Original Research Article

The evaluation and comparison of β-Carotene chemical quality extracted of *Azolla filiculoides* from Anzali wetland in summer and winter seasons via organic solvents

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Abstract

The present research was aimed at determining the quality of natural β-Carotene, comparing it with synthetic β-Carotene and the effect of season on it. *Azolla* was sampled in summer and winter seasons. The treatments included β-carotene derived from *Azolla* through the organic solutions. Synthetic β-Carotene was used as the control. The treatments were kept at 5 °C for one year. The results showed purity, concentration, colorimetric and vitamin A in the experimental and control treatments, revealed significant difference ($p<0.05$). Vitamin A showed significant difference between the experimental treatments ($p<0.05$). β-Carotene amount was higher in summer treatment compared to those sampled of winter ($p<0.05$). The solubility of B-carotene was greatest in tetrahydrofuran, while methanol and acetonitrile exhibited the least solubility. Degradation was greatest in cyclohexanone. The experimental treatments had a desirable chemical quality the end of storage period. As the natural β-Carotene takes precedence over the synthetic one in terms of the food hygiene, it is recommended that β-Carotene extracted from *Azolla* be substituted with synthetic β-Carotene in the food industry.

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Keywords: Anzali Wetland, *Azolla filiculoides*, β-Carotene, Food hygiene, Natural pigment.

Introduction

B-Carotene is a natural pigment with a color ranging from yellow to red which is readily found in most vegetables such as Azolla, certain algae and microorganisms engaged in photosynthesis. The substance was first extracted from carrot by Wakenro in 1931. B-Carotene is currently of wide application as a natural color in various industries including food, cosmetics, live stocks, pharmaceuticals, medical devices and poultry feed. In addition, it is both a strong antioxidant agent and a prerequisite for vitamin A formation in both human and animals. There are considerable interests to produce β-Carotene at commercial scale due to its numerous properties and benefits. It is found in a large number of vegetables as well as *Azolla* which constitute a natural source of β-Carotene [1]. Natural β-Carotene is decomposable and free of any contaminations. Food additives including natural colorants are useful in improving the nutritional qualities of foods and eliminating the complications induced by the use of technology in food production process. Food color and /or color additives can be defined as any sort of dye, pigment or substance that could upgrade the quality or give a better look to either the processed or fresh foods. The experience should show that consumers are highly sensitive to the color of foodstuffs which correlates directly with aspects such as quality and sensory features [2]. Considering the fact that consumers 'initial judgments on the quality of any type of foodstuff is based on the color of the product, it can therefore be stated that color is the factor representing the quality of food which indicates whether or not the foodstuff is of good quality or otherwise. Such as flavor and taste, the form and scale, this factor constitutes one of the most important organoleptic features of foodstuffs. Color is thus, one of the best quality indicators in food which plays a crucial part in food additives. Natural dye compounds are extracted from plant sources such as fruits, vegetables, seeds and roots. These colors are also called herbal color. We consume a considerable amount of these natural pigments in our daily food, particularly anthocyanin, carotenoids and chlorophyll. However, the bodily intake of such substances through consuming food processed with these natural pigments is of high importance. Given the public accessibility and security of food color additives, they have also of other usages such as in pharmaceuticals, cosmetics and medical devices [3, 4]. Artificial food colors are dye compounds produced as the result of synthesis of organic material that has an infinite range of applications in different industries including food industry etc. Considering the growing importance of such compounds and the high usage rate of additives in industrial production units in the world, a lot of research

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have been concentrated on pigments. The results of these research showed that synthetic pigments are devoid of any natural value and that most of the artificial colors are not acceptable for human consumption. Since they tend to cause certain complications such as asthma, urticarial, anaphylactic responses, sleep disorders, hypertension, allergy, reduced vitamin level, carcinogenic condition, liver dysfunction, malignant tumors, interfered brain processes such as hampered learning and weak behavioral functions specifically among children and youngsters, lowered intelligence coefficient as well as precipitating hyperactive states in infants [5]. Moreover, these pigments result in the accumulation of synthetic compounds within food which could finally find their way into human body and affect peoples' health by weakening their body's immune system. Due to the vast problems that the consumption of artificial colors may entail, many restrictions have recently been imposed by a number of international health organizations and/or research institutes, limiting their applications. Thus, a global attempt to search for natural substitutes as color additives began. Natural colorants do not have the adverse effect of their artificial counterparts and their positive impacts on human health have frequently been confirmed by many of the related researches and studies [6].

The photosynthesizing organisms should adapt themselves to the varying environmental factors most noticeably temperature, pH and light which play a crucial role in the formation of photosynthesizing pigments for the primary and secondary metabolisms of the plant life. *Azolla* adaptation to varying environmental light conditions is made possible by *cyanobacteria* coexisting with the plant. Such *cyanobacteria* possess especial strategies to cope with light variations [7].

The majority of *cyanobacteria* are organism already adapted to shade. These microorganisms, particularly organisms exposed to fresh water have suitable mechanisms to counteract harmful impacts of solar rays and light absorption in response to variations in light intensity, quality as well as abundance and accessibility to nutrients. The accumulation of β-Carotene has been indicated in plants which occur in reaction to limited lighting and which may also be linked to changes in cellar proteins. Given the crucial role of sugar in plant's adaptations to low light conditions, the production of glucose in plants via Krebs cycle tend to stimulate the formation of chlorophyll and proteins. The β-Carotene belong to internal compounds of chlorophyll A [8, 9].

Exposure to sunlight leads to the accumulation of verities of proteins belonging to inducible protein family which are liable to be impressed by high photic rate within *cyanobacteria*. In addition to *cyanobacteria*, these proteins are also present in plants and embrace proteins connected to plant chlorophyll a/b1 and primary proteins inducible to light. Considering the fact that carotenoids are compounds dependent upon chlorophyll and these proteins, their being exposed to sunlight may trigger the formation of such proteins and β-Carotene within plants. Corollary to sunlight, plants exposure to ultraviolet rays which occur as the result of rupture in ozone layer may also induce the formation of such proteins and β-Carotene 👌10👌.

The present research was aimed at determining the quality and purity of β-Carotene, comparing it with synthetic β-Carotene and effect of season on quantity of β-Carotene extracted from *Azolla filiculoides* in the Anzali Wetland.

2. Experimental

Materials and Methods

Treatments

In the present study, two experimental treatments and a control group were considered. The experimental treatments involved β-Carotene extraction from wild *Azolla filiculoides* of the Anzali wetland. The control group contained standard synthetic β-Carotene color produced by Sigma Aldridge Corp.

Fig 1. *Azolla filiculoides* from Anzali Wetland

Fig 2. Flowers of *Azolla filiculoides* from Anzali Wetland

Extraction of pigment

Sample collection was in the summer and winter seasons. This research carried out in three replicates. Organic solvents method was used to split β-Carotene from *Azolla* 11. The edible portions collected from a kilogram of *Azolla* was measured. Upon random collection of *Azolla* from Anzali wetland, they were thoroughly rinsed again with tap water and later transferred to hot air oven (50^0C). A sum of 5gr of dried residues was mixed with $mol/25_{mi}$ hydroxide sodium. The mix was later homogenized by a heater. Upon cooling, 500_{ppm} of sodium ascorbate was added to the mix. Next it was followed by 39_{min} pigment extraction period by Soxhlet extractor with $100_{mi/l}$ of petroleum ether. The resultant pigments were purified using solid phase ODS column. The sample was rinsed and the solvent was separated.

Experimental

Materials

All of the chemical materials purchased from the Merck in this study.

Chemical analysis

Quality control were conducted by determination of vitamin composition, solubility and stability in organic solvents, β-Carotene percentage over dried residue weight of *Azolla* (production efficiency), Purity and a comparison of shelf life of the extract.

Determination of vitamin A in samples

This test carried out by Budowski and Bondi method (colorimetric procedure). This involves the conversion of vitamin A to anhydrovitamin A with anhydrous ethanolic - HC1 or p-

toluenesulphonic acid. Wave length is strongly near 480 nm. The reaction is very specific for vitamin A [12, 13, 14].

Purity

To evaluate the quality of the final extract, the purity percentage was determined by HPLC test (involving the injection of liquid to HPLC using micro liter syringes). The chromatography condition of Perkin Elmer program from HPLC included pump of (LC-1000) containing polymeric c18 columns connected with LC250UV/VIS. The peak identification was made possible for HPLC by CSW33 software. In this instrument, β-Carotene measurement from dynamic acetonitrile-methanol-ethyl acetate (88:10:2) was done with a ratio of 1milimeter per min and a wavelength of 250 nm. The ODS colorless acetyl column containing 0.5_{mi} particle size was used in 4_{mm}I.D [15, 16, 17].

Relative solubility of β-Carotene in organic solvents

Approximately 10 mg of β-Carotene was added to 3 mL of each of the solvents such as acetone, acetonitrile, benzene, chloroform, cyclohexane, cyclohexanone, dichloromethane, dimethyl formamide, dimethyl sulfoxide, ethanol absolute, ethyl acetate, ethyl ether, hexane, 2-propanol, methanol, methyl tert-butyl ether, tetrahydrofuran (THF) + BHT and to1uene. Vials were ultrasonically agitated for 5 min. If a clear solution with no residual crystals resulted, additional carotenoid was added until crystalline material remained undissolved. Each solution was then filtered through a 0.2-pm membrane, and appropriate dilutions were made until the absorbance at the wavelength maximum was between 0.5 and 1.0 absorbance unit at ambient temperature. The background absorbance of each solution was subtracted using the appropriate solvent containing no carotenoid. Carotenoid concentration was calculated using Beer's law and the relative absorptivity determined below (Determination of Relative Absorptivity). Measurements were performed in triplicate and the calculation used is (absorbance, at X) (dilution factor)/molar absorptivity, where the subscript s is a given solvent. The measured values were rounded to one significant figure since this experiment was not designed to determine absolute solubility but rather to indicate solubility relative to other solvents [18] .

Stability

Stability was monitored for 10 days at room temperature by measuring absorbance changes at the wavelength maximum according to Table 3 [19, 20, 21].

Color determination

The reading of the β-Carotene obtained through a colorimeter (Hunter Lab) was carried out. The apparatus was calibrated with a standard white ceramic plaque, whereby: the luminosity (L*) represents how light and dark is the sample, varying between 0 (black) and 100 (white). Higher luminosity values indicate whiter colors. The chrome a* values vary from green (-) to red (+); and chrome b* values from blue (-) to yellow (+), in compliance with the International Commission of L'Éclairage [19, 20, 21].

Nutritional decomposition

The *Azolla* needed to prepare β-Carotene was analyzed in terms of nutritional value including protein by distillation method, fat by hydrolysis acid method, moisture by dry oven method, ash by gravimetric method. The prior and post *Azolla* evaluation for pigment also included evaluation in terms of the dried biomass weight [22].

Statistical analysis

The data obtained in spectrometric and chemical tests were analyzed through the use of statistical software (SPSS) and one way ANOVA (Tukey test if required) so as to compare the experimental samples with the control.

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Results and Discussion

Nutritional composition results of *Azolla filiculoides* from Anzali wetland are showed in the Table 1. The colorimetric results of β-Carotene extracted from *Azolla filiculoides* are presented in Table 2. The analysis results of solubility and vitamin composition, purity and β-Carotene amount of samples were exposed in Tables 3 and 4, respectively.

According to Table 1, the nutritional composition of the *Azolla filiculoides* collected in the summer were not significant difference compared with those collected in the winter ($P<0.05$).

Azolla is very rich in protein, fat and the other mineral components. Nutritional value was higher in samples collected in the summer compared with those collected in the winter. That can be due to the herbal growth.

Table 1: Nutritional composition results of *Azolla filiculoides* from Anzali wetland in the summer and winter seasons (%)(Values are mean + standard deviation)

Index	Protein	Moisture	Fat	Ash	Dry weight

Collected Azolla					per 100 gram
In the summer	23.49± 2.13*	32.84±3.15*	19.52±2.18*	24.89±2.98*	89.73±3.15*
In the winter	21.89±2.94*	31.95±2.16*	18.25±1.97*	23.16±2.4*	88.13±2.16*

The different signs in the same column within indicate significant differences ($p<.05$).

As shown in table 2, apart from keeping in dark glass containers, β-Carotene antioxidant property and the use of antioxidant in processing may be cited as some of the effective factors in β-Carotene stability during storage period [23]. Though suitable for non-enzymatic browning reaction, the average moisture in *Azolla* powder may help protect carotenoids from lipid oxidation. In case of using low drying temperature (i.e. 50^0C), most of the carotenoids turn out to be stable and β-Carotene isomerization does not occur during drying process. The no complete removal of cellular walls, cell membranes and destruction of plant tissues in the experimental samples leading to reduction of spectrometry percentage as revealed by Hunter spectrophotometric [24, 25].

As table 2 shows, there was not meaningful difference in calorimetry between test samples. Also, a significant difference was observed between test samples and control samples. According to achieved results, the one year retention time period in a 5^0C did not bring about significant impact on the color in both experimental and control groups ($p>0.05$).

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Table 2: Beta-Carotene colorimetric results extracted from *Azolla filiculoides* during storage at refrigeration (Values are mean + standard deviation)

Treatment	Summer			Winter			Control		
Color spectrum Sampling time (months)	Brightness	Redness-green	Blue jaundice	Brightness	Redness-green	Blue jaundice	Brightness	Redness-green	Blue jaundice
Zero	79.86±2.67*	1.78±1.11*	1.75±1.13*	86.35±3.36*	1.24±1.12*	1.17±0.97*	96.75±3.24*	1.05±0.78*	1.11±0.86*
Three s	79.73±3.14*	1.92±1.24*	1.89±1.18*	86.35±2.28*	1.36±0.96*	1.25±0.85*	96.63±3.14*	1.16±0.98*	1.15±0.67*

Six	79.56±4.12*	2.14±1.43**	2.15±1.22*	86.33±3.16*	1.52±0.99*	1.31±1.12*	96.44±2.98*	1.27±0.93*	1.29±1.13*
Nine months	79.51±3.89*	2.19±1.34*	2.21±1.35*	86.31±4.11*	1.69±1.27*	1.52±0.99*	96.41±2.64*	1.39±0.91*	1.32±0.94*
Twelve	79. 48±3.25*	2.22±1.17*	2.23±1.24*	86.29±3.92*	1.71±0.98*	1.56±1.21*	96.35±3.19*	1.45±1.23*	1.38±0.89*

The different signs in the same column within indicate significant differences ($p<0.05$).

Based on Table 3, the solubility of β-Carotene was greatest in tetrahydrofuran, while methanol and acetonitrile exhibited the least solubility. In the majority of the solvents, initial absorbance decreased by less than 10% during the 10 day period. Degradation was greatest in cyclohexanone. This finding is confirmed by Craft *et al.* study. The difference observed in the solubility of the test samples which might be accounted by not being remove of lipids and chlorophyll from extraction liquid. β-Carotene has most soluble in THF. B-Carotene was least soluble in methanol and acetonitrile. Many existing extraction techniques partition carotenoids into hexane or petroleum ether from aqueous alcoholor acetone. Given the poor solubility of dihydroxy and more polar carotenoids in hexane, this may lead to losses. Diethyl ether has also been used to partition carotenoids from aqueous/polar organic mixtures. Although THF is subject to peroxide formation, it has found increased use for carotenoid extractions due to the high solubility of a wide polarity range of Carotenoids [18, 20].

The differences in solubility values between different solvents can be attributed to differences in the physical condition and purity of the sample, and/or limitations of the experimental technique. Below its melting point (456 K), the solubility varies depending if β-Carotene is in a crystalline or amorphous state. Sample impurities or degradation products from β-Carotene may affect solubility by acting as co- or anti-solvents, thus affecting measured solubility [26].

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Table 3. Relative solubility and stability of β-Carotene in organic solvents

Sampling time	Summer				Winter		
Index Solvent	Wave length Max (nm)	Solubility (mg/l)	Absorptivity (E%, cm^{-1})	molar absorptivity (L mol^{-1} cm^{-1})	Solubility (mg/l)	Absorptivity (E%, cm^{-1})	molar absorptivity (L mol^{-1} cm^{-1})
Acetone	452	190	2516	137423	173	2417	136321
Acetonitrile	452	5	2505	136425	2	2401	135413
Benzene	462	3990	2001	124019	3889	1901	123011
Chloroform	462	1995	2292	125112	1895	2183	124002
Cyclohexane	454	1995	2106	134723	1893	2001	133613
Cyclohexanone	462	1995	2317	126723	1875	2207	125713
Dichloromethane	460	5900	2329	127213	5801	2219	126103
DMF	466	190	2348	128312	170	2237	127212
DMSO	466	20	2215	121315	11	2114	121206
Ethanol	450	20	2526	135811	11	2114	121206
ethyl acetate	452	490	2481	135310	390	2372	134200
ethyl ether	448	990	2617	142809	895	2507	143728
Hexane	448	580	2551	139234	475	2452	138215
2-propanol	450	30	2464	134721	19	2374	132621
Methanol	450	5	2500	136445	2	2401	135413
MTBE	450	990	2543	139067	882	2431	138054
THF	456	9970	2359	128856	9865	2247	128745
toluene	462	3990	2239	121942	3876	2126	121841

As table 4 shows, amount of β-Carotene and vitamin compounds showed meaningful differences between the test samples ($p \leq 0.05$). Purity was observed significant differences in test samples. Furthermore, there was a significant difference in terms of purity, vitamin compounds and β-Carotene solubility between experimental treatments and those of the control ($p \leq 0.05$). According to achieved results, the one year retention time period in a 5^0C did not bring about any significant impact on these factors in both experimental and control groups ($p > 0.05$).

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The formation of β-Carotene is influenced by maturation stages of plant leaves in a way that in the young leaves [27, 28]. Leaves of *Azolla* are young in Summer and is suitable for lycopene synthesis. Lycopene is precursor for β-Carotene synthesis [29, 30, 31]. Therefore, β-Carotene amount was higher in summer compared with winter.

In the experimental groups compared with control samples, lower purity percentage was observed which might be accounted by not being remove of lipids and chlorophyll from extraction liquid prior to identification of β-Carotene level [32, 33, 34].

Based on table 4, the vita, elimination of cellular walls and cell membranes, releasing higher rate of min compounds and spectrometry. β-Carotene density in these treatments ($p \leq 0.05$) are affected by the removal of cellular wall and the destruction of plant tissues. The no complete release of β-Carotene from plant tissues in the experimental samples caused the decrease in β-Carotene density. Considering the formation of two vitamin A molecules that occur as the result of β- Carotene decomposition and that 100% β-Carotene is capable of being converted into vitamin A [35], the decreased β-Carotene value and density in these treatments compare with control samples resulted in the reduction of vitamin compounds [36, 37].

The thermal procedure used for drying *Azolla* no diminished carotenoid because of applying lower temperature for drying. Nevertheless, it caused the destruction of foodstuff enzymes and facilitated solubility and the release of carotenoids and finally increased the accessibility to these compounds. The homogenization carried out for β-Carotene extraction has also led to greater accessibility to β-Carotene [38, 39]. The measures taken in this processing did not result in instability of foodstuff's micro nutrients and did not bring about any changes in the amount of β-Carotene and it's durability under 5^0C temperature (Table 4).

The results obtained in this study were in line with those obtained by Zarreh and Kianirad, that involved extraction of β-Carotene through fermenting the mold - *Blakeslea tripsora* [40], Razavai *et al*. using fermentation method for *poralomyces ruberrimus* H110 via physicochemical processes [41], Baigan *et al*. including the use of algae *Donaliasalina* by altering the culture medium [42] and Moghadasi *et al*. applying soapy method with micro algae *Donaliasalina* [43,44] as well as the findings of Lejeune *et al*. using solvent [44], Vennugopal *et al*. who also applied solvent and Mustafa *et al*. involving Tee method [45, 46]. The results of present study are consistent with results of these researchers.

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The β-Carotene amount of *Azolla filiquidis* showed to be significantly different in comparison to other *Azolla* species reared in other parts of the globe. This may be determined by the genotype, climatic variations, growth and developmental stages of *Azolla* [47].

Table 4: Results of Vitamin composition, purity and β-Carotene amount of β-Carotene produced by alkaline hydrolysis and organic solvents of *Azolla filiculoides* from Anzali wetland during storage at refrigeration (Values are mean + standard deviation)

The different signs in the same column within indicate significant differences ($p<0.05$).

Conclusion

Index	**Vitamin composition**(IU)			**Purity(%)**			**B-Carotene amount** (mg/kg)		
Treatment Sampling time (months)	Summer	Winter	Control	Summer	Winter	Control	Summer	Winter	Control
Zero	9837±2.13***	10893±3.14**	12346±3.49*	89.3±4.17**	79.7±3.18***	99±3.23*	6648±3.48***	7539±3.54**	11863±4.12*
Three	9837±2.45***	10893±3.12**	12346±3.15*	89.3±4.13**	79.7±3.13***	99±2.34*	6648±2.78***	7539±2.97**	11863±4.31*
Six	9837±3.76***	10893±3.16**	12346±2.78*	89.3±4.71**	79.7±3.89***	99±1.89*	6648±2.96***	7539±1.98**	11863±3.26*
Nine	9837±4.12***	10893±3.65**	12346±4.17a*	89.3±3.48**	79.7±3.67***	99±1.99*	6648±2.88***	7539±1.99**	11863±3.91*
Twelve	9837±3.98***	10893±3.49**	12346±3.99*	89.3±3.87**	79.7±2.54***	99±2.95*	6648±2.86***	7539±3.99**	11863±3.65*

Considering the significant difference in the results of chemical factors and the lack of significant difference in durability and shelf life of β-Carotene extracted by organic solutions as compared with that of the control, and significant difference in the amount of β-Carotene extracted between experimental samples, it is safe to point out that it is possible to substitute β-Carotene extracted by organic solutions of *Azolla* from Anzali wetland in the summer, with synthetic β-Carotene in food industry.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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Comment [JSR24]: recheck all the reference and in a smetric manner and also add title, font, page no., etc of the refrence in some references it lacking.

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