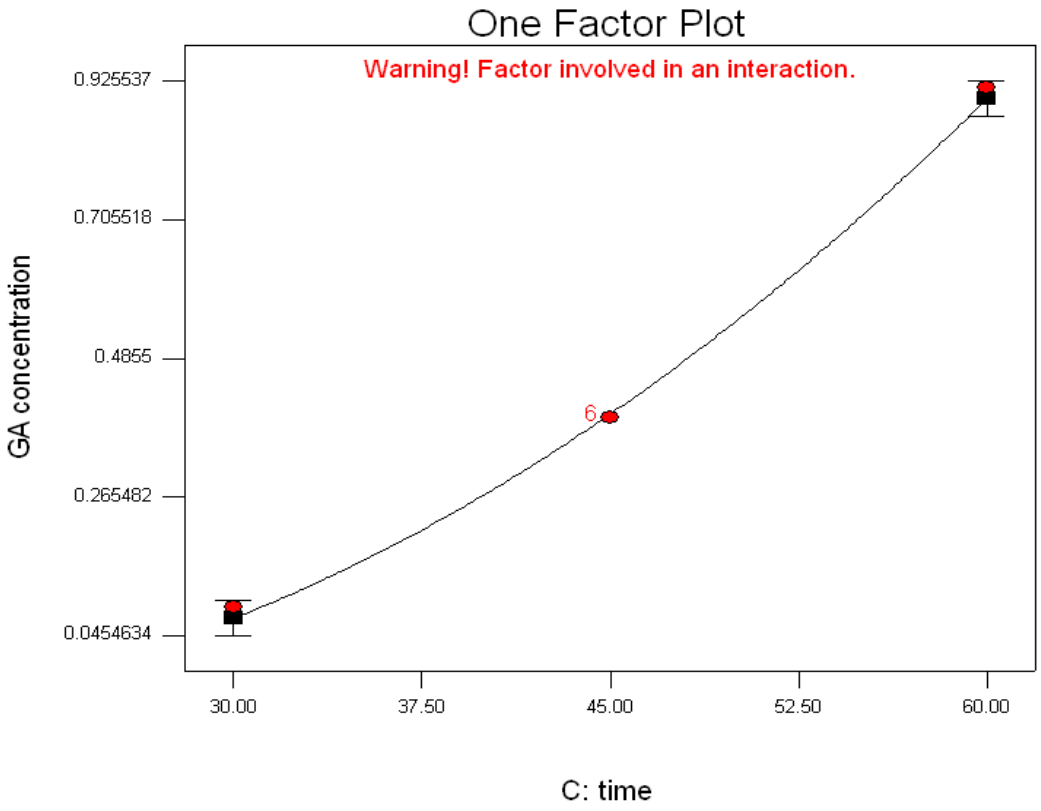

Figure 4.4: Response surface of concentration of glycolic acid as a function of time when temperature and solvent concentration constant at 0.6 °C and 0.6 M

Figure 4.4 shows the linear effect of solvent concentration on the concentration of glycolic acid (GA) from fresh banana peel extract. It can be seen in Figure 4.4, as the time increase the production of glycolic acid also increase when temperature at 60 °C and solvent concentration at 0.6 M. However, too long extraction time could cause degradation of glycolic acid molecules and thus affecting the production of glycolic acid.

4.4 ANALYSIS OF RESPONSE SURFACE

The relationship between concentration of glycolic acid (GA) and independent variables can be illustrated graphically by plotting three-dimensional response surface plots (Figure 4.5).

4.4.1 Effect of Temperature, Solvent Concentration and Extraction Time

The effects of the different extraction conditions on production of glycolic acid (GA) are reported in Table 4.2 by the coefficient of the second order polynomials.

Figure 4.5 shows the contour map for the effect of the independent variables on the concentration of glycolic acid. As shown in Table 4.2, concentration of glycolic acid was positively related to the linear effect of temperature (p=0.0013), solvent concentration (p<0.0001) and extraction time (p<0.0001) and the quadratic terms of these variables were found to be significant resulting in linear increase in production of glycolic acid with 1 M of solvent concentration at all temperature and decrease in production with 0.2 M of solvent concentration as the temperature increase (Figure 4.5(a)). It can be seen in Table 4.2, that there is an interaction effect between solvent concentration and temperature on production of glycolic acid. At the highest level of solvent concentration, the production of glycolic acid was found to increase rapidly with an increase in temperature. At the highest temperature, the production of glycolic acid was also found to increase rapidly with an increase in solvent concentration. The interaction term of solvent concentration and temperature is p<0.0001. One possible explanation for the increase of extraction rate was the improvement of solubility of the extracted glycolic acid by increasing temperature in a certain range of solvent concentration. The maximum production of glycolic acid was reached at optimum conditions (solvent concentration of 1 M and temperature of 80 °C).

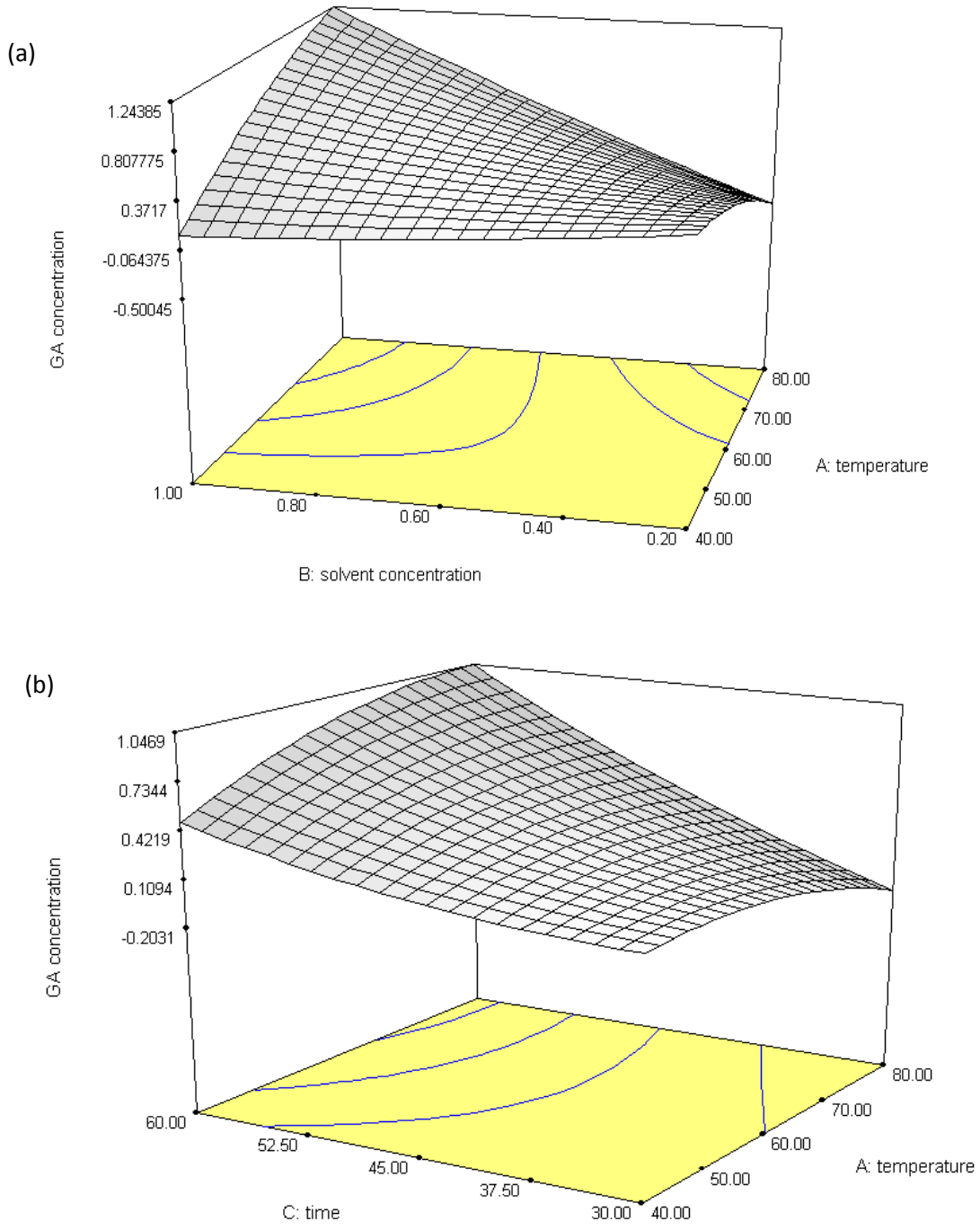

Figure 4.5: Response surface of concentration of glycolic acid as a function of (a) temperature and solvent concentration when time constant at 45 min and (b) temperature and time when solvent concentration at 0.6 M

The response surface in Figure 4.5(b) exhibits the combination effects of extraction time and temperature, indicating that longer extraction time at all temperature led to increase in extraction of glycolic acid. At the longest time, the production of glycolic acid was also found to increase rapidly with an increase in temperature. One of the reason was glycolic acid was not so easy to be dissolved out from plant cell wall if the extraction temperature was too low and extraction time was too short (Qui et al., 2010). The interaction term of extraction time and temperature is $p<0.0001$.

4.5 OPTIMIZATION

There were eighteen solutions generated from numerical optimization that could give maximum levels of the production of glycolic acid (GA). The best combination of variables was found to be at temperature 72.39 °C, solvent concentration at 0.99 M, extraction time at 49.53 min and desirability of 1.000.

The accuracy of the model equation for predicting the optimum response values was tested using the selected optimal conditions. Three additional experiments using the expected optimum conditions for glycolic acid extraction were carried out using modified combination variables (Figure 4.6). The modified variables were temperature at 70 °C, solvent concentration at 1 M and extraction time at 50 min.

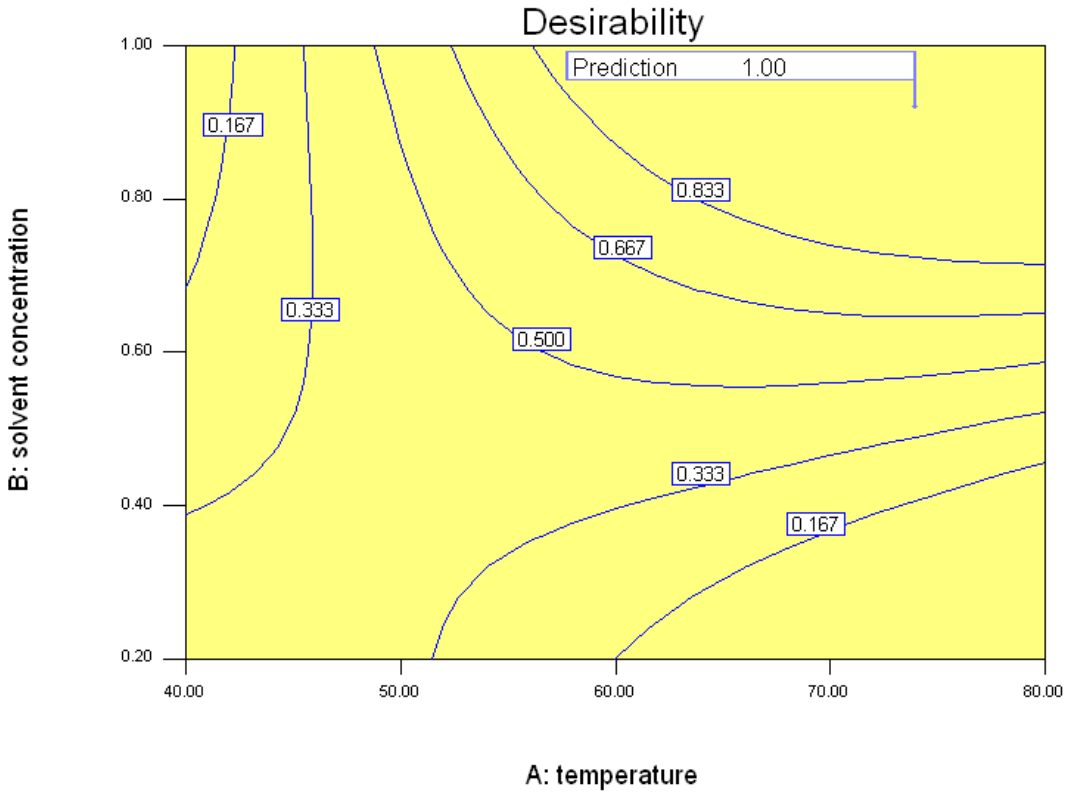

Figure 4.6: The contour plots for optimization of glycolic acid desirability of fresh banana peel keeping the time constant at the central point at 49.53 min

Figure 4.6 shows the contour plots for optimization of glycolic acid desirability of fresh banana peel keeping the time constant at the central point. The zone of optimization, as shown in the contour plot, depicts solvent concentration to be a range of 0.75 M and 1 M and temperature between 58 °C and 80 °C.

4.6 VALIDATION

To validate the practicability and veracity of the equation, validation experiment was done at optimum conditions within the experimental range obtained from the study. This is significantly in agreement with the calculated values (p>0.05).

Figure 4.7 shows the optimum conditions of the clarification process to yield maximum production of glycolic acid, respectively. It was distinguished that the optimum conditions for elucidation were slightly different.

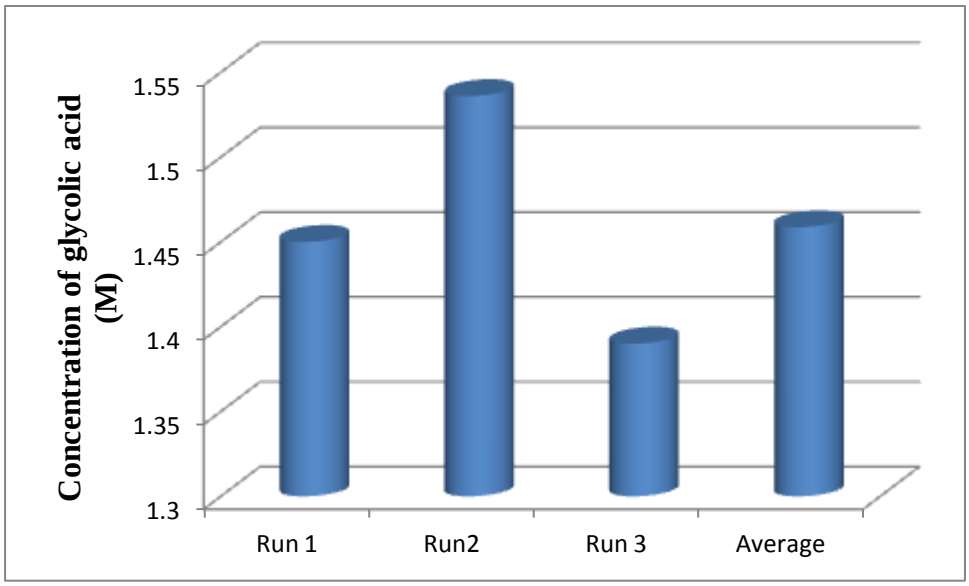

Figure 4.7: Three experiment were carried out using modified variables at temperature 70 ºC, 1 M of solvent concentration and extraction time at 50 min.

The results of analysis confirm that the response models were adequate for reflecting the expected optimization, and the model of equation from ANOVA was satisfactory and accurate.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSION

This research revealed that RSM was an effective tool for estimating the interaction effect of key independence variables in the extraction of glycolic acid (GA) from fresh banana peel. Extraction temperature and solvent concentration showed more significant combined effects on the response value. From ANOVA, equation 4.1 (Section 4.2) was derived.

Present study show that fresh banana peel obtains the highest concentration of GA (0.914 M) compare to other samples. A high correlation of quadratic polynomial mathematical model was obtained and could be employed to optimize the production of glycolic acid from fresh banana peel. According to central composite design in this study, the average value of concentration of glycolic acid from three experiments was 1.46 M, respectively, at temperature 70 ^{o}C, solvent concentration at 1 M and extraction time at 50 min.

5.2 RECOMMENDATIONS

There are a lot of improvements that can be done for the future research. The analysis of samples must be done directly after separation so that glycolic acid (GA) compound would not disappear due to configuration change or react with other

chemical in the samples. The optimization of GA can be further investigated by using different types of solvent, extraction method and ultrasonic frequency.

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APPENDIX A

APPENDIX A1

FORMULA

$$Molarity(M_1) = \frac{density(\rho)\ x\ \%\ x\ 1000}{MW}$$

$$M_1V_1 = M_2V_2$$

$$mass(m) = \frac{mol\ x\ volume(V)\ x\ MW}{1000}$$

APPENDIX A2

PHYSICAL AND CHEMICAL PROPERTIES

ETHYLENE GLYCOL

Physical state and appearance: Liquid. (syrupy)
Odour: Odourless
Taste: Mild sweet
Molecular Weight (MW): 62.07 g/mol
Colour: Clear Colourless
Boiling Point (bp.): 197.6 °C
Melting Point: -13 °C
Specific Gravity (SG): 1.1088
Volatility: Not Available
Solubility:
Soluble in cold water, hot water, acetone. Slightly soluble in diethyl ether. Miscible with lower aliphatic alcohols, glycerol, acetic acid, acetone and similar ketones, aldehydes, pyridine, similar coal tar bases. Practically insoluble in benzene and its homologs, chlorinated hydrocarbons, petroleum ether.

GLYCOLIC ACID

Physical state and appearance: Solid (Crystalline solid)
Odour: Odourless
Taste: Not Available
Molecular Weight (MW): 72.05 g/mol
Colour: White to yellowish
Boiling Point (bp.): Not available
Melting Point: 79 °C
Specific Gravity (SG): 1.49
Volatility: Not Available
Solubility:
Easily soluble in cold water. Soluble in methanol, diethyl ether, acetone.

APPENDIX B

APPENDIX B1

DESIGN EXPERT 6.0.6

DESIGN

	Std	Run	Block	Factor 1 A:temperature deg C	Factor 2 B:solvent conc M	Factor 3 C:time min	Response 1 GA concentrati M
	1	1	Block 1	80.00	1.00	30.00	0.753
	3	2	Block 1	40.00	1.00	60.00	0.2902
	6	3	Block 1	60.00	0.60	45.00	0.3926
	2	4	Block 1	80.00	0.20	60.00	0.2587
	5	5	Block 1	60.00	0.60	45.00	0.3926
	7	6	Block 1	60.00	0.60	45.00	0.3926
	4	7	Block 1	40.00	0.20	30.00	0.2041
	13	8	Block 2	60.00	0.60	60.00	0.9149
	14	9	Block 2	60.00	0.60	45.00	0.3926
	15	10	Block 2	60.00	0.60	45.00	0.3926
	8	11	Block 2	40.00	0.60	45.00	0.2128
	10	12	Block 2	60.00	0.20	45.00	0.0946
	11	13	Block 2	60.00	1.00	45.00	0.811
	12	14	Block 2	60.00	0.60	30.00	0.0911
	9	15	Block 2	80.00	0.60	45.00	0.3515
	16	16	Block 2	60.00	0.60	45.00	0.3926

STATUS

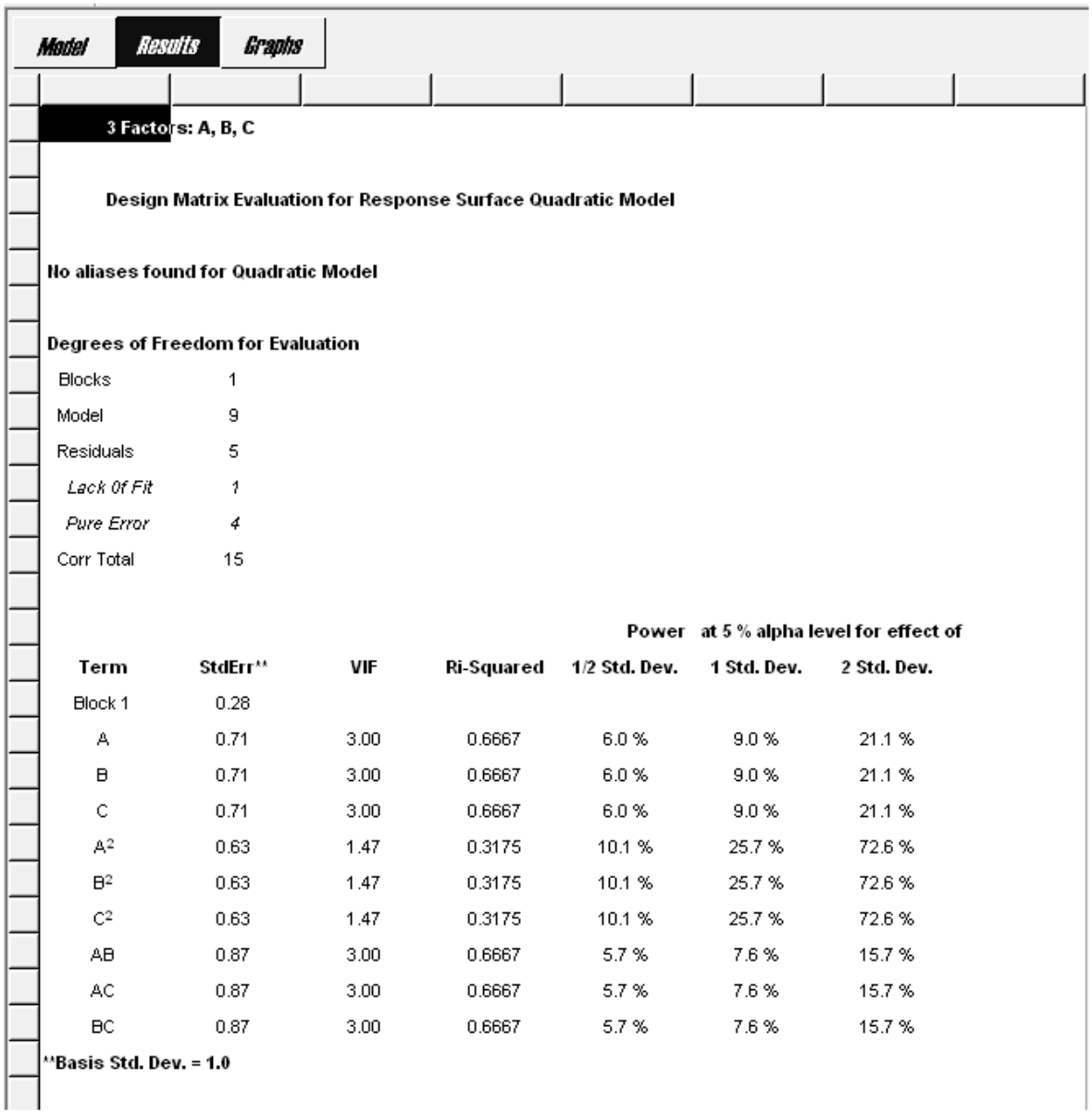
Design Summary

Study Type	Response Surface	**Experiments**	16
Initial Design	Central Composite	**Blocks**	2
Design Model	Quadratic		

Response	**Name**	**Units**	**Obs**	**Minimum**	**Maximum**	**Trans**	**Model**
Y1	GA concentratio	M	16	0.091	0.91	None	Quadratic

Factor	**Name**	**Units**	**Type**	**Low Actual**	**High Actual**	**Low Coded**	**High Coded**
A	temperature	deg C	Numeric	40.00	80.00	-1.000	1.000
B	solvent concenti	M	Numeric	0.20	1.00	-1.000	1.000
C	time	min	Numeric	30.00	60.00	-1.000	1.000

EVALUATION



Model | Results | Graphs

3 Factors: A, B, C

Design Matrix Evaluation for Response Surface Quadratic Model

No aliases found for Quadratic Model

Degrees of Freedom for Evaluation

Blocks	1
Model	9
Residuals	5
Lack Of Fit	*1*
Pure Error	*4*
Corr Total	15

				Power at 5 % alpha level for effect of		
Term	**StdErr****	**VIF**	**Ri-Squared**	**1/2 Std. Dev.**	**1 Std. Dev.**	**2 Std. Dev.**
Block 1	0.28					
A	0.71	3.00	0.6667	6.0 %	9.0 %	21.1 %
B	0.71	3.00	0.6667	6.0 %	9.0 %	21.1 %
C	0.71	3.00	0.6667	6.0 %	9.0 %	21.1 %
A^2	0.63	1.47	0.3175	10.1 %	25.7 %	72.6 %
B^2	0.63	1.47	0.3175	10.1 %	25.7 %	72.6 %
C^2	0.63	1.47	0.3175	10.1 %	25.7 %	72.6 %
AB	0.87	3.00	0.6667	5.7 %	7.6 %	15.7 %
AC	0.87	3.00	0.6667	5.7 %	7.6 %	15.7 %
BC	0.87	3.00	0.6667	5.7 %	7.6 %	15.7 %

****Basis Std. Dev. = 1.0**

Measures Derived From the $(X'X)^{-1}$ Matrix

Std	Leverage	Point Type
1	0.9877	Fact
2	0.9877	Fact
3	0.9877	Fact
4	0.9877	Fact
5	0.3115	Center
6	0.3115	Center
7	0.3115	Center
8	0.9508	Axial
9	0.9508	Axial
10	0.9508	Axial
11	0.9508	Axial
12	0.9508	Axial
13	0.9508	Axial
14	0.1366	Center
15	0.1366	Center
16	0.1366	Center
Average =	**0.6875**	

Maximum Prediction Variance (at a design point) = 0.988

Average Prediction Variance = 0.688

Condition Number of Coefficient Matrix = 12.220

G Efficiency (calculated from the design points) = 69.6 %

Scaled D-optimality Criterion = 4.124

Determinant of $(X'X)^{-1}$ = 3.335E-7

Trace of $(X'X)^{-1}$ = 5.146

Correlation Matrix of Regression Coefficients

	Intercept	Block 1	A	B	C	A^2	B^2
Intercept	1.000						
Block 1	0.408	1.000					
A	-0.000	-0.000	1.000				
B	-0.000	-0.000	-0.000	1.000			
C	-0.000	-0.000	-0.000	-0.000	1.000		
A^2	-0.287	-0.171	0.000	0.000	0.000	1.000	
B^2	-0.287	-0.171	0.000	0.000	0.000	-0.280	1.000
C^2	-0.287	-0.171	-0.000	-0.000	-0.000	-0.280	-0.280
AB	-0.000	-0.000	-0.000	-0.000	0.816	-0.000	-0.000
AC	-0.000	-0.000	-0.000	0.816	-0.000	-0.000	-0.000
BC	-0.000	-0.000	0.816	-0.000	-0.000	-0.000	-0.000

	C^2	AB	AC	BC
C^2	1.000			
AB	-0.000	1.000		
AC	-0.000	-0.000	1.000	
BC	-0.000	-0.000	-0.000	1.000

Correlation Matrix of Factors [Pearson's r]

	Block 1	A	B	C	A^2	B^2	C^2
Block 1	1.000						
A	0.000	1.000					
B	0.000	0.000	1.000				
C	0.000	0.000	0.000	1.000			
A^2	0.358	0.000	0.000	0.000	1.000		
B^2	0.358	0.000	0.000	0.000	0.467	1.000	
C^2	0.358	0.000	0.000	0.000	0.467	0.467	1.000
AB	0.000	0.000	0.000	-0.816	0.000	0.000	0.000
AC	0.000	0.000	-0.816	0.000	0.000	0.000	0.000
BC	0.000	-0.816	0.000	0.000	0.000	0.000	0.000

	AB	AC	BC
AB	1.000		
AC	0.000	1.000	
BC	0.000	0.000	1.000

ANALYSIS

Transform | Fit Summary | Model | ANOVA | Diagnostics | Model Graphs

Response: GA concentrat

*** WARNING: The Cubic Model is Aliased! ***

Sequential Model Sum of Squares

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Mean	2.51	1	2.51			
Block	2.005E-003	1	2.005E-003			
Linear	0.38	3	0.13	2.96	0.0795	
2FI	0.42	3	0.14	20.30	0.0004	Suggested
Quadratic	0.054	3	0.018	78.27	0.0001	Suggested
Cubic	1.144E-003	1	1.144E-003	6.366E+007	< 0.0001	Aliased
Residual	0.000	4	0.000			
Total	3.37	16	0.21			

"Sequential Model Sum of Squares": Select the highest order polynomial where the additional terms are significant and the model is not aliased.

Lack of Fit Tests

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F
Linear	0.47	7	0.067		
2FI	0.055	4	0.014		
Quadratic	1.144E-003	1	1.144E-003		
Cubic	0.000	0			
Pure Error	0.000	4	0.000		

"Lack of Fit Tests": Want the selected model to have insignificant lack-of-fit.

Model Summary Statistics

Source	Std. Dev.	R-Squared	Adjusted R-Squared	Predicted R-Squared	PRESS	
Linear	0.21	0.4463	0.2953	-1.0284	1.73	
2FI	0.083	0.9357	0.8875	0.5618	0.37	Suggested
Quadratic	0.015	0.9987	0.9962	0.3991	0.51	Suggested
Cubic	0.000	1.0000	1.0000		+	Aliased

+ Case(s) with leverage of 1.0000: PRESS statistic not defined

"Model Summary Statistics": Focus on the model maximizing the "Adjusted R-Squared" and the "Predicted R-Squared".

Transform | Fit Summary | Model | ANOVA | Diagnostics | Model Graphs

Response: GA concentration

ANOVA for Response Surface Quadratic Model

Analysis of variance table [Partial sum of squares]

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F
Block	2.005E-003	1	2.005E-003		
Model	0.85	9	0.095	413.88	< 0.0001
A	*9.619E-003*	*1*	*9.619E-003*	*42.05*	*0.0013*
B	*0.26*	*1*	*0.26*	*1121.81*	*< 0.0001*
C	*0.34*	*1*	*0.34*	*1483.38*	*< 0.0001*
A^2	*0.045*	*1*	*0.045*	*197.77*	*< 0.0001*
B^2	*3.638E-003*	*1*	*3.638E-003*	*15.90*	*0.0104*
C^2	*0.020*	*1*	*0.020*	*86.45*	*0.0002*
AB	*0.35*	*1*	*0.35*	*1539.64*	*< 0.0001*
AC	*0.061*	*1*	*0.061*	*264.69*	*< 0.0001*
BC	*4.800E-003*	*1*	*4.800E-003*	*20.98*	*0.0059*
Residual	1.144E-003	5	2.288E-004		
Lack of Fit	*1.144E-003*	*1*	*1.144E-003*		
Pure Error	*0.000*	*4*	*0.000*		
Cor Total	0.86	15			

Std. Dev.	0.015	R-Squared	0.9987
Mean	0.40	Adj R-Squared	0.9962
C.V.	3.82	Pred R-Squared	0.3991
PRESS	0.51	Adeq Precision	65.691

Factor	Coefficient Estimate	DF	Standard Error	95% CI Low	95% CI High	VIF
Intercept	0.40	1	5.755E-003	0.38	0.41	
Block 1	-1.000E-002	1				
Block 2	1.000E-002					
A-temperature	0.069	1	0.011	0.042	0.097	3.00
B-solvent conce	0.36	1	0.011	0.33	0.39	3.00
C-time	0.41	1	0.011	0.38	0.44	3.00
A^2	-0.13	1	9.454E-003	-0.16	-0.11	1.47
B^2	0.038	1	9.454E-003	0.013	0.062	1.47
C^2	0.088	1	9.454E-003	0.064	0.11	1.47
AB	0.51	1	0.013	0.48	0.55	3.00
AC	0.21	1	0.013	0.18	0.25	3.00
BC	-0.060	1	0.013	-0.094	-0.026	3.00

Final Equation in Terms of Coded Factors:

GA concentration =

+0.40

+0.069 * A

+0.36 * B

+0.41 * C

-0.13 * A^2

+0.038 * B^2

+0.088 * C^2

+0.51 * A * B

+0.21 * A * C

-0.060 * B * C

Final Equation in Terms of Actual Factors:

GA concentration =

+2.05660

-0.027159 * temperature

-2.79188 * solvent concentration

-0.044320 * time

-3.32375E-004 * $temperature^2$

+0.23562 * $solvent\ concentration^2$

+3.90667E-004 * $time^2$

+0.064244 * temperature * solvent concentration

+7.10333E-004 * temperature * time

-1.00000E-002 * solvent concentration * time

Diagnostics Case Statistics

Standard Order	Actual Value	Predicted Value	Residual	Leverage	Student Residual	Cook's Distance	Outlier t	Run Order
1	0.75	0.76	-3.750E-003	0.988	-2.236	36.515	0.000	1
2	0.26	0.26	-3.750E-003	0.988	-2.236	36.515	0.000	4
3	0.29	0.29	-3.750E-003	0.988	-2.236	36.515	0.000	2
4	0.20	0.21	-3.750E-003	0.988	-2.236	36.515	0.000	7
5	0.39	0.39	5.000E-003	0.311	0.398	0.007	0.362	5
6	0.39	0.39	5.000E-003	0.311	0.398	0.007	0.362	3
7	0.39	0.39	5.000E-003	0.311	0.398	0.007	0.362	6
8	0.21	0.21	7.500E-003	0.951	2.236	8.788	0.000	11
9	0.35	0.34	7.500E-003	0.951	2.236	8.788	0.000	15
10	0.095	0.087	7.500E-003	0.951	2.236	8.788	0.000	12
11	0.81	0.80	7.500E-003	0.951	2.236	8.788	0.000	13
12	0.091	0.084	7.500E-003	0.951	2.236	8.788	0.000	14
13	0.91	0.91	7.500E-003	0.951	2.236	8.788	0.000	8
14	0.39	0.41	-0.015	0.137	-1.067	0.016	-1.086	9
15	0.39	0.41	-0.015	0.137	-1.067	0.016	-1.086	10
16	0.39	0.41	-0.015	0.137	-1.067	0.016	-1.086	16

Note: Predicted values include block corrections.

NUMERICAL

Criteria | Solutions | Graphs

Solutions 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Constraints

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
temperature	is in range	40	80	1	1	3
solvent concenti	is in range	0.2	1	1	1	3
time	is in range	30	60	1	1	3
GA concentratio	maximize	0.0911	0.9149	1	1	3

Solutions

Number	temperature	solvent concer	time	GA concentrati	Desirability	
1	62.61	0.67	59.70	0.986636	1.000	Selected
2	69.36	0.99	46.18	1.05885	1.000	
3	72.73	0.73	58.07	1.14034	1.000	
4	61.09	0.89	58.65	1.12202	1.000	
5	60.48	0.97	57.00	1.11931	1.000	
6	79.95	0.99	37.00	0.933801	1.000	
7	61.72	0.96	50.46	0.941829	1.000	
8	69.38	0.69	57.83	1.03142	1.000	
9	64.95	0.99	47.08	0.978189	1.000	
10	60.06	0.88	54.30	0.927269	1.000	
11	64.62	0.97	58.99	1.33545	1.000	
12	62.37	0.63	59.06	0.916329	1.000	
13	73.48	0.90	47.04	1.0035	1.000	
14	78.17	0.95	59.79	1.74235	1.000	
15	43.48	0.20	60.00	0.736705	0.784	
16	50.44	0.28	60.00	0.714126	0.756	

16 Solutions found

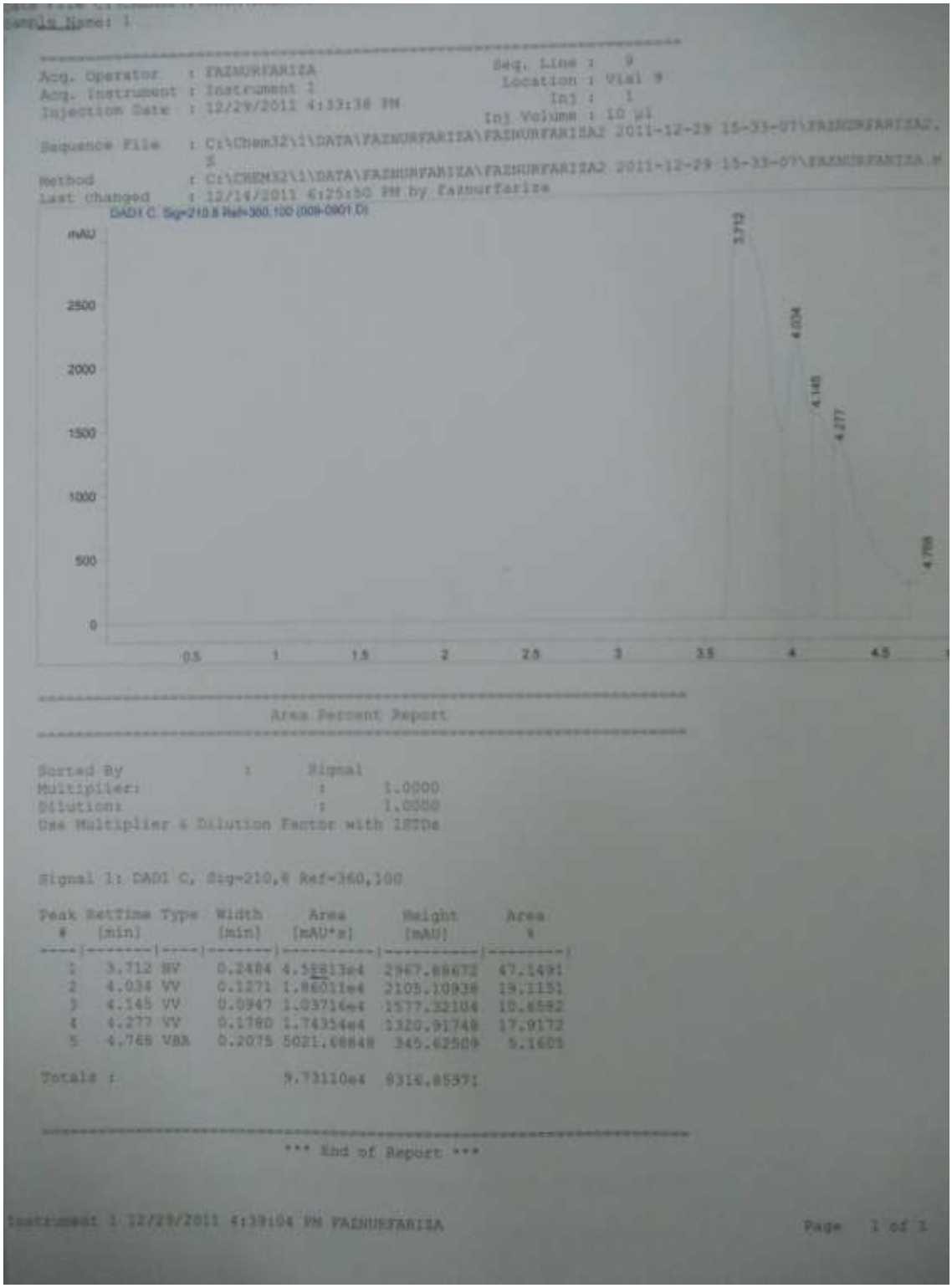
Number of Starting Points 20

temperature	solvent concer	time
63.16	0.37	48.98
75.14	0.77	47.85
40.95	0.45	51.82
40.74	0.92	46.58
60.48	0.97	57.00
76.18	0.91	31.84
50.85	0.72	43.89
69.38	0.69	57.83
60.22	0.67	38.21
67.47	0.78	38.72
56.01	0.69	35.43
79.00	0.40	40.95
41.36	0.99	50.66
47.06	0.69	58.34
61.27	0.21	40.85
78.62	0.70	41.17
44.30	0.21	38.71
44.24	0.46	34.84
47.08	0.24	52.84
78.17	0.95	59.79

APPENDIX C

APPENDIX C1

HPLC RESULT AND CALIBRATION CURVE



Sample Name: 1

Acq. Operator : FAIZNURFARIZA
Acq. Instrument : Instrument 1
Injection Date : 12/29/2011 4:33:38 PM
Seq. Line : 9
Location : Vial 9
Inj : 1
Inj Volume : 10 µl

Sequence File : C:\Chem32\1\DATA\FAIZNURFARIZA\FAIZNURFARIZA2 2011-12-29 15-33-07\FAIZNURFARIZA2.S
Method : C:\CHEM32\1\DATA\FAIZNURFARIZA\FAIZNURFARIZA2 2011-12-29 15-33-07\FAIZNURFARIZA.M
Last changed : 12/14/2011 6:25:50 PM by faiznurfariza

DAD1 C, Sig=210,8 Ref=360,100 (2009-0901.D)

mAU
2500
2000
1500
1000
500
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
3.712
4.034
4.145
4.277
4.765

Area Percent Report

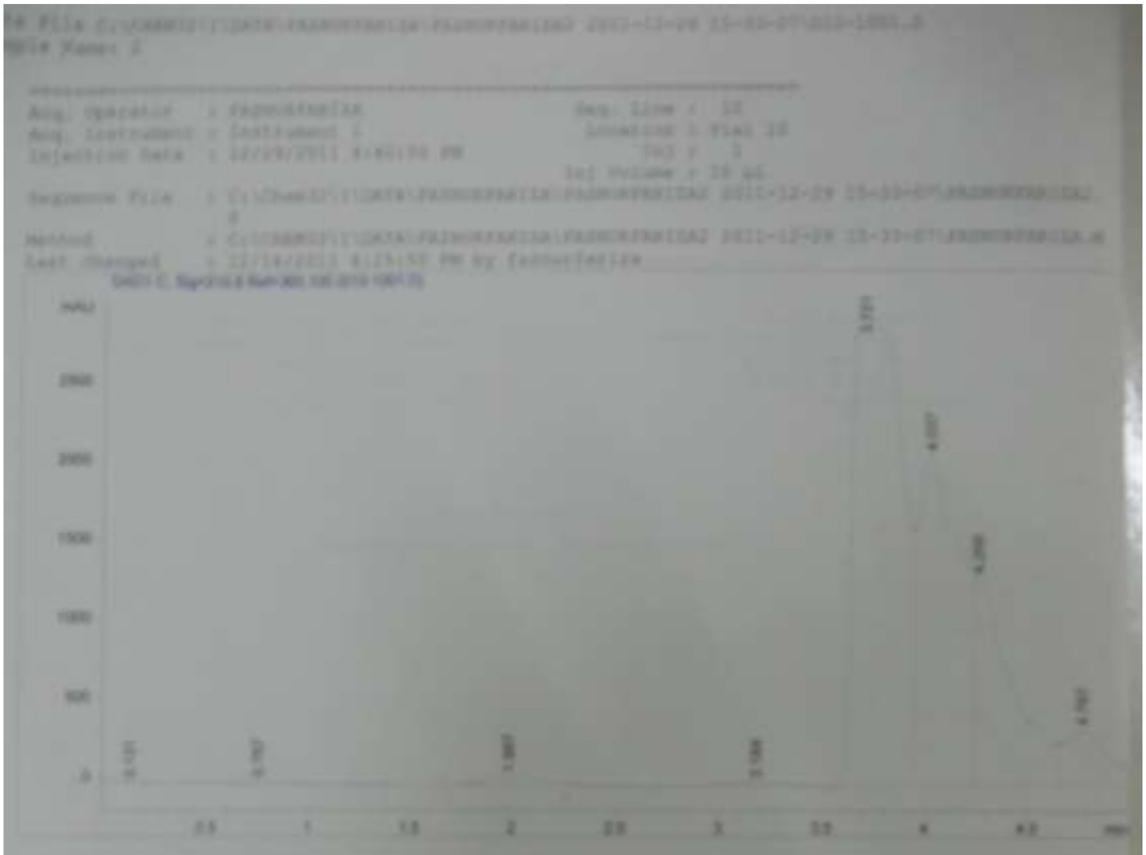
Sorted By : Signal
Multiplier: : 1.0000
Dilution: : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: DAD1 C, Sig=210,8 Ref=360,100

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	3.712	BV	0.2484	4.58813e4	2967.88672	47.1491
2	4.034	VV	0.1271	1.86011e4	2105.10938	19.1151
3	4.145	VV	0.0947	1.03716e4	1577.32104	10.6582
4	4.277	VV	0.1760	1.74354e4	1320.91748	17.9172
5	4.765	VBA	0.2075	5021.68848	345.62509	5.1605
Totals :				9.73110e4	8316.85571	

*** End of Report ***

Instrument 1 12/29/2011 4:39:04 PM FAIZNURFARIZA Page 1 of 1



Area Percent Report

Signal 1: DAD1 C, Sig=210,8 Ref=360,100

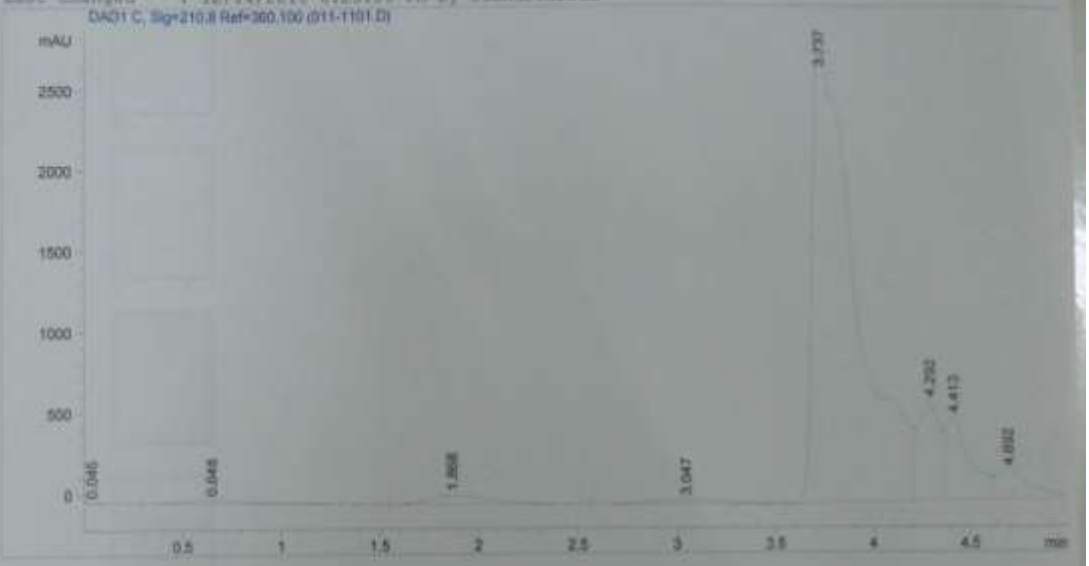
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	0.131	BV	0.1943	34.07095	2.48087	0.3337
2	0.757	VV	0.5388	309.84109	15.96892	0.3057
3	1.987	VB	0.3201	1129.48726	54.44860	1.1176
4	3.184	BV	0.3623	511.49963	22.41283	0.5060
5	3.721	VV	0.2457	4.8[illegible]e4	2938.08032	47.7694
6	4.037	VV	0.1932	3.0[illegible]e4	2048.18724	30.0166
7	4.266	VV	0.2006	1.55890e4	1299.09812	15.4227
8	4.787	VBA	0.2002	4797.29834	341.46041	4.7461

Totals : 1.01078e5 6741.71811

Page 1 of 1

Data File C:\CHEM32\1\DATA\FAZNURFARIZA\FAZNURFARIZA2 2011-12-29 15-33-07\011-1101.D
Sample Name: 3

Acq. Operator : FAZNURFARIZA
Acq. Instrument : Instrument 1
Injection Date : 12/29/2011 4:48:17 PM
Seq. Line : 11
Location : Vial 11
Inj : 1
Inj Volume : 10 µl
Sequence File : C:\Chem32\1\DATA\FAZNURFARIZA\FAZNURFARIZA2 2011-12-29 15-33-07\FAZNURFARIZA2.S
Method : C:\CHEM32\1\DATA\FAZNURFARIZA\FAZNURFARIZA2 2011-12-29 15-33-07\FAZNURFARIZA.M
Last changed : 12/14/2011 6:25:50 PM by faznurfariza



Area Percent Report

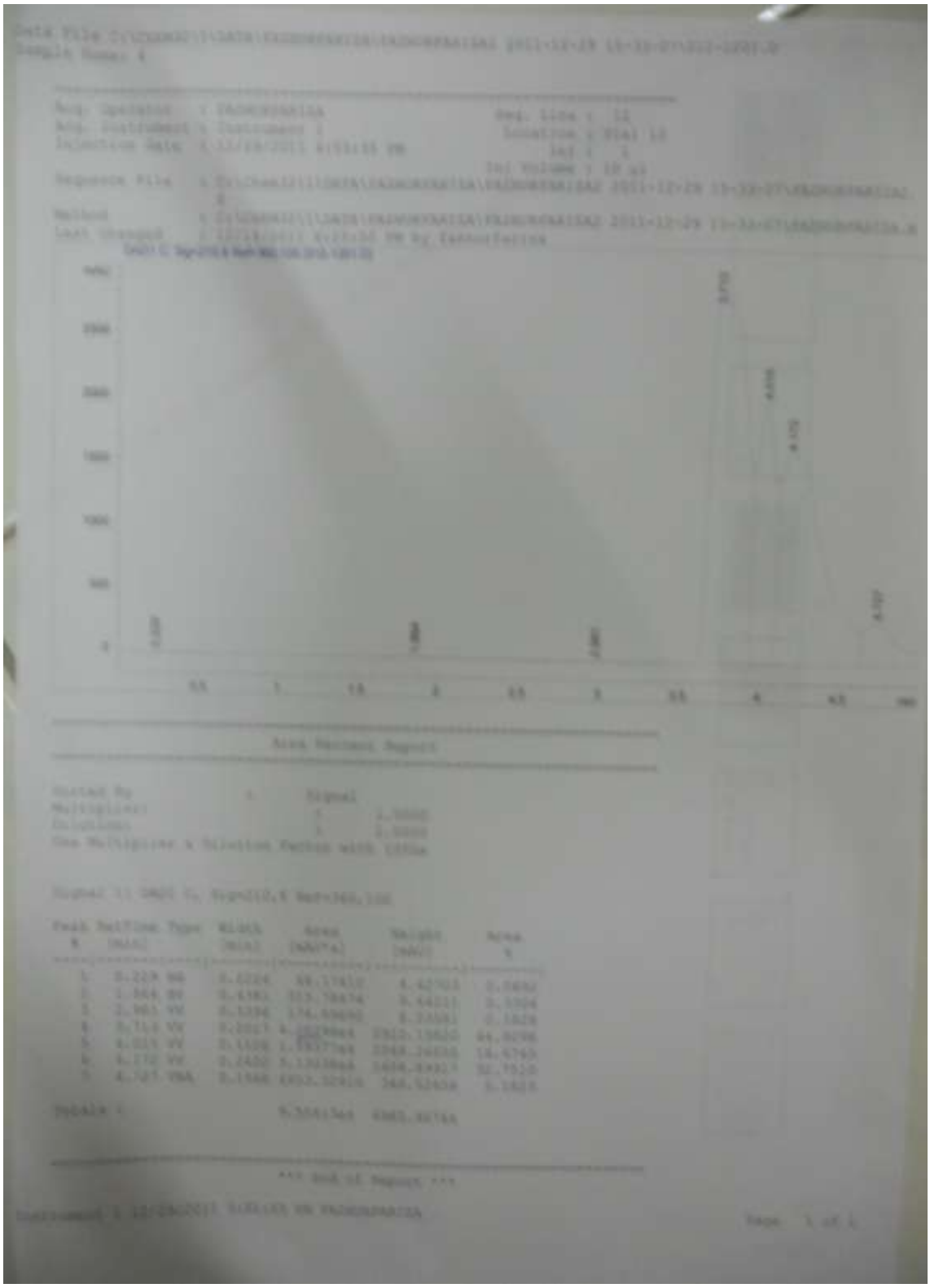
Sorted By : Signal
Multiplier: : 1.0000
Dilution: : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: DAD1 C, Sig=210,8 Ref=360,100

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	0.045	BV	0.1112	6.79188	1.01816	0.0118
2	0.648	VV	0.3274	451.95889	19.12959	0.7850
3	1.866	VB	0.3255	1153.86353	55.29340	2.0041
4	3.047	BV	0.3778	611.94006	25.15919	1.0628
5	3.737	VV	0.2071	4.40003e4	2804.11816	76.4218
6	4.292	VV	0.1120	4748.80176	615.44061	8.2479
7	4.413	VV	0.1215	4455.57275	511.92017	7.7386
8	4.692	VBA	0.1656	2146.35864	179.32156	3.7279
Totals :				5.75756e4	4211.40085	

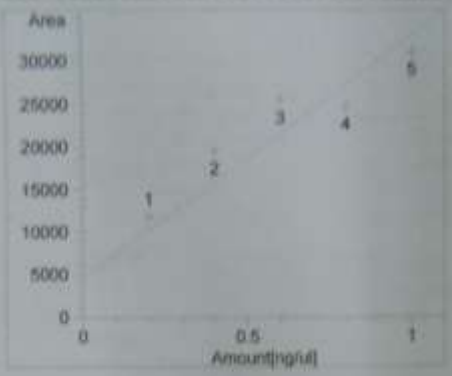
Instrument 1 12/29/2011 4:53:45 PM FAZNURFARIZA

Page 1 of 1



C:\CHEM32\1\METHODS\MORINA.M

Calibration Curves



at exp. RT: 3.826
DAD1 C, Sig=210,8 Ref=360,100
Correlation: 0.94952
Residual Std. Dev.: 3941.90553
Formula: y = mx + b
m: 28520.62305
b: 4482.45768
x: Amount
y: Area

$x = \frac{y - 4482.45768}{28520.62305}$

$x = 3506 \times 10^{-5} - 0.1572$

1: 145 : 1.26
2: 1.536 : 1.22
3: 1.386 : 0.848
4: 1.34 : 1.727