CHAPTER III

A Simple and Effective Isocratic HPLC Method for Fast Identification and Quantification of Surfactin

3.1. Introduction

Surfactants, which can be either chemically or biologically produced, are defined as surface active agents that have wide ranging and attractive properties (Al-Araji *et al.,* 2007). Surfactants produce by biological synthesis are known as biosurfactants and can be generated by a variety of bacteria, yeasts, and fungi through utilization of various carbon feedstocks, such as sugars and oil (Chen *et al.,* 2007). Biosurfactants are usually produced during the stationary phase and excreted as secondary metabolites during growth in microbial culture broth (Georgiou *et al.,* 1992; Wei *et al.,* 2007).

Biosurfactants offer various advantages over chemical surfactants, including being less toxic, but more biodegradable and environmentally friendly, as well as able to maintain their physico-chemical properties at different temperatures and pH (Mulligan, 2005). Due to these attractive properties, there has been increased interest in biosurfactants for applications in various fields, such as food, medical, pharmaceutical, cosmetics, and agriculture industries (Banat *et al.,* 2000). In addition, biosurfactants possess several unique therapeutic properties of biomedical importance (Singh and Cameotra, 2004), and can exert antiadhesive activity against several pathogenic microorganisms (Heinemann *et al.,* 2000). These features make biosurfactants favourable alternatives to chemically synthesised surfactants for a variety of applications.

Research on the formulation, characterization, and pharmacokinetics of surfactin reveal an accurate, effective, quick, and reproducible analytical method for the identification and quantification of surfactin. Identification and quantification of surfactin produced by *Bacillus subtilis* ATCC 21332 is complex since it produces a series of isoforms that differ slightly in their physiochemical properties due to variations in the chain length and branching of its hydroxy fatty acid component

(Hosono and Suzuki, 1983), as well as substitutions of the amino acid components of the peptide ring (Peypoux, 1991).

High-performance liquid chromatography (HPLC) is one of the best methods used to identify and quantify surfactin and has been described in various reports in the literature (Lin *et al.,* 1998; Isa *et al.,* 2007; Wei *et al.,* 2002; Fonseca *et al.,* 2007). Most of the previous methods used employ gradient elution, which causes base line shifting and is time consuming, with a total sampling time of more than 35 minutes (Lin *et al.,* 1998; Isa *et al.,* 2007; Wei *et al.,* 2002; Fonseca *et al.,* 2007). A delay in total sampling time will result in the use of increasing amounts of the mobile phase, which is not necessarily cost effective.

Earlier studies on surfactin identification and quantification involved measuring the surface and interfacial tension of fermentation culture broth or by thin-layer chromatography (Cooper *et al.,* 1981). However, neither of this method is satisfactory for quantitative analysis of surfactin. In contrast, the HPLC assay described in this paper is specific for surfactin identification and quantification and is highly sensitive, quick and reproducible. There is a demand for a quicker and more reliable HPLC method for qualitative and quantitative measurements of surfactin with variable formulation. The objective of the research presented in this paper was to develop an improved HPLC method, which offers a well separated individual peak with the consistent base line that is reproducible, accurate, and has a minimal duration of total elution time. In this study, a novel isocratic HPLC method with variable wavelength detector (VWD) was developed and is considered an improved method for qualitative and quantitative analysis of surfactin in comparison to various methods previously reported.

3.2. Materials and methods

3.2.1. Apparatus

An Agilent 1200 series HPLC system consisting of a vacuum degasser, quaternary pump, autosampler, thermostatted column compartment, and variable wavelength detector was used. Data acquisition was performed using Chemstation software (Agilent Corporation).

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3.2.2. Surfactin standard and chemicals

Surfactin standard of 98% purity was purchased from Sigma-Aldrich (Sigma, USA). Acetonitrile (ACN) and Trifluroacetic acid (TFA) solution of HPLC grade were purchased from RCI Labscan (Thailand) and Merck (Germany), respectively. Deionised water was obtained from the Microbiology Laboratory of Universiti Sains Islam Malaysia (Negeri Sembilan, Malaysia). Deionised water was prepared using a system equipped with 0.2µm filter.

3.2.3. Chromatographic conditions

HPLC system equipped with a Chromolith® high performance RP-18 (100 mm x 4.6 mm, 5 µm) column was operated and maintained at 25° C. A mobile phase mixture consisting of an ACN and 3.8mM TFA solution with ratio of 80:20 were pumped in an isocratic mode with a flow rate of 2.2 ml/min. The injection volume of surfactin was set at 30µl and was detected through a VWD detector at 205 nm. Each analysis was completed within 8 minutes.

3.2.4. Preparation of surfactin stock and standard solutions

Methanolic surfactin stock standard solution was prepared at 5000 mg/l. Later, a series of surfactin solution of 10 to 1000 mg/l were prepared by dilution of the stock solution with methanol and were then stored at 4 °C prior to use.

3.2.5. Preparation of the mobile phase solution

A 3.8 mM TFA solution was prepared in a 1 L volumetric flask filled with deionised water and stirred until complete dissolution. Later, the TFA solution and ACN were filtered through 0.45 µm nylon filters and degassed prior to use.

3.2.6. Method validation

The HPLC assay of the surfactin fraction was validated in terms of linearity, repeatability, intermediate precision, sensitivity and recovery with slight modification and approach based on methodology proposed by previous research (Snydeir *et al.*, 1994; Li *et al.*, 2012).

3.2.6.1. Linearity

Ten standard surfactin solutions were prepared by dilution of the stock solution with the ethanol as solvent. The obtained chromatograms were analysed using Chemstation software. The surfactin calibration curve was constructed by plotting graphs of the total peak area (TPA) against various concentrations of surfactin standard. The slope, y-intercept and linearity of the curve were determined by linear regression (R^2).

3.2.6.2. Repeatability

Instrumental precision (system precision) was determined by analyzing 10 different concentrations of each surfactin catechin in five replicate injections (Snydeir *et al.,* 1994). While, Intra-assay precision (method precision) was determined by analysis of five independent standard solutions of three different concentrations for eachsurfactin (Snydeir *et al.,* 1994).

3.2.6.3. Intermediate Precision

Intra-day repeatability was obtained by analyzing three concentrations of each surfactin at four different times in one day. Inter-day repeatability was determined by analyzing the three concentrations of each surfactin four times on three consecutive days. All injections were carried out in triplicate. The precision of the assays was calculated in terms of percentage of relative standard deviation (% R.S.D) of determinations (Li *et al.,* 2012).

3.2.6.4. Sensitivity

Sensitivity of the HPLC method was determined by the estimation of limit of detection (LOD) and limit of quantification (LOQ) based on the standard deviation of the response (σ) and the slope (S) of the standard curve using the following equations (Eq.) 1 and Eq. 2 (Li *et al.,* 2012):

$$\text{LOD} = 3.3\sigma/S \qquad \text{(Eq. 1)}$$

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3.2.6.5. Recovery

Blank sample prepared by the above method were spiked with a mixture of surfactin standard. The recoveries of each surfactin at three concentrations levels were determined by measuring the percentages of detected concentrations over added concentrations (Li *et al.,* 2012).

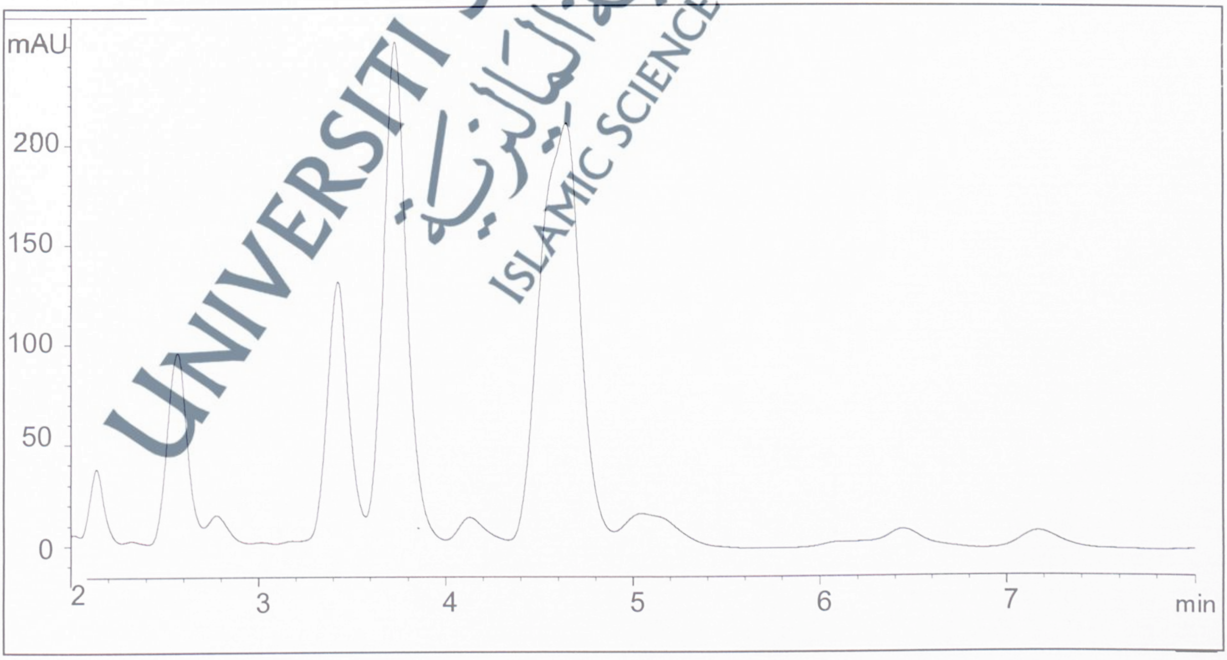
3.2.7. Sample Preparation

The commercial strain of *B. subtilis* ATCC 21332 used in this study was provided by the Microbiology Laboratory, Faculty of Science and Technology, University Sains Islam Malaysia. Bacterial strains were maintained on nutrient agar (NA) at 4 ºC prior to use. Two loopfuls of colonies were inoculated in 100 ml of Cooper's medium (4% (w/v) glucose and mineral salts) (Cooper *et al.,* 1981), prepared in a 200 ml Erlenmeyer flasks, and were incubated in an incubator shaker at 200 rpm at 30 ºC for 24 h. Then, 0.5 ml of the seed culture was inoculated into 200 ml of Cooper's media in a 500 ml Erlenmeyer flasks under same condition (Wei and Chi, 2002) for 96 h. Samples were withdrawn aseptically for further HPLC analysis.

3.3. Result and Discussion

3.3.1 Determination of Suitable Surfactin Separation Condition

Figure 3: HPLC Chromatogram for surfactin standard concentration at 800 mg/l

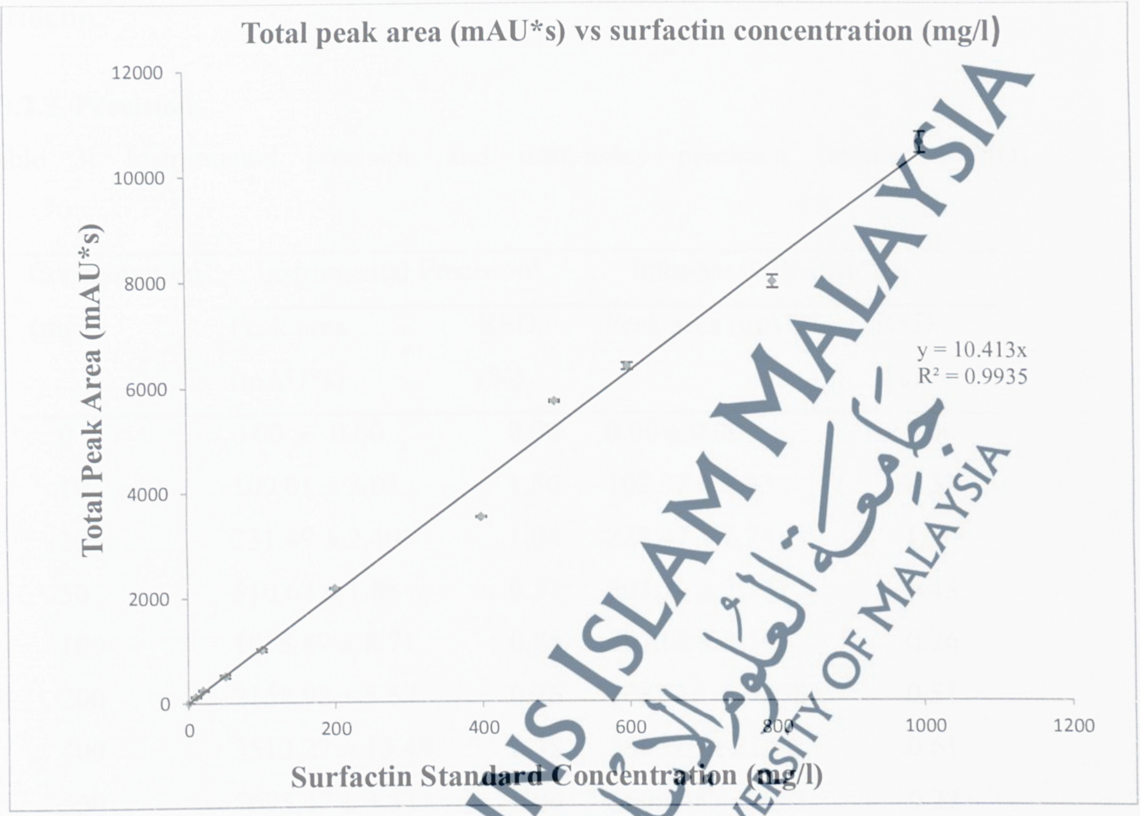


The total elution time and separation of individual surfactin peaks was highly dependent upon the ratio of ACN and 3.8 mM TFA used in the mobile phase. Mixtures of trace amounts of TFA in deionised and ACN are suitable as a mobile phase for surfactin analysis and agrees with similar work on quantitative measurements of surfactin that has been previously reported (Isa *et al.,* 2007; Wei *et al.,* 2002; Fonseca *et al.,* 2007). Acceptable resolution of surfactin peaks was not obtained with ACN and TFA solution ratios of about 60:40, 70:30 or 75:25. The optimum separation of surfactin individual peak was achieved with mobile phase consisting 80:20 ratio of ACN and 3.8mM TFA, respectively. This ratio was also similar to multiple previously reported findings (Isa *et al.,* 2007; Fonseca *et al.,* 2007; Joshi *et al.,* 2008; Hsieh *et al.,* 2004). The presences of TFA in deionized water as mobile phase could suppress the dissociation the structure of surfactin isoforms. The retention times for surfactin were recorded at 2.13, 2.57, 2.78, 3.43, 3.70, 4.14, 4.65, 5.06, 6.27, 6.46 and 7.18 minutes, as shown in Figure 3. The HPLC was run for 8 minutes, which resulted in both satisfactory resolution of surfactin peaks, and quicker total elution time compared to previously reported method (Lin *et al.,* 1998; Isa *et al.,*2007; Wei *et al.,* 2002; Fonseca *et al.,* 2007).

3.3.2. Validation of Method

3.3.2.1. Linearity

Figure 4: Plot of TPA versus various concentration of surfactin standard.



The calibration curve graph for surfactin, shown in Figure 4, was constructed by plotting the total peak area (TPA) against various concentrations of surfactin standard. Satisfactory linearity was obtained in the range of 10 to 1000 mg/l ($y = 10.413x$, $r^2 = 0.9935$, y = TPA, x = amount of surfactin standard in mg/l). The equation derived from the graph represents the relationship of TPA to various concentrations of surfactin. Independent t-test were performed to analyse various concentration of surfactin standard. A significant difference was achieved between the results obtained by HPLC from increasing concentration at 95% confidence level ($P \leq 0.05$). The calibration curve exhibited good linearity over increasing concentrations of surfactin tested, with correlation coefficients (r^2) of 0.9935. The limit of detection (LOD) achieved for the surfactin standards was 5 mg/l per injection volume of 30 µl. The high precision and low LOD indicated that the proposed method was reliable for quantitative analysis of surfactin.

3.3.2.2. Precision

Table 3: Instrumental precision and intra-assay precision (means ± SD).

Concentration (mg/l)	Instrumental Precision[a]		Intra-assay Precision[b]	
	Peak area (mAU*s)	RSD (%)	Peak area (mAU*s)	RSD (%)
0	0.00 ± 0.00	0.00	0.00 ± 0.00	0.00
10	109.01 ± 2.03	1.86	102.37 ± 2.43	2.37
20	231.49 ± 2.40	1.04	228.47 ± 2.74	1.20
50	510.62 ± 1.88	0.37	503.34 ± 2.17	0.43
100	1015.89 ± 8.71	0.86	997.08 ± 2.59	0.26
200	2158.98 ± 5.57	0.26	2082.14 ± 10.53	0.51
400	3512.27 ± 13.49	0.38	3506.61 ± 21.86	0.61
500	5695.47 ± 3.53	0.06	5604.08 ± 12.63	0.22
600	6340.06 ± 22.83	0.36	6291.99 ± 14.36	0.23
800	7858.49 ± 24.58	0.31	7683.14 ± 23.02	0.30
1000	10495.13 ± 14.01	0.13	10473.77 ± 20.54	0.20

[a] Three injection of each sample(n=3)
[b] Three injection of each sample(n=3)

The relative standard deviation (RSD) used to assess instrumental precision and intra-assay precision shown in Table 3. The instrumental precision was measured by repeated injection of the same homogeneous sample while Intra-assay precision was determined by measuring independent surfactin standard solutions of varying concentration (Li *et al.*, 2012). This was repeated for all of the standards. A correlation between the analyte peak area and surfactin standard at various concentrations was observed with $r^2 = 0.9933$ and $r^2 = 0.9929$ for standard curves for instrumental precision and intra-assay precision data as shown in Table 3 respectively, with $r^2 \approx 1$ for both. Meanwhile, the RSD value of both instrumental and intra-assay precision of the surfactin solution were below 2.5%, indicating both HPLC and the proposed method were highly precise, accurate, and reproducible for quantitave analysis of surfactin (Li *et al.*, 2012).

3.3.2.3 Intermediate Precision

Table 4: Intra-day and inter-day reproducibility of the assay (means ± SD, n = 3).

Concentration (mg/l)	Intra-day reproducibility of peak area		Inter-day reproducibility of peak area	
	Average (mAU*s)	RSD(%)	Average(mAU*s)	RSD(%)
0	0.00 ± 0.00	0.00	0.00 ± 0.00	0 .00
10	97.64± 2.52	2.58	96.22 ± 2.64	2.75
20	231.94 ± 2.65	1.14	234.58 ± 4.49	1.90
50	480.66 ± 10.91	2.27	482.30 ± 8.30	1.72
100	984.05 ± 11.08	1.13	947.71 ± 8.76	0.92
200	2137.3 ± 11.24	0.53	2195.96 ± 14.13	0.64
400	3415.75 + 15.60	0.46	3253.88 ± 40.81	1.25
500	5718.64 ± 31.06	0.54	5813.31 ± 13.76	0.24
600	6376.41 ± 53.60	0.84	6439.32± 43.54	0.68
800	7882.73 ± 131.80	1.67	8077.60 ± 37.13	0.46
1000	11035.46 ± 50.67	0.46	12066.19 ± 90. 86	0.75

Intermediate precision of the proposed method was determined by assessing intra-day and inter-day reproducibility. The results of intra- and inter-day reproducibility are listed in Table 4. The RSD values for all tested groups were approximately or less than 3%, which can be considered an acceptable value for quantitative analysis of surfactin (Li *et al.*, 2012).

3.3.2.4. Sensitivity

The sensitivity of this HPLC method was determined by LOD and the limit of quantification (LOQ). LOD is the lowest analyte concentration detectable by HPLC for the proposed method. On the other hand, the LOQ is the lowest concentration that can be quantified accurately by the proposed method. The LOD and LOQ were 5 mg/l and 7 mg/l, respectively. Compared to other reported HPLC methods, the sensitivity of the described method was considerably improved.

3.3.2.5. Recovery

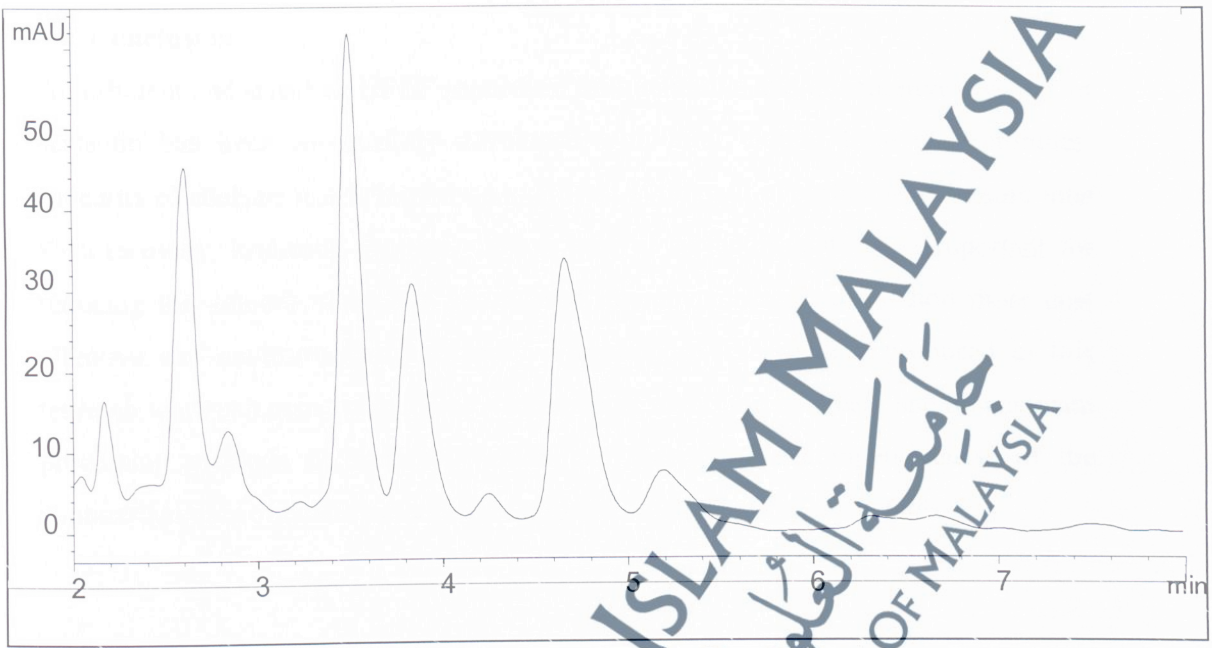
Table 5: Recoveries of surfactin (means ± SD, n=3).

Concentration spike (mg/l)	Concentration Detected (mg/l)	Recovery (%)	RSD (%)
10	9.52 ± 0.16	95.23	1.67
20	20.47 ± 0.29	102.37	1.43
50	47.05 ± 1.13	94.10	2.39
100	96.35 ± 2.05	96.35	2.13
200	204.42 ± 4.96	102.21	2.42
400	400.63± 4.35	100.16	1.08
500	500.91 ± 2.14	100.18	0.43
600	598.88 ± 5.27	99.81	0.88
800	803.76 ± 21.19	100.47	2.64
1000	1005.33 ± 21.17	100.53	2.11

The recovery measures the closeness between the theoretically added amount and the experimental value. It was performed by spiking the surfactin standard solution with a known concentration. The recovery of the sample at different concentrations was above 94% with RSD values below 3%, as shown in Table 5. The results indicated that surfactin can be fully recovered using the column proposed in this study.

3.3.3. Chromatography of surfactin in culture broth of B. subtilis ATCC 21332

Figure 5: HPLC Chromatogram of surfactin in culture broth



The proposed method was also tested with surfactin synthesized from culture broth of *B. subtilis* ATCC 21332. This fermentation broth also contained other fermentation products and residues such as proteins, sugar, lipid compounds, and different types of amino acids (Pursell *et al.,* 2004). The complex nature of *B. subtilis* ATCC 21332 culture broth makes identification and quantification of surfactin difficult. Furthermore, surfactin has different isoforms, which vary in carbon chain length and the peptide sequence (Oka *et al.,* 1993), and depend on the bacterial strain, nutritional supply, and environmental conditions applied during fermentation (Oka *et al.,* 1993). Figure 5 shows a chromatogram of surfactin from culture broth of *B. subtilis* ATCC 21332. It has overall similarity to the surfactin standard chromatogram shown in Figure 3 in terms of the elution time and separation of surfactin individual peak respectively, with a total elution time of approximately 8 minutes. Determination of the surfactin concentration in the culture broth of *B. subtilis* ATCC 21332 was conducted by summing the area under all of the surfactin peaks in the chromatrogram (Figure 3) and then calculated using the equation of $y = 10.413x$, obtained from

Figure 4. The surfactin concentration in the culture broth of *B. subtilis* ATCC 21332 was 168.28 ± 5.49 mg/l, as determined by performing the injection in triplicate.

3.4. Conclusion

An efficient and sensitive HPLC procedure for qualitative and quantitative analysis of surfactin has been successfully developed with total elution time of 8 minutes. Linearity of analysis was achieved up to at least 1000 mg/l of surfactin with more than 95% recovery. Reducing the total elution time of surfactin analysis is important for reducing the amount of mobile phase used, thereby making the method more cost effective and environmentally friendly. Furthermore, the method proposed in this research work can assist in reducing the total cost involving upstream and downstream processing methods of surfactin, which can increase the competitiveness of the commercial uses of biosurfactants in relation to other chemical surfactants.