STUDIES ON THE COLOR RETENTION AND HALF-LIFE OF THE ENZYME EXTRACTED CAROTENOPROTEINS FROM PLANT TISSUES

İnci ÇINAR , Özer ÇINAR

Abstract **- Carotenoproteins, enzymatically extracted from the orange peel, sweet potato and carrot, were stored at 25°C light, 25°C dark, 4°C and 40°C for the color retention and the half-life studies. Rate constants were also calculated from the percentage retention of color vs. time plots by the best mathematical fits. The percentage color retentions of the samples were very good resulting in their promising use in foods. Storage at 4°C resulted in the higher retentions of carotenoid color for all three samples as expected. The highest percentage retention of carotenoid color was in sweet potato followed by orange peel and carrot stored at 4°C with the rate constants of 0.4164, 0,0100 and 0,0166 respectively. The half-lifes of the samples were 97 days in sweet potato, 78 days in orange peel and 50 days in carrot samples stored at 4°C.**

Keywords **- Carotenoids, half life, extraction, cellulase, pectinase.**

Özet **– Portakal kabuğu, tatlı patates ve havuçtan enzimle ekstrakte edilen karotenoidler, 25°C ışık, 25°C karanlık, 4°C and 40°Cde depolanarak yarılanma süreleri ve renk kalıcılığı araştırılmıştır. Reaksiyon sabiteleri % kalan pigmentin zamana karsı çizilen grafiğinden matematiksel olarak hesaplanmıştır. Örneklerdeki yüksek renk kalıcılığı, gıdalarda kullanılması açısından ümit vericidir. Umulduğu üzere % renk kalıcılığı tüm örnekler için 4°C de daha yüksektir. En yüksek renk kalıcılığı 4°Cde depolanan tatlı patateste olup bunu portakal kabuğu ve havuç izlemektedir (reaksiyon sabiteleri sırasıyla 0,4164, 0,0100 ve 0,0166). 4°Cde saklanan örneklerin yarılanma süreleri ise tatlı patateste 97 gün, portakal kabuğunda 78 gün ve havuçta 50 gündür.**

Anahtar Kelimeler **- Karotenoid, yarılanma süresi, ekstraksiyon, selülaz, pektinaz.**

İ. Çınar; Kahramanmaraş Sütçü İmam University, Department of Food Science and Technology, Kahramanmaraş, TURKEY, 46060
E-mail: idogan@uga.edu
Ö. Çınar: Kahramanmaraş Sütçü İmam University, Department of Environmental Engineering, Kahramanmaraş, TURKEY, 46060
E-mail: ocinar@ksu.edu.tr

I. INTRODUCTION

The carotenoids are group of mainly lipid soluble compounds responsible for many of the yellow and red colors of plant and animal products. It has been estimated that the annual production of carotenoids is over a million ton in nature.

The outstanding stability of the color of natural water-dispersible carotenoid-protein complexes is probably due to their ultrastructure. However, remarkably little is known of the nature of the carotenoid-protein linkages. Fruit carotenoids are attached to the proteins, as is obvious from the general properties of the fruit juices. Only few roots contain significant amounts of carotenoproteins, as in carrots and sweet potatoes.

The traditional solvent extraction procedures cause the destruction of natural protein bound structure of carotenoids protecting them from oxidation, isomerization and degradation. In addition, these techniques require harsh grinding and drying. More recent studies investigate the association of carotenoids with the proteins. The enzyme extraction of carotenoid pigments is a totally new approach. In the literature, there are very few researches using enzymes to extract carotenoids. Bryant et al. [1] isolated carotenoproteins from carrot root chromoplasts by lysis and gel filtration and proved the existence of specific carotene-binding protein complex in carrots. Aravantinos-Zafiris et al. [2] reported the usage of enzyme preparation for the orange peel maceration and the pigments were produced by hexane extraction. The enzymatic hydrolysis of alkali-unstable carotenoid esters at neutral pH by cholesterol esterase or lipase was mentioned by Britton [3]. Delgado Vargas and Paredes Lopez [3,4] reported the use of commercial enzyme for the extraction of

carotenoproteins from marigold flowers and quantification was done with the hexane. Chakrabarti [5] used trypsin, pepsin and papain to extract carotenoprotein from brown shrimp waste.

In enzyme extraction, a combination of cellulolytic and pectinolytic enzymes is required to achieve almost complete liquefaction. The sample tissues thus are not only macerated but the cell walls are also largely digested. The use of pectinase and cellulase enzymes distrupts the cell wall of plant sample and releases the carotenoids (carotenoproteins) in the chloroplasts and in cell fluids. These pigments remain in their natural state still bound with proteins. Carotenoids bind with proteins through covalent bonding or weak interactions depending on their structure. This bonded structure prevents pigment oxidation and also affects color [6]. On the other hand, solvent extraction dissociates the pigments from the proteins and causes water insolubility and ease of oxidation [7].

Commercial interest in methods for production of natural carotenoid pigments is increasing due to the consumer demand on more natural food products. So far, an addition of carotenoid including fruit juice concentrates to foods is troublesome in the carriage of the undesired flavor and odor characteristics of the juice plant source to the food products. Enzyme extracted carotenoids on the other hand, are in their natural form, therefore there is no such problem in the use as food colorants. Also, they can be used in the foods as ingredient simply as colorant without needing to be certified. Therefore, it would be useful to study these natural compounds so that industry can develop a stable homogenous pigment and products. The results will be beneficial for both industry and consumer.

Although enzyme extraction provides more stable carotenoid pigments, the retention of carotenoids during storage is a very important objective to make the final product attractive and acceptable. Degradation of carotenoids not only affects the attractive color of foods but also their nutritive value and flavor.

The objectives of this study were to provide an effective extraction method for carotenoids and to study the color retention of extracted pigments under different storage conditions.

II. MATERIALS AND METHODS

II.1 Sample Preparation

Three different materials, navel orange peels, sweet potatoes and carrots, were used in this research, since they are very good sources of carotenoid pigments. All materials were purchased from a local grocery store in Clemson, South Carolina, USA. Commercial pectinase and cellulase from *Aspergillus niger* were purchased from Sigma Chemical Co., St. Louis, MO. Hexane obtained from Baxter Health Care Corporation, Musketon, MI was of analytical reagent grade and was used without further purification. Celite ® Filter Cell was purchased from Fluka Chemical Corp., Ronkonkoma, NY.

After washing under the tap water, carrots were skinned and oranges and sweet potatoes were peeled. Orange peels were abraded by using a small wire screen to remove the white colored albedo layer. All materials were diced into approximately 0,5 cm^3, thoroughly mixed, weighed and used immediately for further processing. The samples were homogenized in a laboratory Waring blender for 2 minutes with distilled water to increase the surface area for efficient enzyme treatment.

II.2 Pigment Extraction

Homogenized materials were placed in a large beaker. Commercial pectinase and cellulase enzymes were added in the ratio of 2,5 ml of pectinase and 2 g of cellulase for the 100 g of food sample in 200 ml of distilled water. Samples were stirred on the Corning PC 351 brand magnetic stirrer at medium speed for 24 hours at room temperature. Following the enzyme treatment, a celite bed was prepared on a porcelain buchner funnel on #1 Whatman filter paper. Approximately 2g of celite was added to the enzyme treated pigment mixture and let stand for 10 minutes. The mixture was vacuum filtered through the celite bed. The first filtrate was the water soluble pigment extract. The vacuum flask was changed and the celite bed containing carotenoid pigments was washed with 95 % ethanol. The washing procedure was repeated until the celite bed turned its original gray color. Distilled water was added to the ethanol extract until pigment precipitation occurred. The ethanol then was evaporated by using a Buchi EL 130 brand rotary evaporator at 50°C. The remaining residue was carotenoid pigments (lipid soluble extract) and water.

II.3 Color Retention Studies

Pigment extracts were analyzed for color retention by using a Perkin-Elmer brand UV-visible spectrophotometer. Since quantification of carotenoid pigments from the samples is out of the focus of this study, only the spectrophotometric measurements of the pigment color intensity were used for the comparisons among the different storage conditions. For the color retention studies, the effects of light, dark and temperature were investigated because of water insolubility and instability of pigments in the presence of

light, oxygen and heat were very well reported in the literature [8].
For each condition, pigment extracts were stored in small bottles. Dark conditions were provided by wrapping a glass bottle with several layers of aluminum foil while samples were exposed to fluorescent room light during the day for the light effect. For the temperature effect, 25°C (light and dark), 4°C and 40°C were used. For the absorbance measurements, 1 ml of aqueous extracts was taken and placed into long test tubes. Forty-five ml of hexane was added to dissolve the pigment. Tubes were shaken and let stand for two phase separation. The lipid soluble extract was taken into the hexane phase Absorbance was measured with UV-visible spectrophotometer at 450 nm due to the fact that the carotenoid pigments have sharp bands in the visible range of 400 to 600 nm. All samples were duplicated. The absorbance values of samples were converted to percentage retention for accurate comparison among the storage conditions.

Half-life and rate constant of pigments were further used to express the color retention of pigments. Half-life was defined as the day at which the pigment retention was 50 %. For the rate constant calculations, the percentage of retention of color was related with time in days by the different mathematical fits. In each treatment, the plot which gave the best correlation was selected and the slope of the regression line was the rate constant of the pigments [9].

II.4 Statistical Analysis

All data from sample stability studies were further subjected to Analysis of Variance (ANOVA) using a Randomized Block Design procedure. Experimental variables were treatments (25°C light and dark, 4°C and 40°C) and percentage retention of pigments. Mean differences among storage conditions and percentage retention of pigments were tested for significance using LSD (Least Significant Difference).

III. RESULTS AND DISCUSSION

The retention of orange peel pigments was 21,50 % for the samples stored at 4°C after over 120 days, while there was no pigment retention for 25°C light, 25°C dark and 40°C samples after 84 days, 89 days and 76 days respectively as shown in Figure 1. Statistical data indicated that 4°C and 25°C dark samples of orange pigments were significantly different than others ($p< 0.05$), while there was no significant difference between 25°C light and 40°C samples.

For the carrot samples, the retention of pigment after 120 days storage period was 12,27 % for the 4°C samples. All color was lost after 26 days for 25°C light and 25°C dark samples, after 21 days for 40°C samples and differences among these three treatments were not significant (Figure 2), but storing the carrot samples at 4°C significantly improved pigment retention ($p< 0.05$).

Similarly, sweet potato pigment retention was 42.76 % for the samples stored at 4°C after 120 days. Zero retention was observed after 85 days for 25°C light and dark samples and after 42 days for 40°C samples as shown in Figure 3. There was no significant difference between 25°C light and dark samples, while the difference was significant for 4°C and 40°C samples.

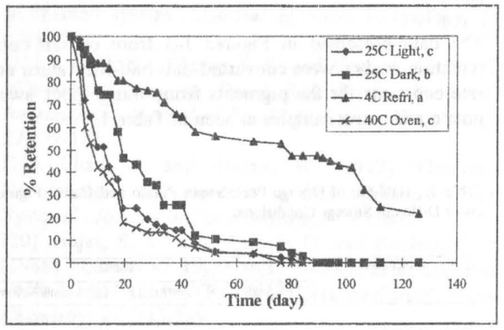

Figure 1. % Retention of Orange Peel Pigments at Different Storage Temperatures. (The same letters in the legend are not significantly different).

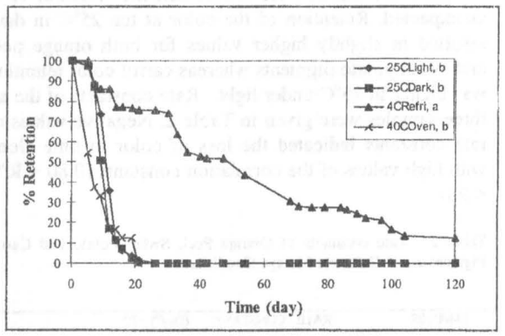

Figure 2. % Retention of Carrot Pigments at Different Storage Temperatures (The same letters in the legend are not significantly different).

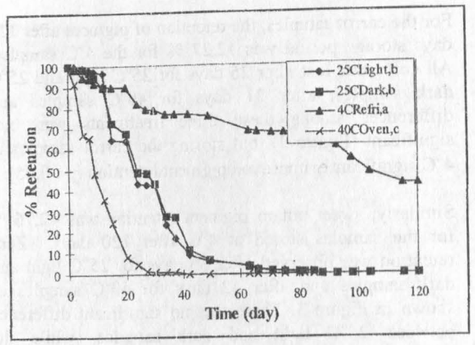

Figure 3. % Retention of Sweet Potato Pigments at Different Storage Temperatures (The same letters in the legend are not significantly different).

The data presented in Figures 1-3 from overall color retention studies were converted into half-life values and rate constants for the pigments from orange peel, sweet potato and carrot samples as seen in Table 1.

Table 1. Half-life of Orange Peel, Sweet Potato and Carrot Pigment under Different Storage Conditions.

SAMPLES	HALF LIFE (days)			
	25°C Light	25°C Dark	4°C Refrig	40°C Oven
ORANGE PEEL	11	18	78	8
SWEET POTATO	22	23	97	8
CARROT	11	9	50	6

All three samples had the highest half-life values when stored at 4°C and had the lowest half-life stored at 40°C as expected. Retention of the color at the 25°C in dark resulted in slightly higher values for both orange peel and sweet potato pigments whereas carrot color retention was higher in 25°C under light. Rate constants of the all three samples were given in Table 2. Negative values of rate constants indicated the loss of color in time along with high values of the correlation constants ($0.80 < R^2 < 98$).

Table 2. Rate constants of Orange Peel, Sweet Potato and Carrot Pigment under Different Storage Conditions.

SAMPLES	RATE CONSTANT (Day^{-1}) : R^2			
	25°C Light	25°C Dark	4°C Refrig	40°C Oven
ORANGE PEEL	-0.0533 : 0.98	-0.0384 : 0.97	-0.0100 : 0.93	-0.0636 : 0.94
SWEET POTATO	-0.0521 : 0.95	-0.0542 : 0.93	-0.4164 : 0.95	-0.1344 : 0.94
CARROT	-0.1564 : 0.87	-0.1787 : 0.80	-0.0166 : 0.97	-0.1252 : 0.95

In overall treatments, it was concluded that sweet potato pigments were the most stable followed by orange peel pigments. The highest retention was reached at 4°C for all conditions, the lowest being 40°C. For carrot and sweet potato samples, light and dark conditions were not significantly different, indicating potential for use in transparent packaged foods. This can be explained by the relatively higher stability of β-carotene to light since these samples contain mainly β-carotene.

Most investigators have reported the stability of solvent extracted pigments in hours or some in 1 to 2 days. Craft [10] spectrophometrically monitored 32 % to 97 % retention of β-carotene depending on the extraction solvent type over a 10 day period. By comparison, enzyme extracted pigments had much higher stability especially compared to pigments extracted in cyclohexanone. These findings were consistent with these reported by [11, 12] as a general trend. Tsimidou and Tsatsaroni [13] stated that the half-life of the saffron pigments in aqueous solutions was 59 hours in the dark at 40°C and 32 days at 4°C. It is apparent that enzyme extracted pigments are much more stable. According to Pesek and Warthesen [14], the carotenoids in a carrot juice system retained only 25 % of the original color after 4 days of light exposure at 4°C. Enzyme extracted carrot pigments on the other hand, retained 94.08 % of the color under the same conditions.

It has been proven that water content has a protective role in stability by directly affecting free radicals produced during pigment oxidation. Free radical content can be reduced significantly by interaction with water [15]. These results were in agreement with those reported by several researchers [16, 17, 18, 19] who concluded that in model systems, water has a protective influence on the autoxidation of carotenoids. Since enzyme extracted pigments were kept in an aqueous system, they were more stable to all storage and processing conditions. Degradation, isomerization and oxidation reactions which result in lower color intensity and stability occured at higher temperatures. Light and dark conditions had no significant effect except for the orange peel pigments, although Najar et al. [20] stated that light has a strong destructive effect on pigments.

IV. CONCLUSION

Enzyme extraction of the orange peel, sweet potato and carrot samples resulted in the high percentage retention of the carotenoids under all storage conditions when compared with the solvent extraction from the literature. Storage at 4°C provided the most retention of the pigment color. Also, samples stored at 25°C light and 25°C dark did not have statistical difference, and that simply has important potential use in transparent packages when needed. Enzyme extracted pigments had long half-life in all storage conditions especially for the samples stored at 4°C. These pigments can be stored for long periods of time prior to use in the food products. Since extracted pigments were isolated from the fruit juices, comparing the fruit or vegetable juice concentrates, these pigments will have lower volumes in the storage room giving the same level of coloring intensity.

REFERENCES

[1]. Bryant, J. D., McCord, J. D., Unlu, L. K., and Erdman, J. W. (1992) "Isolation and Partial Characterization of Alpha-Carotene-Containing and Beta-Carotene-Containing Carotenoprotein from Carrot (Daucus-Carota L.) Root Chromoplasts", *Journal of Agricultural and Food Chemistry, 40, 545-549.*

[2]. Aravantinos-Zafiris, G., Oreopoulou, V., Tzia, C. and Thomopoulos, C. D. (1992) "Utilization of Orange By-products - Orange Peel Carotenoids", *J. Sci. Food Agric., 59*, 77-79.

[3]. Britton, G., Liaaen-Jensen, S. and Pfander, H. (1995). Carotenoids. Basel, Boston, Birkhauser Verlag.

[3]. Delgado Vargas, F. and Paredes Lopez, O. (1997a) "Enzymatic treatment to enhance carotenoid content in dehydrated marigold flower meal", *Plant Foods for Human Nutrition, 50,* 163-169.

[4]. DelgadoVargas, F. and Paredes Lopez, O. (1997b) "Effects of enzymatic treatments on carotenoid extraction from marigold flowers (Tagetes erecta)", *Food Chemistry, 58,* 255-258.

[5]. Chakrabarti, R. (2002) "Carotenoprotein from tropical brown shrimp shell waste by enzymatic process", *Food Biotechnology, 16,* 81-90.

[6]. Fennema, O. W. (1985). Food Chemistry. Marcel Dekker Inc. New York.

[7]. Bassi, R., Pineau, B., Dainese, P. and Marquardt, J. (1993) "Carotenoid-binding proteins of Photosystem II", *European Journal of Biochemistry, 212*, 297-303.

[8]. Giddings, J. C., Grushka, E., Cazes, J. and Brown, P. R. (1983). Advances in Chromotography". Edited by J. C. Giddings. Volume: 22, Marcel Dekker Inc. New York.

[9]. Labuza, T. P. (1984) "Application of Chemical Kinetics to Deterioration of Foods", *J. Chem. Ed., 61*, 348-358.

[10].Craft, N. E. (1992) "Relative Solubility, Stability and Absorptivity of Luteine and Beta-carotene in Organic Solvents", *Journal of Agricultural and Food Chemistry, 40*, 431-434.

[11]. Pesek, C. A. and Warthesen, J. J. (1988) "Characterization of the photodegradation of β-carotene in Aqueous Model Systems" *Journal of Food Science, 53,* 1517-1520.

[12]. Kopas, L. M. and Warthesen, J. J. (1995) "Carotenoid Photostability in Raw Spinach and Carrots During Cold Storage", *Journal of Food Science, 60,* 773-776.

[13]. Tsimidou, M. and Tsatsaroni, E. (1993) "Stability of Saffron Pigments in Aqueous Extracts", *Journal of Food Science, 58,* 1073-1075.

[14]. Pesek, C. A. and Warthesen, J. J. (1987) "Photodegradation of Carotenoids in a Vegetable Juice System", *Journal of Food Science, 53,* 744-746.

[15]. Labuza, T. P., Tannenbaum, S. R. and Karel, M. (1970) "Water content and Stability of Low and Intermediate Moisture Foods", *Food Technology, 24,* 543.

[16]. Ramakrishnan, T. V. and Francis, F. J. (1979) "Stability of Carotenoids in Model Aqueous Systems", *Journal of Food Quality, 2*, 177-189.

[17]. Arya, S. S. ; Natesan, V. ; Parihar, D. B. and Vijayaraghavan, P. K. (1979) "Stability of Carotenoids in Isolated system", *Journal of Food Technology, 14,* 571-578.

[18]. Lee, D. S. ; Chung, S. K. and Yam, K. L. (1992) "Carotenoid Loss in Dried Red Pepper Products", *International Journal of Food Science and Technology, 27,*179-185.

[19]. Chau, H. and Breene, W. (1972) "Oxidative Decoloration of β-carotene in Low Moisture Model Systems", *Journal of Food Science, 37,* 66-68.

[20]. Najar, S. V. ; Bobbio, F. O. and Bobbio, P. A. (1988) "Effects of Light, Air, Anti-oxidants and Pro-oxidants on Annatto Extracts (Bixa orellana)", *Food Chemistry, 29,* 283-289.