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Article

Protocol of a Rapid Method to Quantify Beta-Carotene in Human Sera

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Abstract: Beta-carotene is a fat-soluble antioxidant commonly found in foods such as fruits, vegetables, and palm oil. Despite various liquid chromatography methods are proposed to detect and measure the sera carotenoid level, a long retention time to elute this marker is needed. This study attempted to develop a rapid reverse phase method in eluding beta-carotene in human sera. The researchers managed to elute the antioxidant in 2.2 minutes by applying a combination of a C8 column, mobile phase comprising of acetonitrile mixed with methanol in 70:30 proportion, and an ultra-high performance liquid chromatography system. The outputs had a good calibration curve (R^2=0.959) and low coefficient of variation (0.2%), suggesting this protocol is reliable. A column with a lower carbon chain such as C8 allows the beta-carotene molecule to flow through the column faster. Besides, selecting solvents with high elution strength coupled with an ultra-high performance liquid chromatography system which equipped with high pressure can force the beta-carotene through the column in a shorter duration compared to previous reported methods. Therefore, it is recommended to adopt this protocol in epidemiological studies where beta-carotene is screened as a dietary biomarker associated with disease of interest by using human sera in the population level.

Keywords: rapid; beta-carotene; UHPLC; human sera; Malaysia

Introduction

Non-communicable diseases contributes to 71% of total mortality cases worldwide [1]. Oxidative stress is responsible for the progression of systemic inflammation [2], which leads to non-communicable diseases such as diabetes mellitus, cardiovascular disease, and cancers [3]. Several studies reported that dietary antioxidant carotenoids, in particular beta-carotene was observed to reduce the risk of overweight or obese [4]. It was also effective in reducing the overall mortality risk [3,5].

Carotenoids are commonly found in fruits, vegetables, and palm oil [6] which are commonly consumed by human and being circulated in human sera as beta-carotene. Furthermore, Martín-Pozuelo *et al*. reported that these fat-soluble antioxidants react towards lipids via an absorption and transportation process in-vitro due to their fat-soluble properties [7], suggesting the similar metabolism could occur in human as well.

Various methods have been applied to detect the carotenoids, such as thin-layer chromatography, high-performance liquid chromatography (HPLC) or a combination of HPLC with mass spectrometry [8]. However, the most used method for identifying and quantifying carotenoids utilizes HPLC. Besides, a recent ultra-high performance liquid chromatography (UHPLC) approach was proven to provide significant higher details in peak width and shorter analysis times for detecting carotenoids [9].

Carotenoid separation can be carried out using both normal and reversed-phase HPLC. However, the normal phase HPLC is not ideal for separating non-polar carotenoids [8]. Additionally, the normal phase HPLC usually utilizes aggressive chemicals like n-hexane to elute carotenoids. This chemical is harmful for routine bioanalytical workflow without an adequate ventilated environment.

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In contrast, the reverse phase HPLC enables a significant increase in the interaction between the analyte and non-polar stationary phase leading to an enhanced resolution of the carotenoids. It should be noted that the elution of carotenoids via an HPLC system depends on several factors, including types of columns in stationary phase and varieties of hydro-organics used in mobile phases [10].

There is an increase in epidemiological studies that incorporate antioxidants in predicting or associating with chronic illness. Therefore, a rapid and accurate detection protocol on this antioxidant is required in view that epidemiological studies usually involve hundreds to thousands of individuals, which can incur significantly higher costs and time for sample analyses. Hence, this study attempts to develop a protocol to reduce the retention time needed for eluting beta-carotene without compromising its eluted resolution quality.

Materials and Methods

Human sera sample preparation

Five human sera were randomly selected to develop the protocol. Human sera (stored in a -80°C freezer) were thawed at room temperature for 10 minutes. A total of 100uL sera sample was pipetted into a microcentrifuge tube, followed by adding 100uL of absolute ethanol (Merck). The mixture was vortexed for 10 seconds, and a 500uL of n-hexane (Merck) was added, and the mixture was further vortexed for one minute. Next, the sample was centrifuged for 10 minutes at 1500g. The supernatant was then extracted twice with n-hexane. The combined supernatant was subjected for solvent evaporation using a SpeedVac (Heto CT110) at 30°C and followed by centrifuge for 15 minutes at 1500g. The residue was resuspended in 100uL eluent consisting of acetonitrile (Merck) and methanol (Merck) in a ratio of 70:30. The solution was then filtered using a 0.2 µm PTFE 4mm syringe filter (Phenex) into an amber vial (Phenomenex) equipped with a 250 uL vial insert (Phenomenex). Samples were immediately stored at -20°C and subjected for UHPLC analysis on the next day to minimise the compound loss due to oxidation.

Optimizing protocol for detecting beta-carotene in human sera

A duplicate beta-carotene standard with a concentration of 100 µg/ml (Dr. Ehrenstorfer, purity 95.3%) was used to optimize the protocol. The analysis was carried out using a UHPLC approach (Agilent 1290 Infinity II LC System) with acetonitrile and methanol (70:30, v/v) as mobile phase, flow rate of 1.0 ml/min, isocratic for 10 min and injection sample volume of 20uL. The detection wavelength was set at 450 nm, and the separation was carried out with a Kinetex C8 column (2.6 µm X 100X 4.6mm) (Phenomenex) at 40°C. As a confirmation test, an additional run time of 120 minutes was conducted on a beta-carotene standard for ensuring the retention peak corresponds to the beta-carotene compound. Upon confirmation on the compound's retention time, duplicates of beta-carotene standards concentrations (0.5, 1.0, 5.0, 10.0, and 20.0 µg/ml) and human sera were run according to the protocol.

Statistical analysis

A linear regression based on five different average beta-carotene standards was plotted. An R^2 at 0.95 and above is considered adequate for estimating samples' concentration [11]. Additionally, the coefficient of variation (CoV) of the retention time (minutes) both intra (samples analysed on the same day) and inter-tests (same samples analysed on other days) were calculated. The percentage of CoV less than 10% is considered minimal variations across analyses [11].

Ethical consideration

Ethics clearance was obtained from the University Malaya Medical Centre (UMMC) Medical Research Ethical Committee (Reference number: 950.1).

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Result and Discussion

According to Figure 1, the retention time for both beta-carotene standard (A) and a comparative human serum (B) excited at 450nm wavelength, recorded approximately 2 minutes and 10 seconds. The retention time of this compound was consistent, whereby no additional peaks observed despite the extension of UHPLC running time to 120 minutes (Figure 2).

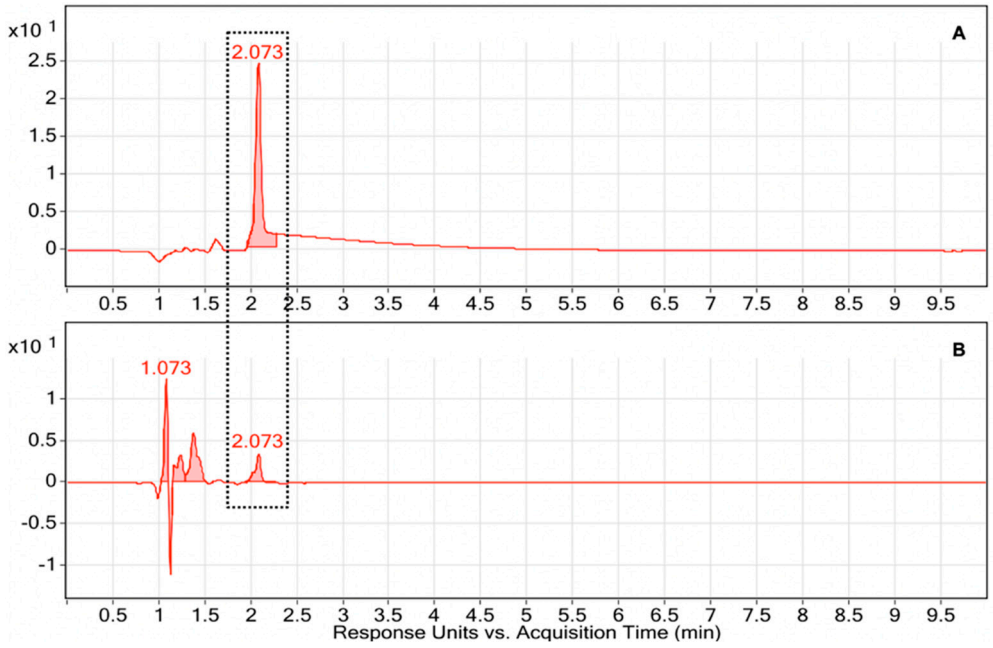

Figure 1. A comparative of chromatograms on beta-carotene compound's retention time (x-axis) against response units (y-axis) between the standard and a human sera sample A) beta-carotene standard; B) human sera sample.

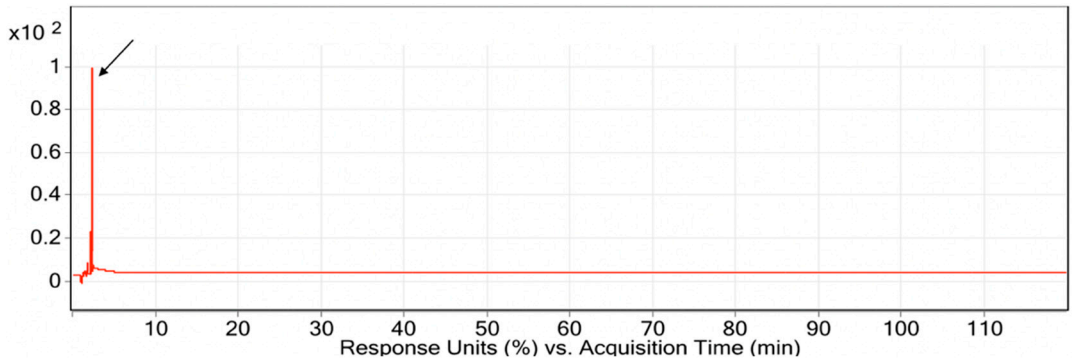

Figure 2. Chromatograms on beta-carotene compound's retention time (x-axis) (arrow) against its reponse units (%) (y-axis) between the standard extended to 120 minutes running time (a confirmation test).

Table 1 documented the consistency of retention time of the beta-carotene standard concentrations, as measured by parameters such as mean, standard deviation, coefficient of variations, and relative retention time corresponding to the standard beta-carotene compound. The average and standard deviation of retention times for 1st measurement and 2nd measurement were 2.077 minutes (Standard deviation: 0.005) and 2.24 minutes (Standard deviation: 0.002), respectively, whereby the coefficients of variation on the two tests were in the range from 0.1-0.2%. Although there was a slight variation in retention time of these two tests, the coefficient of variations inter-tests was also minimal (0.2%), which was much lower compared to the minimum variations between tests as

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demonstrated by Bower's study [11]. The relative retention time intra and inter tests were estimated in a range from 1.00 to 1.08 suggesting no severe deviation in retention time relative to the reference.

Table 1. Coefficient of variation of the beta-carotene standards' retention time (minutes) between intra and inter UHPLC tests.

	Mean	Standard deviation	Coefficient of variation
Intra test (1)	2.077	0.005	0.2%
Intra test (2)	2.241	0.002	0.1%
Inter test	2.159	0.004	0.2%

The linear regression line plotted from five beta-carotene concentrations was illustrated in Figure 3. The linear equation constructed was y = 38.967 x – 42.262, where y: area under the curve; x: sample concentration. The straight-line equation explained approximately 95.9% of the total variance. By applying this equation, the five human sera samples' concentrations were estimated at an average of 1.67 µg/ml, or equivalent to 3.10 µmol/l after unit conversion (Table 2). Since all estimated samples' beta-carotene concentrations were within the normal reported range in human blood [12], this suggests that the linear equation in Figure 3 can adequately estimate the human sera beta-carotene concentration level.

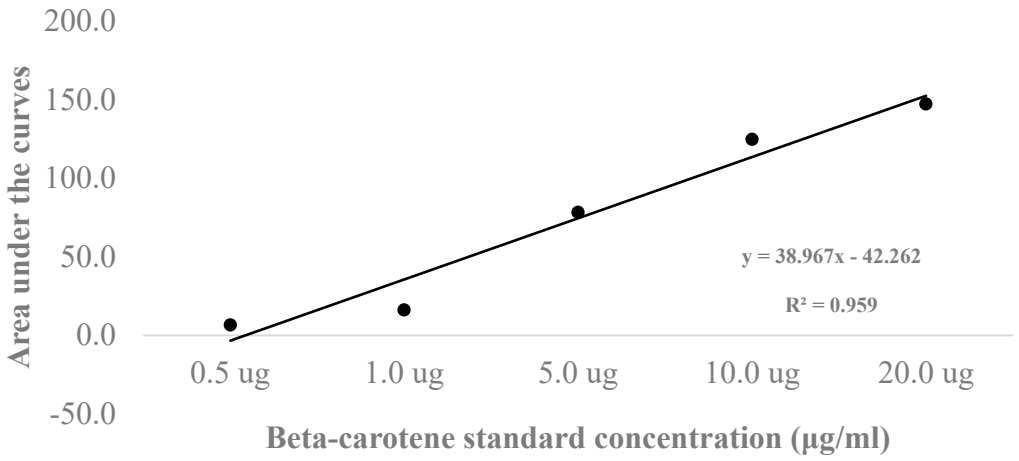

Figure 3. Regression line plot on beta-carotene standard concentration (µg/ml) against area under the curves estimated by the UHPLC system.

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Table 2. Sample concentration of tested human sera according to the protocol.

Human sera	Average area under the curve	Concentration (µg/ml)	Concentration (µmol/l)
1	17.86	1.54	2.87
2	22.34	1.66	3.08
3	58.43	2.58	4.81
4	3.35	1.17	2.18
5	11.52	1.38	2.57
Average		1.67	3.10

The researchers managed to detect beta-carotene compounds at approximately 2 minutes using a UHPLC system. Comparing our findings with several other similar studies, only two studies eluted beta-carotene compound with retention times similar with ours, which recorded at 1.2 minutes [13] and three minutes [14], respectively. On the other hand, other researchers documented beta-carotene retention times ranging from 10 to 27 minutes [15–22]. Inter-study variations, such as the types of LC systems applied, types of organic chemicals used in the mobile phase, and types of columns, were some of the factors possibly affecting the retention time of beta-carotene in human sera.

Although ordinary HPLC systems are still popular among researchers, the UHPLC system outperform the existing system in eluting beta-carotene in a shorter time, saving solvent and providing good retention peaks [23]. Indeed, those studies that managed elute beta-carotene rapidly were utilizing the UHPLC system. Compared to the ordinary HPLC system, the UHPLC can operate at significantly higher pressure (maximum pressure at 2.5X compared to the HPLC system). Additionally, UHPLC also allows the use of a smaller particle size column (as low as 1.8 um), which reduces the beta-carotene's retention time to one third of the time used compared to the HPLC system [23,24]. Hence, combining these two features may explain how the UHPLC system shorten the mobile and stationary phases, resulting the beta-carotene to be eluted faster in the chromatogram.

Apart from the LC systems, types of solvents also affect the retention time of beta-carotene through the mobile and stationary phases. The property of the organic chemicals such as corresponding eluotropic strength is one of the determining factors of which solvent to be selected in eluding the compound of interest. Of all organic solvents, acetonitrile and methanol are commonly used in reversed-phase liquid chromatography. The aqueous component of the mobile phase (e.g., ultrapure water) has a weak analyte elution strength while the organic component has a higher elution strength. Hence, a relative proportion of aqueous and organic can be adjusted to control analyte retention. For instance, acetonitrile has a higher elution strength than methanol for reversed-phase chromatography. Therefore, when these two solvents are used in combination, a shorter

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analyte retention can be expected. In addition, relying on acetonitrile solvent alone will likely lead to overlapping compounds, hence could not accurately identify the unique peak of beta-carotene. Thus, a combination of both acetonitrile and methanol may have better separation on this unique compound in human sera samples.

The beta-carotene component is hydrophobic and requires ample time to elute when the C18 and C30 HPLC columns are used. Notably, the compound's retention time for the C18 column is about 13 minutes and C30 column required approximately three times (42 minutes) the retention time to elute the same compound [10]. Compared to the C18 and C30 columns, the C8 column used in our protocol can significantly reduce the retention time of beta-carotene. The C18 column has 18 carbon atoms compared to the C8, which only contains eight carbon atoms [26]. The C18 has an increased surface area, delaying the molecule (beta-carotene) in the mobile phase to travel across the stationary phase column. In addition, the column pore size of above 100 A could potentially affect the retention parameters among carotenoids [10]. However, as the current study's column has a pore size at 00 A, thus, this is not a contributing factor towards the retention behaviour of beta-carotene.

It should be noted that there was a slight retention time swift in beta-carotene elution despite the coefficient of variation on the time swift was within the acceptance range (<0.5%) [27]. While column temperature and sample flow rate may cause sample retention time swift [27]. However, these parameters were kept constant throughout the protocol development, hence the slight retention variation was probably not affected by these two factors. Besides, one of the most common causes of shifts in retention time in reversed-phase LC separations is a minor change in the concentration of the organic solvent, either methanol or acetonitrile. This happens when a minor error in formulation or a change in the mobile-phase composition if one solvent evaporates over time. Hence, utilizing the same apparatus and consistent with solvent preparation protocol is recommended to minimize the variation in its concentration across tests.

Conclusion

Through this protocol aiming to detect beta-carotene in human sera, a method that incorporated the utilization of a UHPLC system, less carbon density C8 stationary phase and an ideal solvent with a mixture of acetonitrile and methanol is proposed. Combining these three indicators can improve the retention time of beta-carotene and reproducible with the low coefficient of variation. Hence, this protocol is recommended for routine screenings or epidemiological or population studies that involve huge sample sizes.

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