STUDY ON FACTORS AFFECTING EXTRACTION OF CAROTENE FROM CARROT BY USING SOXHLET EXTRACTION METHOD

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ABSTRACT

In this research, carotene in the carrot (*Daucus carota L.*) was extracted by using Soxhlet extraction method. Carotene as a pigment that naturally contained in most vegetables is important source of nutrients. The main constituent of the carotenes which is beta-carotene was investigated. Two variables were varied in order to investigate the effect of drying time and solid-to-solvent ratio on the extraction and beta-carotene yield. Samples with different water content were prepared according to drying time of 3 h, 6 h, 9 h, 12 h, and 24 h at 60°C. The solvent used in the extraction process is 2-propanol with solid-to-solvent ratio of 1:4, 1:6, 1:8, and 1:10. After the extraction process, the mixture of carotene and solvent was separated using rotary evaporator in order to get extracted carotene. Then, the extracted carotene was analyzed using HPLC to determine the beta-carotene yield. From HPLC analysis, it shows the composition of beta-carotene in the extracted carotene increase as increasing drying time and solid-to-solvent ratio. This is due to less presence of water content in the sample and the excess of solvent used enhance the extraction process. The study found that optimal operating condition for this carotene extraction process was obtained at 12 h of drying time and 1:10 of solid-to-solvent ratio.

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LIST OF ABBREVIATIONS

HPLC	- High Performance Liquid Chromatography
DAD	- Agilent Photodiode Array Detector
h	- Hour
%	- Percentage
°C	- Degree of Celsius
psi	- Pound per square inch
g	- Gram
cm	- Centimeter
µl	- Microliter
ml	- Milliliter

1 INTRODUCTION

1.1 Motivation and statement of problem

Nearly a century, fruits and vegetables have been recognized as a significant source of human nutrition such as vitamins and minerals. They have been especially valuable for their ability to prevent vitamin C and vitamin A deficiencies. They remain an important source of nutrients in many parts of the world, and offer advantages over dietary supplements because of low cost and wide availability. Furthermore, fruits and vegetables in the daily diet have been strongly associated with reduced risk for some forms of cancer, heart disease, stroke, and other chronic diseases (Prior and Cao, 2000). It is because some biologically active compounds found in fruits and vegetables are strong antioxidants and function to modify the metabolic activation and detoxification of carcinogens, or even influence processes that alter the course of the tumor cell (Wargovich, 2000).

Carrot (*Daucus carota L.*) is a root vegetable, usually orange in colour, belongs to the group of common edible vegetables. The important nutrients contained in carrot include beta-carotene, vitamin A, and minerals that can provide most of the health benefits. Beta-carotene is the most significant one since it makes the carrot orange and it will converted by the liver into vitamin A. According to Chen *et al.*, 1995, beta-carotene constitutes a large portion (60-80%) of the carotenoids in carrots followed by alpha carotene (10-40%) and lutein (1-5%). Beta-carotene that appears like an orange pigment or colorant found in carrots is best known for its role as antioxidant. Consequently, anti-cancer activity and other health benefits provided by beta-carotene include the protection against cardiovascular disease or cataract prevention (Dietmar & Bamedi 2001).

The extraction of carotene from fruits and vegetables has been performed a few years ago. Nowadays, there are many extraction methods available to extract beta-carotene such as Soxhlet extraction, microwave-assisted extraction, solvent extraction and Supercritical Fluid Extraction. However, Soxhlet extraction method is preferred in this project since it is the simplest extraction technique compared to other techniques. Besides that, the Soxhlet extraction has been used for a long time and this assertion has been supported by the fact that it is a standard technique during more than one century (Castro and Ayuso, 1998). In this project, the several factors that affect the extraction yield have been analyzed. These factors including solid-to-solvent ratio and moisture content of carrot sample which may affecting the quality of beta-carotene. Therefore, the extraction of carotene from carrot also aimed to identify the optimum process parameters that can be applied in the real carotene extraction process.

Although synthetic antioxidants are approved as food additives, the international regulations tend to establish more and more restrictions so that the uses of synthetic antioxidants can be reduced. The consumers are recommended to choose natural antioxidant instead of synthetic antioxidant for their dietary supplement. This is because consumers who use the synthetic antioxidants probably easier to suffer side effects compared to natural antioxidants which are much safer. There has been an interest by the industry and a desire by consumers to replace synthetic antioxidants with natural antioxidants. It is because the use of natural sources of antioxidants necessarily can offer better immune system to our body. Furthermore, Malaysia is better to produce beta-carotene from natural materials rather than making synthetic beta-carotene because they rich with the natural plant.

1.2 Objectives

The objective of this research is to determine the optimal operating condition to extract carotene from carrot by using Soxhlet extraction method and to study the effect of drying time and solid-to-solvent ratio on beta-carotene yield.

1.3 Scope of this research

The following are the scope of this research:

i) Extract carotene from carrot using Soxhlet extractor.
ii) Investigate the effect of drying times of 3 h, 6 h, 9 h, 12 h and 24 h on extraction yield by controlling.
iii) Study the effect of solid-to-solvent ratio (1:4, 1:6, 1:8 and 1:10) on the extraction yield.
iv) Analyze beta-carotene yield using high-performance liquid chromatography (HPLC).

1.4 Main contribution of this work

Currently carrot becomes popular since the people know about the nutritious value inside the fruit. With high level of antioxidant content and lower price in market, this fruit is potential as a good alternative for natural antioxidant. This will expand the usage of carrot and also can reduce the production and usage of synthetic antioxidant in Malaysia. In addition, based on the knowledge of the optimal process parameters gained through this research will enable for the development and technology transfer to the local producers. Since the knowledge has been established, it can contribute to the local pharmaceutical industry in this country.

1.5 Organisation of this thesis

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

Chapter 2 provides a description on the beta-carotene identification and its properties, advantages and disadvantages of taking it as supplement. A general description on the Soxhlet extraction process as well as the method principles from the previous work is presented. This chapter also provides a brief discussion on the process variables which can affect the efficiency of the Soxhlet extraction process and also extraction yield.

Chapter 3 gives a guideline for the extraction of carotene from carrot based on Soxhlet extraction method. All the materials and equipment used in the extraction process are listed. The carotene extraction consists of 4 main process including sample preparation process, extraction process, separation process and analysis process. The summary of experimental procedure also provided for a better understanding.

Chapter 4 is devoted to discuss the result of the research that has been conducted. All the results have met the objectives of the study and every discussion made is based on the facts or even previous work. 2 factors affecting the extraction yield have been discussed involving drying time and solid-to-solvent ratio. Also, the optimal operating parameter needed from the research has been determined. Furthermore, the analysis of beta-carotene which can support the experimental result was discussed based on HPLC data.

Chapter 5 draws together a summary of the thesis and outlines the future work which might be derived from the model developed in this work.

2 LITERATURE REVIEW

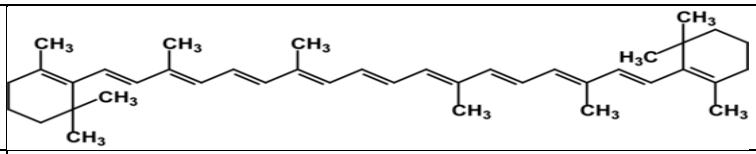
2.1 Overview

This chapter presents the previous work on the carotene extraction process based on Soxhlet extraction technique. Beta-carotene content that available in carrot is discussed regarding to their role as antioxidants. This review on beta-carotene is made including their chemical and physical properties, advantages and disadvantages. Soxhlet extraction method is one of simplest technique used in this carotene extraction. This technique is most suitable for studying purpose about the extraction under laboratory scale. The general operation of the Soxhlet extractor is outlined in order to know the basic mechanism in conducting that equipment. Besides, the outstanding advantages for using this technique are highlighted and some comparison is made with other conventional extraction method. The factors of drying time and solid-to-solvent ratio that affecting on carotene extraction yield are discussed according to previous research. In this case, the effect of drying time is explained based on water content in the sample and long drying time can destroy some of the antioxidant compounds. Conversely, the solid-to-solvent ratio is discussed in the context of diffusion rate of that compound from solid to solvent. Lastly, the high-performance liquid chromatography (HPLC) that used in this research is reviewed including its principles and analysis the data.

2.2 Beta-carotene as Antioxidant

Beta-carotene is a coloured red-orange pigment contained in plants and fruits. It is an organic compound and classified in hydrocarbon group which is of considerable interest due to their antioxidant properties. Beta-carotene is distinguished from other carotenoids by having beta-rings at both ends of the molecule structure. The details of beta carotene and its properties are shown in figure 2.1. Beta-carotene is the most abundant form of provitamin A in fruit and vegetables (Ross AC, 1999). It is an effective source of vitamin A in both conventional foods and dietary supplements. Beta-carotene is a non-polar compound, and it should be separated with a non-polar solvent.

Table 2.1: Properties of beta-carotene (adapted from www.wolframalpha.com)

Structure diagram	
Name	beta-carotene
CAS number	7235-40-7
Formula	$C_{40}H_{56}$
IUPAC name	3,7,12,16-tetramethyl-1,18-bis(2,6,6-trimethyl-1-cyclohexenyl)octadeca-1,3,5,7,9,11,13,15,17-nonaene
Molecular weight	536.873 g/mol
Phase	solid (at STP)
Melting point	181^{o}C
Flash point	346 ^{o}C
Density	1 g/cm^{3}
Vapor pressure	3×10^{-16} mmHg
Solubility	insoluble in water
RTECS classes	mutagen

Epidemiological studies have shown that people with high dietary intakes of beta-carotene or high blood levels of this nutrient have a reduced risk of various diseases, including cancer and heart disease (Sies and Stahl, 1995). However, the excessive dietary intakes of beta carotene can cause a conspicuous orange skin tint arising from deposition of the carotenoid in the outermost layer of the epidermis (Stahl W, Heinrich U, *et al*., 1998). If vitamin A in the body is high, the conversion of beta-carotene into vitamin A by liver will be reduced. Therefore, the excess beta carotene is predominantly stored in the fat tissues of the body and leads to yellowish skin, but it is quickly recover upon discontinuation of intake.

2.3 Soxhlet Extraction Method

Soxhlet extraction method is one of the simplest extraction techniques and mostly used for a long time (Luque de Castro & Garcia-Ayuso, 1998). This solvent extraction is commonly known as solid-liquid extraction where it is a process of removing solute from a solid by using of liquid solvent. The general operation of the soxhlet extraction is the solvent will condensed by heating the boiling flask and is allowed to drip back onto the thimble. The liquid condense that drips out onto the sample perform the extraction which then passes through the container and back into boiling flask. The cycle is repeated continuously as long as needed. As it progress, the extracts are concentrated in the flask. This technique is adequate for both initial and bulk extraction.

The concept of the Soxhlet extraction is organic compound are extracted by repeated washing with an organic solvent under reflux in special glassware as shown in Figure 2.2. Generally, the setup consists of round bottom flask containing the solvent, an extraction chamber and a condenser. The solid used are the consistency of small particle like powder or soil. It is stated in several extraction studies, the raw materials are grounded before the extraction can be preceded (Barriada-Pereira *et al.*, 2002). The smaller the size of particles will increase contact area between the solid and solvent. Hence, it will increase the mass transfer of active component into the solvent.

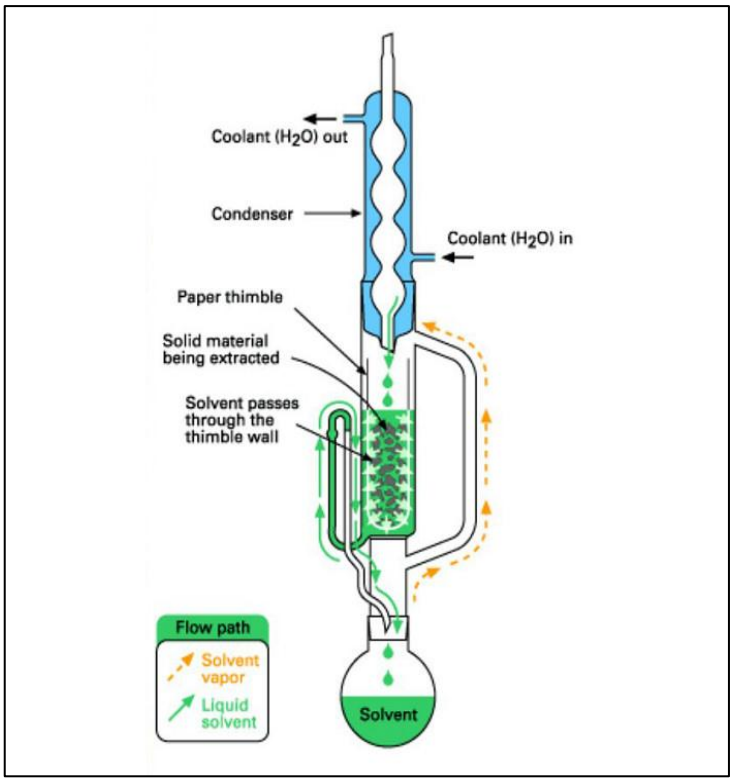


Figure 2.1: Schematic Diagram of a Soxhlet extractor

According to previous studies on the Soxhlet extraction method, there are most outstanding advantages of this conventional extraction method. In the soxhlet extraction, sample is repeatedly brought into contact with fresh solvent, thereby helping to displace the transfer equilibrium. The temperature of the system remains high since the heat applied to distillation flask reaches the extraction capacity to some extent. Furthermore, no filtration is required after the extraction process. Soxhlet extraction method also is a very simple methodology which needs little specialized training since the basic equipment is inexpensive. This conventional method also has the possibility to extract more sample mass compare to other methods like microwave extraction (Luque-Garcia & Luque de Castro, 2004). Based on the advantages of the soxhlet extraction, this conventional method has been a standard leaching technique in extraction process.

2.4 Factors Affecting Extraction Process

a) Drying Time

In the Soxhlet extraction method, the sample should be dried and crushed first before starts the extraction. The drying of the sample is intended to decrease the water activity which ultimately retards the microbial growth and helps to conserve the desirable qualities. During the drying, the enzymatic processes of the fresh plant tissues may occur and it may lead to significant changes in the composition of phytochemicals (Capecka, Mareczeek and Leja, 2005). It was reported that high drying temperatures might destroy some of the antioxidant compounds. The lower drying temperature also did not inactivate the oxidative enzymes completely, which may results some oxidation of the antioxidant substances and lower the antioxidant content (Garau *et al.,* 2001). Due to this problem, it is important to know optimum drying temperature for high extraction yield. From the previous studies, it was found that optimum temperature for most of bioactive compounds extraction is at 60^{o}C including beta-carotene itself and it can be explained by a good release of that compounds from the disturbed texture of the samples at 60°C (Fikselova M. *et al*., 2008).

Time allocated for drying process also important because long drying time might destroy some of the antioxidant compounds. The drying process would generally result in a depletion of naturally occurring antioxidants in raw materials from plants. Intense and prolonged thermal treatment may be responsible for a significant loss of natural antioxidants, as most of these compounds are relatively unstable (Lim & Murtijaya, 2007). In this situation, the longer the time taken will results in less water content in the sample due to water removal during the drying process. The study on the drying time is conducted to know the optimal time required for drying process according to extraction yield.

b) Solid-To-Solvent Ratio

A high solid-to-solvent ratio was found to be favourable in extraction of antioxidant compounds. These results were consistent with mass transfer principles where the driving force for mass transfer is considered to be the concentration gradient between the solid and the solvent. A high solid-to-solvent ratio could promote an increasing concentration gradient, resulting in an increase of diffusion rate that allows greater extraction of solids by solvent (Cacace and Mazza, 2003). In addition, the chance of bio-active components coming into contact with extracting solvent expanded with increase amount of extraction solvent, leading to higher leaching-out rates (Zhang *et al*., 2007). However, active component yields will not continue to increase once equilibrium is reached. The solid-to-solvent ratio could significantly affect the equilibrium constant and characterized the relationship between yield and solvent use as a steep exponential increase followed by a steady state to give the maximum yield (Hamdam *et al.*, 2008).

A solid's solubility is affected by changes in the activity coefficient, which varies with the temperature and composition of the solution (Frank *et al*., 1999). Interactions of the compounds with solvent could have modified the activity coefficient and thus the solubility of the compounds to the solvent. Overall, the main effect of the solid-to-solvent ratio was to modify the solubility and equilibrium constant and thus increase the extraction yields to a maximum at the highest solid-to-solvent ratio (Cacace and Mazza, 2003). Although amount of antioxidant compounds generally increased with increase of solid-to-solvent ratio, the increase in yield of that compound may not be directly proportional. Thus, it is important to evaluate the influence of solid-to-solvent ratio during optimisation of extraction of phytochemicals from different plant materials. Furthermore, use of high solid-to-solvent ratios would result in dilute solutions (Ho *et al*., 2008).

2.5 High Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography (HPLC) is a type of chromatographic technique which used to separate, identify and quantify compounds that dissolved in a solution. Theoretically, this technique involves a liquid sample being passed through a solid adsorbent material packed into a column using a flow of liquid solvent. The compounds of the sample are separated from each other due to their different degrees of interaction with the adsorbent particles. These interactions are physical in nature, such as hydrophobic (dispersive), dipole-dipole and ionic, most often a combination thereof. Each compound in the sample interacts slightly different with the adsorbent material, thus retarding the flow of the compound. If the interaction is weak, the compound flow off the column in a short amount of time, and if the interaction is strong, then the elution time is long.

In HPLC, a sample is injected into a mobile liquid phase and it passes along a stationary phase. Although manual injection of samples is still possible, most HPLCs are fully automated and controlled by a PC, allowing up to 200 or more samples to be injected. The stationary phase comprises a column which is usually stainless steel and packed with silica particles bonded with alkyl chains. The length of the chain depends on the type of molecule being analysed. For example, for large protein molecules a C4 column could be used but for smaller molecules C8 or even C18 may be more appropriate.

Figure 2.2: HPLC column

After the sample passes over the column, it is detected by ultraviolet absorption. The sample and the mobile phase are collected as waste and the absorption spectrum is outputted as a chromatogram. This process is fully automated and controlled by a PC. The time taken for a sample to pass through the system is recorded as its retention time and is one of the characteristics used to identify a compound. From the chromatogram, the area under a peak is used for calculating the concentration of a sample.

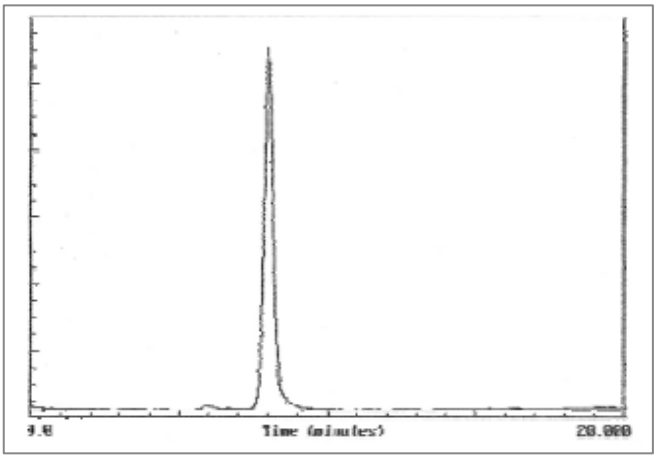


Figure 2.3: HPLC chromatogram

The concentration of compounds can be calculated by first running a series of standards at known concentrations. A curve is then plotted of the concentration of the standards (x-axis) versus their peak area (y-axis). From the chromatogram peak areas, it is possible to calculate the concentration of the compounds in sample.

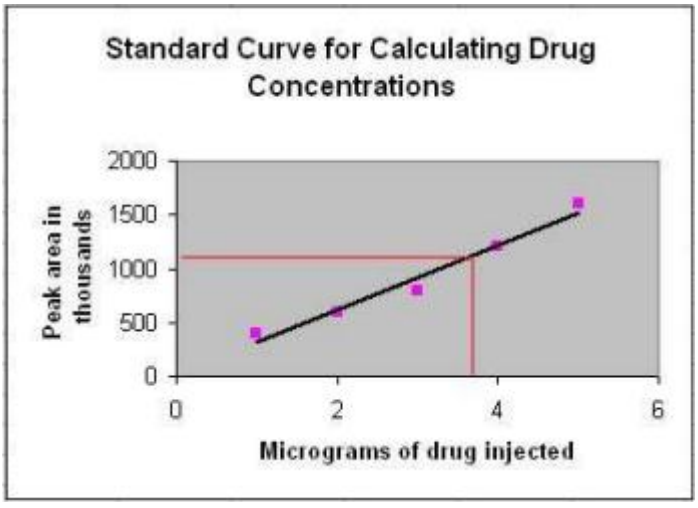


Figure 2.4: Standard curve used to calculate concentrations

2.6 Summary

In this chapter, the explanation on carotene extraction process, Soxhlet extraction method, factors affecting extraction process and HPLC principles are reviewed based on the previous research. All these information and methods applied in the previous research have been used as a reference in this research especially for discussion on the factors affecting on the carotene extraction and HPLC analysis. Based on this literature review, it shown that research on beta-carotene had been done for a long time ago and every improvement with respect to extraction process is made to ensure the competency of that process.

3 MATERIALS AND METHODS

3.1 Overview

This chapter will discuss the experimental work on the study of carotene extraction using Soxhlet extraction method. There are four stages involved in completing the experiment including sample preparation process, extraction process, separation process and lastly analysis process. In the sample preparation process, carrot samples were prepared by controlling drying time for 3h, 6h, 9h, 12h and 24h at 60^{o}C. Then, extraction is made among three samples with highest percentage water removal in extraction process. The extracted carotene was separated from solvent at separation process. Lastly, the beta-carotene yield in the extracted carotene is determined by using High Performance Liquid Chromatographic (HPLC) under analysis process. The extraction yield was compared in order to know the best time to be used in the drying process. The sample with the best time of drying process is used for next study on the effect of solid-to-solvent ratio.

3.2 Materials

Fresh carrot is used as a sample in this extraction process. The solvent of 2-propanol (purity of 99.8%) is used along with the sample in the extraction process. The HPLC grade of methanol, acetonitrile and dichloromethane are used to prepare mobile phase for HPLC use.

3.3 Apparatus

In the sample preparation part, a microwave oven is used to dry the sample. Then, the dry sample is blended by using a blender to become powder. An electronic balance is used to weight the sample and product. The Soxhlet extraction apparatus consisting of boiling flask, Soxhlet extractor and condenser are used in the extraction part. The extracted carotene is separated from solvent using rotary evaporator. High Performance Liquid Chromatography (HPLC) is used to analyse the beta-carotene yields.

3.4 Experimental Procedure

a) Sample Preparation Process

In this process, the carrot powder is prepared before it used in the extraction process. The carrot is sliced and weighed then it placed in the oven for drying process at temperature of 60°C. The sliced carrot is left in the oven for 3 hours, 6 hours, 9 hours, 12 hours and 24 hours respectively. After drying process, the dried carrot is weighed and inserted in the airtight containers and keeps in a dry place. The dried carrot should be grinded into powder by using blender before it can be used in the extraction process.

Figure 3.1: Microwave oven used in drying of carrot samples

b) Extraction Process

The carotene extraction is performed by using Soxhlet extraction method. The 25 grams of carrot powder is placed in porous cellulose thimble. The thimble is placed in extraction chamber of the Soxhlet extractor, which is located between the boiling flask at bottom and condenser at the top. The round boiling flask is filled with 100 ml, 150 ml, 200 ml and 250 ml of solvent 2-propanol respectively. Then, the water source is opened and channeled from bottom condenser and exit at the top of condenser. The extraction process is performed at 82°C which is boiling point of 2-propanol. The extraction time for the process is 5 hours for each run.

Figure 3.2: Soxhlet extractor used in carotene extraction process

c) Separation Process

The separation of carotene from solvent is performed after the extraction method. The carotene and solvent is separated by using rotary evaporator. The first step in this solvent separation method is removing the suspended solid in the mixture by using filter paper. Then, the mixture is placed in the rotary flask before it is attached to the rotary evaporator equipment. The temperature used onto this equipment is 82°C which is boiling point of the 2-propanol. Lastly, carotene obtained after the separation is weighted using analytical balance and placed in the sample bottle.

Figure 3.3: Rotary evaporator used for separation process

d) Analysis Process

The analysis of beta-carotene yield on the extracted carotene is performed by using equipment of High Performance Liquid Chromatography (HPLC) coupled with a column of ZORBAX Eclipse XDB C18 and an Agilent Photodiode Array Detector (DAD). Firstly, 1 litre of mobile phase solution is prepared by mixing Acetonitrile, Dichloromethane and Methanol by the ratio of 70:20:10 respectively. Then, the solution is sonificated in water to remove bubbles for 1 hour before it transferred into HPLC. Concurrently, the extracted carotene is filtered using nylon filter and filled in HPLC vials. Then, the vials are transferred into HPLC and it is ready for analysis. HPLC calibrated by running mobile phase at the rate of 1 ml/min. Wavelength is fixed at 452 nm. The pressure of the column is kept 1800-2000 psi. Injection volume of 20 μL is used. The beta carotene yield is analysed based on the percentage peak area on the data from the HPLC. The higher percentage peak area, the higher concentration of beta-carotene. In addition, the optimal process parameters for the extraction process are determined based on the percentage of beta-carotene yield.

Figure 3.4: High performance liquid chromatography (HPLC) used for analyzing beta-carotene