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Scheme of obtaining β-carotene standard from pumpkin (*Cucurbita moschata*) flesh

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Abstract: Characterization and quantification of carotenoid compound is complicated, costly and time-consuming. The accuracy and reliability of the data depend solely on the standard and to High Performance Liquid Chromatography (HPLC) analysis but the major constraint is to acquire and to maintain the pure standards. Carotenoid standards are commercially available but they are expensive and are prone to isomerization and oxidation. Thus, the purpose of this study is to establish an analytical method for isolating β-carotene by using open column chromatography (OCC) from pumpkin (*Cucurbita moschata*) to be used as one of the carotenoid standards for determination of total and individual carotenoid. Pumpkin with orange flesh has been chosen due to the non-seasonal nature and its availability all year-round. This study demonstrated that the purity of β-carotene standard; determined by HPLC was ranged from 92.21 to 97.95%. The standard curves with five different concentrations of β-carotene extract from pumpkins in triplicate were constructed by plotting the peak area against the concentration. The coefficient of correlation was 0.9936. Therefore, this study established that pumpkin can be a reliable source of beta-carotene standard as it is cheap and commonly available throughout the year.

Keywords: Carotenoids, ß-carotene, HPLC, *Cucurbita moschata*

Introduction

Reliable data on carotenoid analysis is urgently needed as this research area is developing its own potential to human health and also is important to the food and pharmaceutical industry. Carotenoid is recognized as a phytochemical compound which is responsible to reduce the risk of some degenerative diseases such as cancer and disease related to cardiovascular (Ames *et al*., 1993). Generally, carotenoid is a tetraterpenoid organic pigment which occurs naturally in the chloroplasts and chromoplasts of plants including some photosynthetic organisms like algae, some fungus, bacteria and at least one species of aphid (plant lice) (Nancy and Tyler, 2010).

In nature, more than 600 carotenoids, not including *cis* and *trans* isomers, have been isolated and characterized from natural sources (Pfander, 1987). Most of the carotenoid compounds can be isolated from deep color vegetables and fruits as these sources are known to have rich amount of phenolic compounds, including flavonoids, anthocyanins, and carotenoids (Qian *et al*., 2004; Sass-Kiss *et al*., 2005; Trappey *et al*., 2005; Cieslik, 2006). In pumpkins, carotenoid is a natural plant pigment which is responsible to give the orange colour. In some studies done by Azizah *et al*. (2009) and Amotz and Fishler (1998) reported that pumpkins consist of β-carotene and lycopene.

Most of the carotenoids in food can be reliably determined either by using an open column chromatography (OCC) or high performance liquid chromatography (HPLC) (Adewusi Bradbury, 1993; Carvalho *et al*., 1993). Both of these techniques have their own advantages and disadvantages. OCC for instance, does not require a constant supply of carotenoid standards. This is because the separated fractions can be determined and quantified directly by *uv-vis* spectrophotometer by referring to the published coefficients of absorption data. However, the sample throughput is low, and the results obtained totally depend on the handling approach of the analyst. On the other hand, HPLC analysis is expensive and the results obtained depend on the accuracy of standardization. One of the major problems in HPLC is the difficulties to obtain and maintain pure standards as the carotenoid compound is prone to isomerization and oxidation and also expensive especially to those who have difficulties to purchase them from overseas (Miekoand Rodriguez-Amaya, 2002).

According to Zaharah *et al*. (2006), the area for

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pumpkins plantation in Malaysia was around 138 ha, where pumpkin is abundantly planted in Kelantan (79.6 ha), Terengganu (59.6 ha) and Johor (93.5 ha). The aim of this study is to isolate ß-carotene compound from pumpkin which is a natural source abundantly planted in Malaysia to be used as a standard in carotenoid analysis by using the method of OCC and quantification by HPLC.

Materials and Methods

Sample preparation

Pumpkins were obtained from Agricultural District Office, Kelang, Malaysia. Samples were cut and grinded to reduce the size and dried for further analysis. For individual carotenoid identification in pumpkin flesh, fresh samples of pumpkin flesh were cut to reduce size and directly extracted and analyzed.

Sample extraction

Samples were extracted with a mixture of acetone and petroleum ether with a ratio of 1:1 at room temperature overnight. This step was repeated until the grinded pumpkin flesh becomes colourless. The crude extracts were filtered, evaporated in rotary evaporator and re-suspended in petroleum ether (Ren and Zhang, 2008).

Purification

Samples were purified using silica gel in gravity column. 2-3 ml of sample were put onto the top of column and eluted with approximately 100 ml of 10% (v/v) acetone in petroleum ether. The elution was collected, evaporated, dissolved and made up to a volume of 10 ml with petroleum ether and kept in a dark bottle at the temperature of -20°C, in a freezer until next analysis.

Total carotenoid content

The dried carotenoid with nitrogen gas was re-suspended in 250 µl of ethyl acetate for determination of total Carotenoid. 50 µl of the re-dissolved sample was then diluted with 950 µl chloroform for spectrophotometric analysis. Carotenoid containing solutions were measured at three different wavelengths: λ 480 nm, 648 nm, and 666 nm using UV-VIS - VARIAN 50 Conc UV-visible spectrophotometer. The Wellburn Equation in chloroform was applied in this study to obtain total carotenoid content as described below (Wellburn, 1994):

$C_a = 10.91A666 – 1.2A648$

$C_b = 16.36A648 – 4.57A666$

$C_{x+c} = (1000A480 – 1.42\ C_a – 46.09\ C_b) / 202$ (µg/ml)

C_a = Concentration at 666 nm

C_b = Concentration at 648 nm

C_{x+c} = Total carotenoid concentration at 480 nm

HPLC analysis

The HPLC analysis of carotenoids was performed using Agilent model 2100 series which comprised of a binary pump with autosampler injector, micro vacuum de-gassers, thermostat column compartment and a diode array detector. The column used was a HPLC column: ZORBAX Eclipse XDB-C18, analytical 4.6 x 150 nm (5 micron) end capped 5 µm. The solvents used were (A) acetonitrile: water (9:1 v/v) and (B) ethyl acetate. The solvent gradient used was developed as follows: 0-40% solvent B (0-20 min), 40-60% solvent B (20-25 min), 60-100% solvent B (25-25.1 mins), 100% solvent B (25.1-35 min) and 100-0% solvent B (35-35.1 min) at a flow rate of 1.0 ml min^{-1}. The temperature of the column was maintained at 20ºC.

The injection volume was 10 µl. Carotenoid standards violaxanthin and neoxanthin were isolated from *Eruca sativa* (roquette or rocket salad) by open column chromatography as described by Kimura and Rodriguez-Amaya (2002), whereas the standard for β-carotene, lutein and zeaxanthin were obtained commercially from Sigma-Aldrich. Detection of individual carotenoids was made at the wavelengths of maximum absorption of the carotenoids in the mobile phase: neoxanthin (438 nm), violaxanthin (441 nm), lutein (447 nm), zeaxanthin (452 nm) and β-carotene (454 nm).

Carotenoid compounds were identified by co-chromatography with standards and by elucidation of their spectral characteristics using a photo-diode array detector. Detection for carotenoid peaks were in the range of 350 to 550 nm. Individual carotenoid concentrations were calculated by comparing their relative proportions, as reflected by integrated HPLC peak areas, to total carotenoid content determined by spectrophotometer.

Preparation of the ß-carotene standard solutions from Cucurbita moschata and construction of the standard curves

All aliquots were dried and dissolved in 1 ml ethyl acetate (HPLC grade). 100 µl were filtered through 0.45 µm syringe filter and only 10 µl were injected into HPLC and purity was verified. For standard curves, triplicate aliquots of 1,2,3,4 and 5 ml were transferred into culture tubes and dried to completion

under a gentle stream of oxygen-free nitrogen. Curves constructed with five different concentrations in triplicate were confirmed as linear with a correlation coefficient ≥ 0.90. The purity of the standard solution was calculated as follows:

$$\% \text{ purity: } \frac{\text{Area under the standard peak}}{\text{Total area of all peaks}} \times 100$$

Once the desired purity was obtained (≥90%), the concentrations of the pure standards were determined spectrophotometrically, using the following $A_{1\,cm}^{1\%}$ values = ß-carotene , 2550 in ethanol, 2396 in chloroform, and 2592 in petroleum ether. A sample of at least 1.0 mg of the pure crystalline substance was weighed accurately (to 3 decimal places) and dissolved in an accurately measured volume of a suitable solvent. The concentration of each pure standards were calculated according to the following formula:

$$C\ (\mu g/ml) = \frac{\text{absorbance} \times 10^4}{A_{1\,cm}^{1\%}}$$

The concentrations with the respective standard's % purity then were corrected:

$$\text{Corrected C } (\mu g/ml) = \frac{\text{C } (\mu g/ml) \times \%\ \text{purity}}{100}$$

Result and Discussion

Determination of total carotenoid content and HPLC analysis of its composition in Cucurbita moschata

The total carotenoid content in flesh of *Cucurbita moschata* was 121.21±0.64 µg/g of fresh weight (FW). Carotenoid analysis performed by HPLC system detected at least three major carotenoid peaks: violaxanthin, lutein and β-carotene. In Figure 1 below, HPLC chromatogram showed that the retention time of violaxanthin, lutein and β-carotene were 2.359, 8.491 and 27.986 minutes respectively.

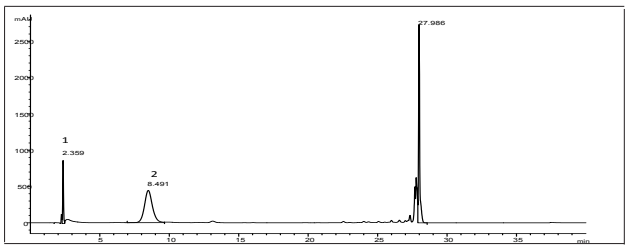

Figure 1. HPLC chromatogram of individual carotenoids from *Cucurbita moschata*.1=violaxanthin, 2=Lutein, 3= ß-carotene

Tee and Lim (1991) also detected three major carotenoids in pumpkin (*Cucurbita maxima)* which were lutein (0.94 µg/g), α-carotene (0.756 µg/g), and ß-carotene (0.578µg/g) which is similar with the results obtained in this study except for α-carotene, while Murkovic *et al*. (2002) reported that three species of pumpkins (*Cucurbita pepo, Cucurbita maxima and Cucurbita moschata)* consist of beta-carotene (0.06 -7.4 mg / 100 g, α-carotene (0-7.5 mg / 100 g) and lutein (0.17 mg / 100g). According to some studies in squashes and pumpkins, some cultivars have α- and ß-carotene as principal carotenoids, whereas ß-carotene predominates in others (Rodriguez-Amaya, 1990, 1999; Arima and Rodriguez-Amaya, 1988; Azevedo-Meleiro and Rodriguez-Amaya, 2002).

These results demonstrated that the carotenoid composition and accumulation level vary with extraction method and environmental factors. Understanding the mechanism that controls carotenoid biosynthesis and exploring the diversity of carotenoid compounds in a wider range of germplasm will contribute greatly to the enhancement of β-carotene production or other carotenoids in pumpkins.

Isolation of ß-carotene from Cucurbita moschata *by OCC*

Figure 2 shows that the purity of the ß-carotene isolated from *Cucurbita moschata* at 1 day by using OCC method was 97.95% and the standard curves obtained from a one point calibration showed linearity with coefficients of correlation of 0.9999. The standard deviations between calibrations were around 0.01 to 0.20. The coefficient of correlation standard curves obtained by commercial reference standard was 0.9950. Full calibration was also applied for day 1, 10 and 40 to observe the stability of ß-carotene isolated from *Cucurbita moschata*.

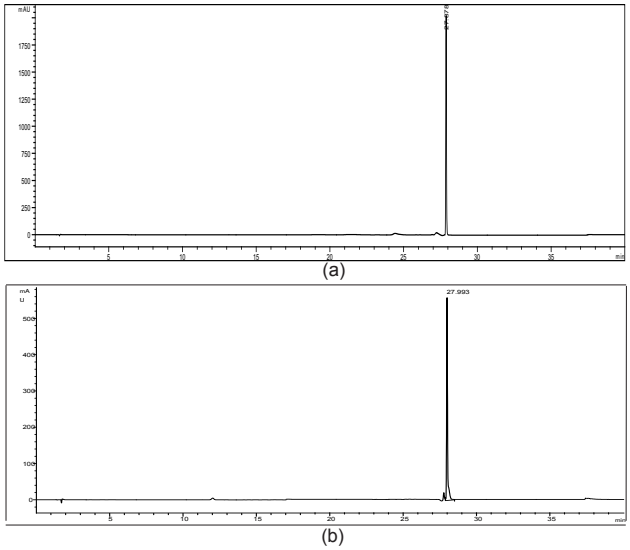

Figure 2. HPLC chromatogram of (a) beta carotene standard and (b) isolated beta carotene from pumpkin (*Cucurbita moschata*) at 1 day

From this study, the purity calculated as the percentage of the carotenoid's peak area relative to

total area was 97.95% for day 0, 96.13% for day 10 and 92.12% for day 40. The purity of ß-carotene isolated from this source decreased by time from day 0 to day 40. Kimura and Rodriguez-Amaya (2002) reported that one study by Quackenbush, and Smallidge (1986) showed that commercial ß-carotene had purity by spectral absorbance range from 2.4% to 95.6% where the degradation was associated to autoxidation after packaging. According to Rodriguez Amaya and Kimura (2002), the purity percentage of ß-carotene isolated from lettuce was 90–97% using MgO:Hyflosupercel column. As compared to this study, ß-carotene from *Cucurbita moschata* which was isolated and purified using silica gel OCC was range from 92.12% to 97.15%. Even though silica gel is not a popular adsorbent because of its characteristic to inherent acidity which may cause carotenoid isomerization and degradation (Taylor, 1983) the purity percentages obtained were substantially high and reliable to be used as a standard.

Table 1. Comparison of ß-carotene composition (µg/g) of *Cucurbita moschata* flesh obtained by one point recalibration, straight line equation, standard deviation and covarians between calibrations

Sample number[a]	Purity (%)	One-point calibration	Straight line equation	SD between calibration	Covarians between calibration
1	97.95	9.13	9.59	0.00	0.00
2	96.13	8.11	8.82	0.20	0.03
3	92.21	9.13	10.07	0.06	0.02

[a]Sample were analysed at 0(1) days, 10(2) days, and 40(3) days

Conclusion

The study on the scheme of obtaining β-carotene standard from pumpkin flesh demonstrated that the purity of β-carotene standard obtained and quantified by HPLC was 92.21 - 97.95%. The standard curves with five different concentrations for β-carotene in triplicate were constructed by plotting the area against the concentration. The coefficient of correlation was 0.9936. This study established that pumpkin can be a reliable source of beta-carotene standard which is cheap and commonly available throughout the year.

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