Construction of a Tributyrin Calibration Curve using High Performance Liquid Chromatography

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Abstract. Most of the explored research works on Triglycerides or any of the Triglycerides families was based on the profiling identiﬁcation than a quantiﬁcation. To quantify them, a calibration curve needs to be constructed at a certain range of concentration. In this study, the calibration curve for the shortest chain of Triglycerides namely Tributyrin was accomplished using high performance liquid ultraviolet detector with two variables parameters. The composition of mobile phase for tributyrin was found optimum using 50:50 acetonitrile: acetone while the optimum ﬂowrate for tributyrin was 0.55 mL/min using a 20 μL injection, constant condition at 35°C and 215 nm wavelength. The calibration curve of standard tributyrin was found linear from 0.0025 - 0.0375 mM with the correlation coefﬁcient (R2) of 0.986. The developed calibration curve could serve as a future platform for the quantification of TB in real samples with various matrixes such as food and serum.

1. Introduction

Triglycerides (TGs) are important in human metabolism as an energy source for transportation of dietary fat. TG is an ester derived from glycerols that contain three hydroxyl (OH) groups and three molecules of fatty acid with a carboxyl group (COOH). It includes other individual TGs, which are tributyrin (1,2,3-tributyrylglycerol), tricaprylin (1,2,3-trioctanoylglycerol), tripalmitin (1,2,3-trihexadecanoylglycerol), tristearin (1,2,3-trioleoylglycerol), triolein (1,2,3-tri-[c-9]-octadecenoylglycerol), and trilinolein (1,2,3-tri-[c9,c12]-octadecadienoylglycerol), depending on the number of carbon associated with the number of fatty acid attached to one molecule of glycerol. However, an excess of TGs in food and blood may cause coronary heart disease with a permitted concentration of 40 to 160 mg dL−1 in men and 35–135 mg dL−1 in women [1]. Among the TGs, tributyrin (TB) which is the shortest medium-chain TGs and hydrophobic in nature [2, 3], has been used as a model of TGs substrate in a biosensor development [4,5]. The major advantage of quantification of TG analysis is it allows food-related authorities and professionals to establish information in providing sufficient knowledge to the public, therefore, many efforts have been done on TGs using HPLC.

The selection of the detector in HPLC is a crucial factor in TG profiling analysis. Refractive detector (RI), ultraviolet detector (UV), mass detector (MS), and evaporative light scattering detector (ELSD) are the common detector in HPLC which owe their advantages and disadvantageous to TGs detection. For example, RI was found unsuitable for a gradient elution system which requires longer time for
stabilization. Even though it is not affected by the polarity of the mobile phase nature, it is found as a suitable detector for the fractionation and quantification of the TGs peaks [6]. Another approach in TGs chromatography analysis involved HPLC-ELSD due to its stability which is not strongly affected by the polarity of the mobile phase and is available for gradient elution [2,7,8]. Even though this detector is likely to be a universal detector for TGs detection, it is not suitable for the quantitative analysis and the fractionation of the peaks because the signal for calibration curves produced by this detector could not produce a linear equation [6]. Attention has been focused on HPLC-MS system as another universal detector and a new method for the TGs molecular species analysis due to its ability to identify the structure of each peak from the fragmentation pattern along with quantification. However, this instrument is known to be expensive which requires highly-skilled personnel and time investment for personnel training [9]. HPLC-UV is influenced by the nature of the mobile phase and is able to analyze compounds containing chromophore through absorption at selective wavelengths such as 210, 215, and 255 nm. However, a study by Naviglio et al. [10] on TGs using HPLC-UV found that detection still does not involve quantification analyte but requires transesterification of triglycerides as phenethyl esters before being examined through this detector to create a chromophore that allows detection using UV detection methods. This is well supported by Lee et al. [7] who agreed that the UV detector has limitations on species with the absence of many double bonds such as TGs and other lipid species. The preparation of samples containing fat or oil required a simple method as reported by Naviglio et al. [10]. In the work, anhydrous fat was dissolved in hexane, and 1 μL of this mixture was injected prior to profiling analysis. However, the analysis on quantification was not reported, therefore developing an urgency to study the conditions for producing a calibration curve before quantitation of TG can be performed. TGs can be effectively eluted by HPLC using octadecyl groups (RP-18) as a stationary phase as has been reported in many research. To observe the elution, good criteria of mobile phase are highly needed which would enhance the polarity of the mobile phase, increasing peak sensitivity and reducing retention times. This can be achieved with the incorporation of acetonitrile with an organic modifier such as acetone that improves the solubility of TGs [11]. Although there were reported uses of other mobile phases such as diethyl ether, alcohol, tetrahydrofuran, and hexane to enhance the solubility of TGs, the improvement on the sensitivity is almost absent in HPLC-UV detection due to the absence of the chromophore in the mobile phase. Retention time is the time required for a compound to travel in the stationary phase before being detected by a detector. Reversed-phase high-performance liquid chromatography (RP-HPLC) that utilized the polar mobile phase and nonpolar stationary phase has been widely explored for the analysis of the TG mixtures where the retention time is mostly affected by the total length of the acyl chains together with the degree of unsaturation [12]. Retention time is influenced by the polarity and affinity of a compound against the mobile phase and stationary phase. A long period of analysis involving HPLC will usually increase the cost of the consumption of the mobile phase. In addition, at the end of a long elution, a blank run should be performed using a mobile phase for at least 30 minutes to ensure optimal column cleaning between samples as agreed by King et al. [13]. Due to the many compounds in TGs, elution involving a typical integration for 15 families of triglycerides normally require almost half an hour to complete one elution [6, 14, 15]. It is found to be impractical if a researcher only needs one compound from TGs especially if it involves components in earlier elution such as TB. Most of the research works on TGs are on profiling identification instead of quantification that has been explored using ELSD [2] MS [7] UV [10] and RI [11]. Quantification had been successfully performed with a combination of two detectors which are charged aerosol detection (CAD) in line with the UV detection and MS that permits the detection and quantification of both short-chain volatile molecules and non-volatile complex TGs [13]. However, the calibration curve for individual standards of each triglyceride to assess linearity was not employed in those studies. In this study, the development of the calibration curve of individual TGs containing the shortest carbon chain, which is TB as a model, was performed through a variable affecting the TB significant peaks. These changes are a composition of the mobile phase and mobile phase flow rate that contribute to the significant TB in HPLC peak.
2. Research Method

2.1. Chemicals and instrumentation
N-hexane, acetone, and acetonitrile with 99.9% purity were used as a mobile phase (Merck, Germany). The standard solution of tributyrin with 99.9% purity was purchased from Sigma, Germany. The solvents and prepared standards were degassed in an ultrasonic bath prior to HPLC analysis and filtered through a vacuum filter to remove possible particulate matter and prevent clogging. A HPLC-UV instrument was used in this study. This instrument consists of carbon 18 column (150 mm × 4.6 mm, 5 µm) equipped with a quaternary pump with a 20 µL loop injector and UV detector (Agilent Technologies 7820A). The temperature of the column and the detector was set at 35 °C respectively. The mobile phase is acetonitrile/acetone at a certain ratio and a flow rate was used for optimization. The wavelength was set at 215 nm.

2.2 Preparation of stock, standard solution and calibration curve of Tributyrin
The stock standard solution was prepared in 1617 µL hexane containing 382 µL of tributyrin standard. To prepare the serial standard solution, the stock was diluted with a certain volume of hexane to obtain 0.3 mM, 0.15 mM, 0.075 mM, 0.0375 mM, 0.018 mM, 0.009 mM, 0.0045 mM, and 0.00225 mM. Using the optimized condition, 20 µL of each concentration was injected three times into the injector loop. The calibration curve was plotted using the peak area as the y-axis and concentration as the x-axis. The correlation coefficient and equation on calibration curves were constructed using the formula from Microsoft Excel.

2.3 Effect of mobile phase composition and flowrate
To further the experiment, five different mobile-phase compositions were optimized which are 70:30, 60:40, 50:50, 40:60, and 30:70 of acetonitrile: acetone. The study was done by using 0.018 mM standard tributyrin at 0.75 mL/min flowrate and 215 nm wavelength. Next, studies on various flow rates were performed to identify the optimum flow rate of acetonitrile and acetone. In this analysis, five flow rates which were 0.35, 0.55, 0.75, 0.95, and 1.00 mL/min have been used throughout the experiments. The wavelength was set at 215 nm. The optimization process was done by using 0.018 mM standard tributyrin at 50:50 acetonitrile: acetone mobile phase composition.

3. Result and Discussion

3.1. Effect of mobile phase composition
Five compositions of mobile phase were used in the analysis of tributyrin starting from the most polar to the least polar solvent which is acetonitrile: acetone (70:30), (60:40), (50:50), (40:60), and (30:70). The effect of the mobile phase composition of acetonitrile: acetone to the peak area of tributyrin using 0.018 mM and wavelength of 215 nm is shown in figure 1. From the graph, the highest peak area was found using 50:50 acetonitrile: acetone and started to decrease at 40:60 acetonitrile: acetone and 30:70 acetonitrile: acetone. Therefore, the composition of 50:50 acetonitrile: acetone was selected for subsequent optimization. TB is a polar compound with the presence of ester linkage. It is more polar compared to other TG due to its shortest chain of fatty acid. The equal combination of acetonitrile and acetone composition is suitable possibly due to the characteristic of the medium polarity solvent for both solvents with a polarity index of 5.1 and 5.8, respectively. Acetonitrile is one of the known solvents that has successfully improved the solubility of TG, peak selectivity, retention time, and selectivity of TG compound with the use of another organic modifiers such as acetone [11].
Figure 1. Effect of mobile phase composition of acetonitrile: acetone to the peak area of tributyrin using 0.018 mM and wavelength of 215 nm.

3.2. Effect of flowrate
Various flow rates reduce retention time as a higher flow rate will sweep the elution faster. Figure 2 shows the effect of flow rates on the peak area of tributyrin. The peak area was found to increase when the flow rate was increased from 0.35 mL/min to 0.55 mL/min. However, the peak area was found to start decreasing at flow rates of 0.75, 0.95, and 1.0 mL/min. Therefore, the flow rate of 0.55 mL/min was chosen for the subsequent experiments due to the optimum peak area compared to the other flow rates. This finding was almost similar to Zeb and Murkovic’s [16] result who obtained a flow rate of 0.6 mL/min using the MS detector. However, this contradicts the mentioned flowrates of 1.4 mL/min [11] and 1.5 mL/min [17].

Figure 2. Effect of flowrate to the peak area of 0.018 mM Tributyrin at wavelength of 215 nm.

3.3. Calibration curve
To investigate the linearity of the HPLC-UV towards the TB standard, several concentrations of TB were injected to the injector which were 0.3 mM, 0.15 mM, 0.075 mM, 0.0375 mM, 0.018 mM, 0.009 mM, 0.0045 mM, 0.00225 mM, and blank. Figure 3 shows the chromatogram of the TB at different concentrations. A sharp peak that was successfully obtained indicates that the optimized parameters are suitable for the elution of tributyrin. Figure 4 shows the calibration curve of the TB standard at 0.55 mL/min and 50:50 mobile phase composition with a correlation coefficient of 0.986. Based on the graph shown, the peak area was found proportionally linear to the concentration of standard tributyrin at a range of 0.025–0.0375 mM. Concentrations higher than 0.0375 mM were not included in the calibration curve since it reaches a saturated point in the chromatogram. A similar study has been explored by Rombout et al. [8] but using gradient elution with acetone, acetonitrile, and dichloromethane as the mobile phase which requires a higher cost than the present study. This present study was also better than
reported by Aoki et al. [6] who obtained difficulties to get a linear calibration of TGs curve through gradient elution using HPLC-UV. So far, the study to quantify TGs is a huge challenge as quantification of complex TGs is difficult due to the large possible variation in the carbon number of the fatty acids at each position on the glycerol backbone and the presence of the configuration of cis and trans to the double bonds on each fatty acid (Wei et al., 2021). Therefore, an exploration of the fundamentals of the calibration curve is highly demanded prior to quantification. A calibration curve is made possible in the present study due to the reverse phase chromatography which used carbon 18 as a column. A similar column was reported by Chen et al. (2020) on TG profiling of species in human milk. In the future, the approach of quantifying serum samples using the developed calibration curve of TGs could be a benchmark to support the past available profiling study. This is very important since the heart and cardiovascular disease become a worldwide concern that may be contributed by elevated TGs in human serum (Ritchey et al., 2019). To express the importance of quantification, to date, another advanced technique has been explored by Bin Peng et al. (2021) using the enzymatic esterification of oil samples to elucidate the effects of different TGs on lipid accumulation in oleic acid-induced human hepatic cells culture.

![HPLC chromatogram of A) blank, B) 0.00225 mM, C) 0.0045 mM, D) 0.009 mM, E) 0.018 mM and F) 0.0375 mM. Condition: Wavelength was set at 215 nm.](image)

**Figure 3.** HPLC chromatogram of A) blank, B) 0.00225 mM, C) 0.0045 mM, D) 0.009 mM, E) 0.018 mM and F) 0.0375 mM. Condition: Wavelength was set at 215 nm.
4. Conclusion

The composition of 50:50 acetonitrile: acetone and flow rate of 0.55 mL/min were used to develop the calibration curve of tributyrin. The calibration curve was successfully constructed at 0.0025–0.0375 mM based on the best condition of the variable on the mobile phase composition and flow rate. This finding can be a future platform for the quantification of TB in real samples with various matrixes such as food and serum.

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