IOSR Journal Of Pharmacy
(e)-ISSN: 2250-3013, (p)-ISSN: 2319-4219
Volume 10, Issue 12 Series. I (December 2020), PP. 21-27
www.iosrphr.org

Determine β–carotene in carrot (*Daucus carota* L.) by using HPLC and GC–MS

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Received 28 December 2020; Accepted 09 January 2020

Abstract
Context: β–carotene has been used extensively in food, industry, and medicine. β–carotene extracted by chemical solvent, so that β–carotene is of high economic value. **Aims**: To extract β–carotene without chemical solvent and to determine β–carotene using UHPLC and GC–MS. **Settings and Design**: This research was laboratory experiment. Carrot is obatained from local supermarket. **Methods and Material**: Approximately 300 g carrots, grind, separate from that matrix. To the filtrate was then, added some chemical salt, centrifuged 5 000 × *g* for 30 min to have pellet. The pellets evaporated 40 ^{0}C for 48 h. β–carotene pellets are measured with UV–Vis, HPLC and GC–MS. Mobile phase was chloroform–methanol (95:5) with a flow rate of 1 mL min^{-1} at a wavelength of 460 nm UV–Vis. **Statistical analysis used**: There is no sample size were performed. Analysis data observed from the capacity of Instrument to perform the time peak of evaluation. **Result**: The retention time examination of β–carotene and confirm standard (sigma β–carotene) using HPLC and GC–MS were 3.85 min and 9.80 min respectively. GC–MS examination showed ions and molecules of carotenoid. **Conclusion**: Research showed that this method can be used to be developed for the withdrawal of β–carotene in carrots and others, without any need for organic solvents.
Keywords: Antioxidant, carotenoid, extraction

I. INTRODUCTION

Antioxidants have recently been discussed not only among scientists but also in societies that are increasingly aware of their benefits.[1] Besides being known as a powerful weapon to ward off a variety of diseases, antioxidants are also believed to be able to keep young.[2] Along with the times, causing changes in lifestyle people who tend to live an unhealthy lifestyle such as smoking, drinking liquor, consuming junk food and being exposed to excessive ultraviolet light. As a result, there are so many free radicals is obtained, can lead to harm to the body. These free radical compounds can be formed as a result of the chemical processes, that occur in the body, such as oxidation, metabolism and inflammation. [2],[3]

Free radicals are defined as atoms or molecules that contain one or more unpaired electrons that can be occur in the cellular level of whole of the body. Free radical-induced injury can explain many clinical conditions The toxicity of many xenobiotics is associated with the metabolic activation of foreign compounds to form free radicals or with the production of reactive oxygen species as superoxide anion, hydroxyl radicals or hydrogen peroxide which are responsible for the tissue damaging effects as lipid peroxidation, and DNA and protein damage.

Oxidative stress associated with production of reactive oxygen species is believed to be involved not only in the toxicity of xenobiotics but also in the pathophysiology of aging, and various age-related diseases, including cataracts, atherosclerosis, neoplastic diseases, diabetes, diabetic retinopathy, chronic inflammatory diseases of the gastrointestinal tract, aging of skin, diseases associated with cartilage, Alzheimer's disease, and other neurologic disorders. The cellular sources of free radicals and reactive oxygen species, the biological targets of free radicals, and clinical conditions which are associated with free radical production and tissue damage are reviewed. In addition, potential therapeutic approaches to the prevention of free radical damage are considered.[4] .

To protect the body from attacks by free radicals, the body needs antioxidants, including consisting of β–carotene, vitamin E, vitamin C and selenium.[4] The β–carotene is one of the antioxidants that can prevent form disease. This antioxidant compound is able to neutralized free radicals in the body which is a trigger source for various diseases, like as, metabolic degenerative, as well as cancer diseases. Naturally β–carotene is abundant in fruits such as pumpkin (*Cucurbita pepo*), palm oils (*Elaeis guineensis* Jacq.), red fruit (*Pandanus conoideus* Lam.), watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai), mangoes (*Mangifera indica* L.),

tomatoes (*Solanum lycopersicum* L.), melons (*Cucumis melo* L.) and carrot (*Daucus carota* L.). One of the main sources of β–carotene is carrots, as high–value compound. In recent years carotenoids have represented a good alternative for the pharmaceutical and food industries and especially for the human health, carotenoids prevent different diseases, such cancer, macular degradation and cataract.[5]

Carrots are classified as a group of vegetables, contains carotenoid groups. The level of β–carotene that found in carrots is also varies. Differences in the content of β–carotene in carrots remembering that β–carotene is a useful of antioxidant compound, there fore, to know the content of β–carotene and to develop the methods of extraction is needed.[3],[6] In this study, the isolation of β–carotene were carried out using a high performance liquid chromatograph that used reverse phase and Gas Chromatograph (GC–MS). This study also to got the right and careful method in determining β–carotene, and let to know the possibilty of this methods to determine β–carotene in carrots. The main aim of the study was to isolation and concentrate β–carotene from carrot extract made by calsium salts.

II. MATERIALS AND METHODS

Material

Carrots, which are obtained from the super market in Palembang, Indonesia β–carotene ingredients, chloroform (Sigma), tetrahidrofuran (THF), methanol (Sigma), aquabidest (Sigma), acetonitrile sigma grade HPLC. Standard β–carotene Sigma Aldrich.

High performance liquid chromatography using (ultimate U–HPLC thermo 3 000 series), GC–MS series, ultraviolet–visible spectrophotometer (analytic jena specord 200), analytical scales (sartorius dragon), micro scales (sartorius dragon), blender (philips), rotavapor (buchi V–800), separating funnels, glassware, whatmann filter paper number 40.

Methods

determination of optimum Ultra–HPLC conditions

i) Determination of the maximum absorption wave length of β–carotene.

A total of 20 mg β–carotene of isolated, is inserted into a 50 mL measuring flask dissolved and diluted with chloroform until the mark. Then piped 2.5 mL into 10 mL pumpkin measuring, diluted with chloroform until the mark. Then the spectrum was made using a UV–Vis spectrophotometer at a wavelength of 420 nm to 500 nm.[7],[8]

ii) Selection of the mobile phase and flow rate.

A total of 20 mg β–carotene of isolated is put into a 50 mL measuring flask dissolved and diluted with chloroform to the mark line. Then piped 2.5 mL, was put into a pumpkin measuring 10 mL diluted with chloroform until the mark. Inject 20 µL into the HPLC tool using the mobile phase of methanol–chloroform (95:5); methanol–tetrahydrofuran–water (67:27:6); chloroform–tetrahidrofuran–water (67:27:6); acetonitrile–chloroform (92:8); chloroform–methanol (95:5) and chloroform–tetrahidrofuran–methanol (70:25:5) with a flow rate of 0.5 mL min^{-1} and 1 mL min^{-1}. The mobile phase and the selected flow rate provide the best separation with a not too long retention time.[9]

identification of β–carotene in carrots

i) Extracts/isolates:

Fresh carrots that have been cut and mashed, weighed 300 g (carrots), 300 g of blended using 15 mL water solvents, then filtered. The bulk is seperated. The filtrate was then rinsed with the calcium salts, centrifuged 3 000 mg kg^{-1} to 5 000 mg kg^{-1} 15 min. The residue as a pellet is seperated from the filtrate. The pellet was then removed from the filtrate. Rinse the pellet to removed the calsium salt. The pellet was then drying with nitrogen vacuum, or rotary evaporator at temperature 40 ^{0}C. Then, stored the pellet in cold temperatures.

ii) Standar solution standard solutions:

A total of 10 mg β–carotene is put into a 50 mL measuring flask dissolved and diluted with chloroform until the mark. Then pipette 2.5 mL was put into a 10 mL measuring flask diluted with chloroform to the mark line (standard β–carotene).

iii) How to identify:

A number of test solutions were injected as much as 20 µL into the HPLC tool, then compared the retention time with the standard retention time of β–carotene. [10]

A number of test solutions were injected as much as 50 µL into the GC–MS tool, set the GC–MS 310 ^{0}C, start set from 60 ^{0}C, injector temperature 205 ^{0}C then compared the retention time with the standard retention time of reference. [10]

quantitative analysis by high performance liquid chromatography

i) System suitability test

System suitability tests are conducted to find out whether tools, methods and conditions form a single analysis system. 20 µL of the standard β–carotene solution is injected five times into the HPLC tool, then the peak area is measured with the optimum HPLC condition, then the relative standard deviation values are calculated.

ii) Determination of levels of β–carotene in HPC carrot extract.

The test solution was sonicated for 10 min. Each injected 20 µL into the HPLC tool and measured its peak area with optimum HPLC conditions. Levels of β–carotene isolates can be calculated into Formula (1).

$$\mu g/g = \frac{(Au/Ab) \times Cb \times fp \times VL}{B \; (Formula)^1}$$

Where :
Au = area under the curve sample
Ab = area of content
Cb = concentration β–carotene (µg mL^{-1})
fp = factor correction (mL)
VL = volume sample (mL)
B = weight of sample (g)
(Serlahwaty, at al.)[11]

III. RESULT

The standard β–carotene identification doing by Serlahwaty[11] can be seen in Figure 1, Figure 2, and Figure 3. The identification of raw β–carotene in carrots of this study that obtained from the measurement testing using HPLC and GC–MS is depected in figured (Figure 4, Figure 5, and Figure 6). The standard chromatogram of β–carotene with a retention time of 1 309 min show in Figure 1, with flow rate 1 mL min^{-1}. In Figure 2 depicted the standard β–carotene chromatogram with a retention time of 2.86 min with flow rate of mobile phase 0.5 mL min^{-1}. Figure 3 is the standard material for UV–Vis β–carotene cromatogrph.[11] Figure 4 is a UV–Vis β–carotene of carrots. Figure 5 is a chromatogram of β–carotene showed retention time 3.85 min produced by U–HPLC.

Analytical conditions: GC–MS (EI) was carried out on a Perkin Elmer (Auto System XL) gas chromatograph coupled to the MS detector (Perkin Elmer TurboMass) in the electron impact mode (70 eV). The column was PE–5HT (30 m × 0.25 mm × 0.1 µm); carrier gas: Helium (1 mL min^{-1}); the inlet mode was split: 50:1; and the injector temperature was 250 ^{0}C. The initial column temperature was 60 ^{0}C, and after 1 min the temperature was raised to 310 ^{0}C with 4 ^{0}C min^{-1}. Thereafter, the conditions were held for 6 min. The mass spectrometer measurement was scanned from 40 m z^{-1} to 600 m z^{-1}. According to the literature, the GC–MS method was successfully applied in many cases to analyze products with lower molecular weight than retinol. [10]The major metathesis products were hexadecanoic acid, methyl ester, pentadecanoic acid, methyl ester, hexadecanoic acid, methyl ester as determined by GC–MS (Figure 6).

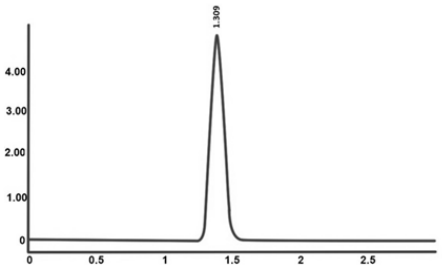

Figure 1.Chromatograph of standard β–carotene produced: RT 1 309 min inflow rate 0.5 mL min^{-1} with phase mobile chloroform: THF: Methanol = 70:20:5).[11]

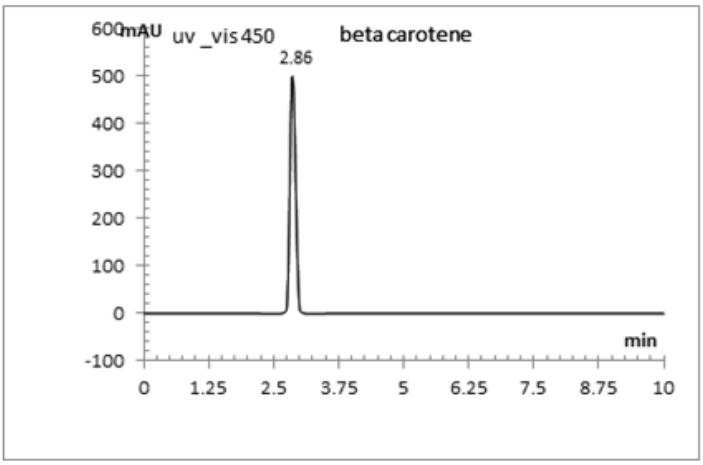

Figure 2. Chromatograph of standard β–carotene produced: Retention time RT 2.86 min flow rate 0.5 mL min^{-1} with phase mobile chloroform: THF: Methanol = 70:20:5). (Serlahwaty, et al.)[11]

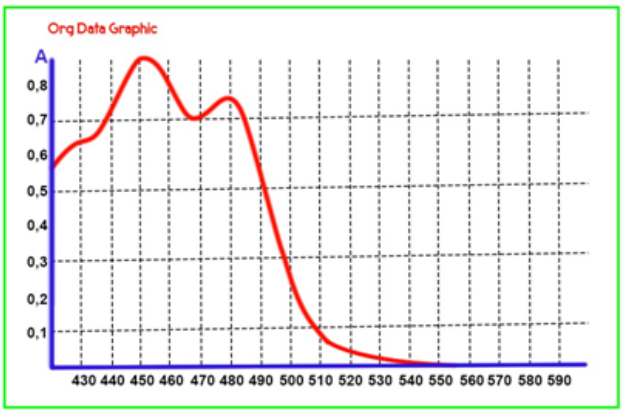

Figure 3. Chromatograph of β–carotene UV–Vis in wave length 450 nm. (Serlahwaty, et al.)[11]

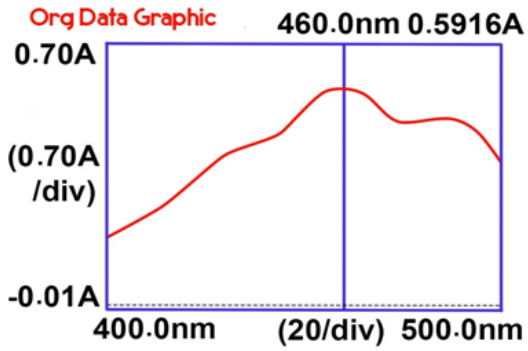

Figure 4. Chromatograph spectra of β–carotene sample tested on UV–Vis at 460 nm wave length in chloroform solvent.

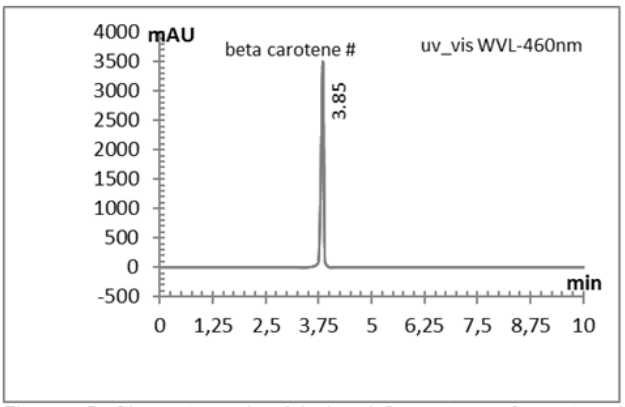

Figure 5. Chromatograph of Isolated β–carotene of carrot and standard (Sigma) betacarotene. The retention time is 3.85 min in mobile phase chloroform: Methanol (95:5) with detector UV–Vis 460 nm.

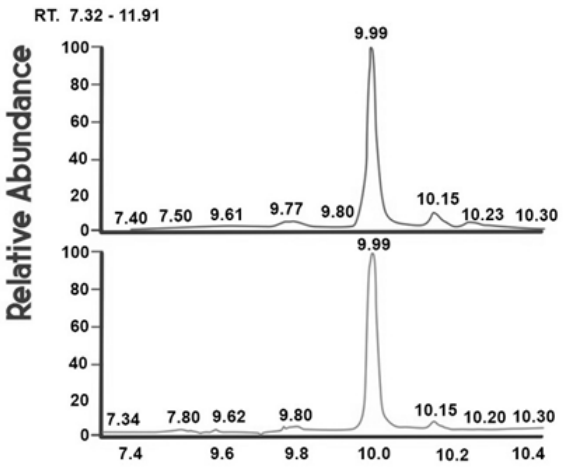

Figure 6. Cromatograph of β–carotene in carrots using GC–MS to produced hexadecanoic acid, methyl ester; pentadecanoic acid, methyl ester and hexadecanoic acid.

IV. DISCUSSION

β–carotene is a groups of carotenoids, isoprenoid, hydrophobic, so it is called soluble in organic solvents. The chemical properties of β–carotene are mainly due to the multiple containing double bonds.[5],[6] Organic solvents that often used to extrcted β–carotene are mainly chloroform, petroleum ether, dichloromethane, and hexane. The isolation of β–carotene from carrots with organic solvents has been carried out,[9] one of them is the Fikselova study[12] investigating the relationship of solvent combinations and temperature of loss. In this finding due to the isolation of β–carotene from carrots was used without organic solvents, the principle of go to green. The double bond in β–carotene can settle with calcium salts so this principle is used in research to get β–carotene from carrot juice. The separation of precipitate is used by centrifuge. The pellets obtained are as orange β–carotene. Mostly in references, doing to extract of β–carotene almost with organic solvents. Reversely, in this study, did not used of organic solvents, but we can sure that with the principle of precipitation according to the nature and principles of the reaction of calcium salt with in β–carotene. The subsequent β–carotene as standard β–carotene (Sigma Aldrich) is treated by using Ultra HPLC. The result is compared if any difference from the standard β–carotene and β–carotene tested. Seems it is only best doing by HPLC to find of β–carotene compare to GC–MS, because of the β–carotene reveal is unstable to heat in running by GC–MS. Some researcher can be found the mass spectra of β–carotene peak in MS 540, therefore not fully complete to understand because of the limitation of material and time to course.

Analytical conditions: GC–MS (EI) was carried out on a Perkin Elmer (AutoSystem XL) gas chromatograph coupled to the MS detector (Perkin Elmer TurboMass) in the electron impact mode (70 eV). The column was PE–5HT (30 m × 0.25 mm × 0.1 µm); carrier gas: Helium (1 mL min^{-1}); the inlet mode was split: 50:1; and the injector temperature was 250 ^{0}C. The initial column temperature was 60 ^{0}C, and after 1 min the temperature was raised to 310 ^{0}C with 4 ^{0}C per min. Thereafter, the conditions were held for 6 min. The mass spectrometer measurement was scanned from 40 m z^{-1} to 600 m z^{-1}. According to the literature, the GC–MS method was successfully applied in many cases to analyze products with lower molecular weight than retinol. The major metathesis products were hexadecanoic acid, methyl ester, pentadecanoic acid, methyl ester, hexadecanoic acid, methyl ester as determined by GC–MS.

From the results of the optimum conditions, it was shown that β–carotene provided maximum absorption at a wavelength of 460 nm with chloroform solvents (Figure 4). The wavelength shift from 450 nm to 460 nm is possible influenced by the solvent factors used that are different when measuring than the reference.[7],[8] Based on the experimental results, the best comparison is chloroform–methanol (95:5) with a flow rate of 1 mL min^{-1}, according with retention times obtained faster and the resolution is good. The mobile phase commonly used according to conditions is chloroform, alcohol, hexane, methanol, acetonitrile, test grade HPLC[9],[10]. Furthermore, it can be given a supporting solution such as THF.[11] The results of wavelength determination can be seen in (Figure 5); the results of the selection of the mobile phase and flow rate are shown in (Figure 5). Although several studies have shown different β–carotene (retention time) results from this study, the mobile phases performed are different, different flow rates and different HPLC devices.[7],[12] So that each RT measurement is an optimal condition carried out by researchers in producing a chromatogram of β–carotene to produce a relatively fast RT and clear peak images.[6],[13] The content of carotenoids in plants is influenced by several factors, these may be genetic, environmental, or strategies used to manage the crop during its growth. The latter can result in an increase in the concentration of carotenoids.[6],[7],[8]

In this study, the retention time of β–carotene was 3.85 min using a flow rate of 1 mL min^{-1} with the mobile phase of chloroform: Methanol (95:5). With in the regard a β–carotene peak was detected between only 2 min to 4 min. It is better in 2 min to 3 min using by HPLC, said some resercher.[9] The sample was further observed at various wavelengths thus detect impurities with absorbance maximum different from 440 nm, no considerable disturbances in the β–carotene region were observed. Peaks indicating other substance (e.g. Impurities) was eluted and thus detected earlier in the chromatogram (Figure 4). Using a comparatorly flow rate of 1 mL min^{-1} standard β–carotene can be separated at retention time 3.85 min. Improvement of peak chromatogram can be continued by adding a combination of mobile phase solution with THF combination to obtain a sharper peak.[13],[14] From the results of the linearity test (Figure 1), the value for the standard solution of β–carotene is 0.9 971. The r value shows the ideal value because it is close to one, so the correlation coefficient between the solution concentration and the peak area detected by HPLC is good and can be used for research. The research of Serlahwaty[11]; Fikselova[12] has provided inspiration in this study, especially in the use of raw data on β–carotene. Further research is being carried out for the preparation of formulations and preparation of standard β–carotene.

V. CONCLUSION

The used of method to isolation, has produced β–carotene. The proof was carried out by Ultra High Performance Liquid Chromatography (UHPLC) and GC–MS compare to standard β–carotene. Suggest to have β–carotene in general.

ACKNOWLEDGEMENT

Acknowledgment were delivered to Polytechnic of Health Palembang, Indonesia also The Palembang Health Laboratory of their helping in using the HPLC and UGM Life Health Science Laboratory for using GC–MS.

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