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Research Article

ANALYSIS OF SPINACH (*SPINACIA OLERACEA*) PIGMENTS USING CHROMATOGRAPHY AND UV-VISIBLE SPECTROSCOPY

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Abstract The aim of the study is to access the determination of qualitative and quantitative changes in (raw, boiled and freeze) spinach pigments. Pigments are large group of natural compounds in plant kingdom. Chlorophyll and carotenoids are two significant pigments in spinach. These compounds can be identified by Column chromatography, Thin layer chromatography (TLC) and UV-visible spectroscopy. Antioxidant activity was detected by using DPPH (1,1-diphenyl-2-picrylhydrazyl) and Dot blot assay. It was examined that boiled spinach is highly beneficial as it contains pigments like (chlorophyll-a, chlorophyll-b, carotene, xanthophyll, lycopene), large antioxidants as compared to raw and freeze extract. Spinach has natural antioxidants to suppress the oxidative stress and to cure many health problems such as cancer and heart disease.

Keywords: Spinach, antioxidant, pigments, chromatography, UV-visible spectroscopy.

Introduction

Spinach *(Spinacia oleracea)* belongs to Chenopodiaceae (Goosefoot) family which also includes Swiss chard and Beets. Spinach is a low growing fleshly leaved annual that forms a heavy rosette of either smooth or wrinkled leaves. Spinach prefers cool climate. The minimum temperature for seed germination is 2^0C with a maximum germination temperature of 3^0C and an optimum range of 7 to 20^0C Young plants can withstand at -90°C. Spinach bolts rapidly when days are both long and hot (Zvalo *et al*., 2008). Color is one of the main attributes that contribute to the sensory quality of vegetables. The green color of spinach is an indication of the "freshness" of the product. The vegetables are colored due to the presence of various pigments primarily green chlorophylls, yellow, orange, and red carotenoids. In green leafy vegetables such as spinach, only the green chlorophylls are seen because they mask the bright colors of the carotenoids. An antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons or hydrogen from a substance to an oxidizing agent. When antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions. They do this by being oxidized themselves, antioxidants are often reducing agent such as thiols, ascorbic acid (Helmut *et al.,* 1997). Antioxidants are widely used in dietary supplements and have been investigated for the prevention of diseases such as cancer and even altitude sickness (Baillie *et al*., 2009). In raw spinach, β-carotene, lutein, violaxanthin, and neoxanthin are the four most abundant carotenoids (Salomon *et al*., 1991). Apart from acting as pigments with a photo protective function, the carotenoids also have an impact on the nutritional quality and the health-benefit properties of spinach. Some of the carotenoids possess provitamin- A activity as they are converted to vitamin A in the human body. In leafy vegetables, including spinach, the vitamin A effect is vital because of β-carotene (Gross *et al*., 1991). In addition, there is considerable evidence that some of the carotenoids have protective properties against cancers, cardiovascular diseases and eye problems such as age-related macular degeneration (Poppel *et al*., 1996). Spinach is richer in fat-soluble vitamins. Considerable work has been done since then to differentiate between the growth producing and anthracitic factors in this fat, thus focusing attention on the saponifiable extract of spinach (Osborne *et al.*, 1999).

Material and Methods

2.1 Sample preparation

The green spinach was purchased from local market located in Sirhind. To check the effect of processing on spinach, two treatments *i.e.* boiling and freezing was given. Boiled sample was prepared by taking 100 mg of spinach and was boiled in boiling water. Feezed sample was prepared by freezing in refrigerator at 0℃ for 2 hours simultaneously and control was maintained without any treatment, named as raw sample. Take 1.0 g of treated spinach leaves and make a fine paste by adding 1-2 ml of acetone. If the acetone evaporates, then add an additional 1 ml more acetone and transfer the paste using a Pasteur pipette along with acetone to a centrifuge tube. Add 3 ml of hexane and 3 ml of water to the above liquid and cap the centrifuge tube. Mixed the contents and centrifuged again. Removed the organic layer using a Pasteur pipette and place it in a clean dry test tube. Repeat steps with the remaining aqueous layer. concentrate the organic layer by using a rot evaporator. Seal the flask of extract and for further analysis.

2.2 Phytochemical analysis:

2.2.1 Column chromatography (Quach *et al.,* 1999)

In column chromatography, 1gm of anhydrous sodium sulfate and activated silica was added in column and filled it carefully. For pigment extraction, transfer 0.5 ml of spinach extract in column. The extract drained off; pigments begin to separate into a yellow carotene band. Added 3.0 ml of hexane (non polar) to the top of the column. Continuously added hexane until the yellow band was completely eluted. Similarly added 4.0 ml of acetone (polar solvent) for the separation of polar pigments. Green band is drained off through polar solvent. Cover and save these fractions.

2.2.2 Thin layer chromatography (TLC) (Robert *et al.,* 2004)

The TLC plates were prepared by using silica gel adsorbent. Thick slurry of silica gel was poured on glass plates. The resultant plates were then dried and activated by heating in an oven for 1 hour at 110°C. The thickness was measured around 0.1-0.25 mm. Different samples of extraction solution was applied as a small dot in a plate with the help of capillary tube about 1cm from the base. In the TLC chamber hexane and acetone was mixed in the ratio of 7:3 (v/v) as solvent. The plates were dipped and developed in saturated chamber to the distance of 75 mm. After 15 minutes of air-drying, spots were visible they indicated the separation of pigments on the basis of retardation factor.

2.2.3 Determination of antioxidant activity in different spinach samples:

2.2.3.1 Dot blot assay (Solar *et al*., 2000)

The prepared TLC plates were used and different samples were applied plate with the help of capillary tube about 1cm from the base. The TLC plates were air drying about 15 minutes in saturated chamber after that the plates were sprayed by 0.04% DPPH solution for 5 seconds. The bands were observed under visible light after 2 minutes of spraying. The appearance of light yellow against purple background indicated the radical scavenging activity.

2.2.3.2 DPPH assay (Srinivasan *et al.,* 2007)

Different aliquots of 25 mg/ml, 50 mg/ml and 100 mg/ml were pipette out of (BHA) butylated hydroxide anisole and samples. The final volume was made to 1ml with methanol.50 µl of above each concentration was pipette out into second set of test tubes. 5 ml of DPPH was added to each test tube and then reaction mixture was incubated at room temperature for 30 minutes. After 30 minutes, discoloration was observed. The absorbance was measured at 517 nm against methanol. The percentage of discoloration was calculated for each concentration by using formula.

Calculations:

% DPPH Reduction=

[(A Blank- A Sample/ A Blank) x 100]

A = Absorbance at 517nm

Blank = 0.004 % DPPH reagent

The percentage of the standard and sample was compared by plotting graph of antioxidant concentration against % free radical scavenging activity.

2.3 UV visible spectroscopy:

A double beam systronics UV-visible spectrophotometer, model UV-2201 (India) with a spectral bandwidth of 1 nm, wavelength accuracy of 0.5 nm and a pair of 1cm quartz cells were used to measure the absorbance of resulting solutions.

Preparation of solvent for study analysis

Acetone (v/v) solvent used for analysis of spectra by UV-vis spectrophotometer. Instrument should have a cuvet containing acetone in the reference beam and acetone extract solution in the sample beam. The complete spectrum of extract in the range, 400-800 nm. This can be compared to the spectrum of each of

individual sample (raw, freeze and boiled).

Results and Discussion

3.1 Column chromatography:

In column by adding the hexane (non polar) solvent. This observed that yellow band separation from green extract. This band is moves relatively quickly and acetone (polar) solvent is used. The band separation by column chromatography as described in table 1.

Table 1 Represents band separation by Column chromatography.

Bands	Raw Spinach	Boiled Spinach	Freeze Spinach
Dark Yellow	+	+	+
Light Yellow	+	+	-
Dark Green	+	-	+
Light Green	+	+	-

+ positive, - negative

In raw spinach sample four bands (dark yellow, light yellow, dark green, light green) was separate out, in boiled spinach three bands (dark yellow, light yellow, light green) were separate out but the dark green band was missing because chlorophyll-a was degraded during boiling process. In freeze spinach two bands were separate out (dark yellow, dark green) light green and light green was missing because pheophytins degraded during freezing process due to pheophytinization.

Figure 1 Separation of pigments in different spinach extracts

William *et al*., (2008) isolated ß-carotene, chlorophyll-a, and chlorophyll-b from spinach using column chromatography. Spinach was dehydrated using ethanol and pigments were extracted with hexane and acetone. Then, the pigments were run through a column using a non-polar solvent, hexane. The polar absorbent material in the column separated the different pigments by allowing the least polar molecules to travel through the column faster than the more polar molecules. The different pigment layers were collected. ß-carotene was the least polar molecule, and it traveled through the column faster than the chlorophylls. Chlorophyll-a was next to travel through the column followed by chlorophyll-b because chlorophyll-a was more polar than ß-carotene and less polar than chlorophyll-b, this observation is reasonable.

3.2 Thin layer chromatography (TLC):

Table 1 Represents R_f value of different spinach samples by thin layer chromatography.

	R_f Value of three sample		
Band	Raw	Boiled	Freeze
Dark Green	0.6	0.67	0.63
Light Green	0.7	-	-
Dark Yellow	0.87	0.81	0.88
Light Yellow	0.80	0.83	-

Raw spinach

Dark green Light green Dark yellow Light yellow

Figure 2 Separation of pigments in raw spinach.

As shown in figure 2 raw samples contains all the pigments and separated as dark green, light green, dark yellow and light-yellow band by thin layer chromatography.

Boiled spinach:

Dark yellow Dark green Light yellow

Figure 3 Separation of pigments in boiled spinach.

As shown in figure 3 in boiled sample light green band is missing because chlorophyll-a is degraded during boiling process but carotenoids pigments are more stable than chlorophyll pigments during blanching. (Kidmose *et al*., 2002) determined the content of individual chlorophyll and carotenoid pigments in three spinach varieties (Lorelei, Springfield, and Ballet) after processing. Increasing blanching time resulted in decreased contents of chlorophyll a and b, and carotenoid pigments are more stable than the chlorophyll pigments during blanching. Violaxanthin is also significantly reduced during blanching.

Freeze spinach:

Dark yellow Dark green

Figure 4 Separation of pigments in freeze spinach.

As shown in figure 4 in freeze sample light yellow and light green band is missing because pheophytins degraded during freezing process due to pheophytinization. (Lajolo *et al*., 2003) determine the effect of spinach pigments during freezing process. During freezing process chlorophyll, a-b degraded and also olive-brown colored pheophytins less formed due to pheophytinization.

3.3 Determination of antioxidant activity on different spinach samples:

3.3.1 Dot blot assay.

As shown in figure 5, yellow band formed against the purple background helps us to determine the antioxidant activity. Higher the yellow band more is the antioxidant activity.The order was Boiled spinach > Raw spinach > Freeze spinach.

Raw Spinach Boiled spinach Freeze spinach

Figure 5 Dot blot assay of spinach samples.

3.3.2 DPPH assay.

Raw spinach and boiled spinach have more antioxidant activity as compared to standard whereas Freeze spinach and standard showed almost similar activity. All the samples shows increase in antioxidant activity with increase in concentration of sample as shown in figure 2. Results of Dot blot assay and DPPH assay for spinach extracts shows dose dependent results followed the same order of antioxidant activity.

Table 2: % DPPH reduction in spinach samples with standard

Conc.of spinach extracts (mg/ml)	BHA	Raw Spinach	Boiled Spinach	Freeze Spinach
25	1.42	2.7	12.1	1.3
50	3.6	3.9	10.1	2.7
100	7.7	11.9	20.5	6.1

The DPPH % reduction of spinach extracts was determined by DPPH assay and BHA used as standard as shown in figure 6. The order was Boiled spinach > Raw spinach > Freeze spinach.

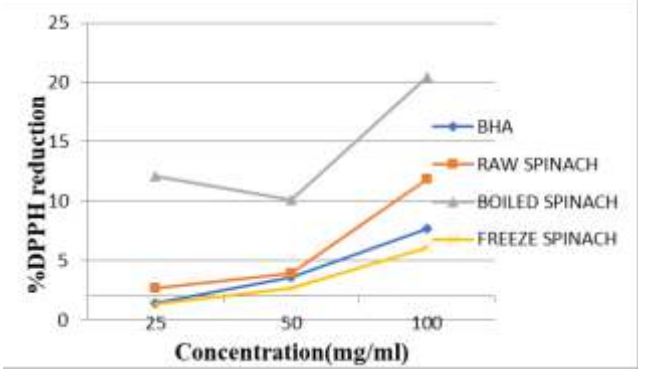

Figure 6: % DPPH reduction in spinach samples

The scavenging activity of DPPH radical and H_2O_2 were increased depending on the concentration (Shon *et al*., 2004). the cooking process increased antioxidant activity by 16% (Sultana *et al.,* 2008). The author reported contradicting results found by most researchers as there was a significant ($p<0.05$) increase in reducing power as a result of frying. However, boiling and microwave cooking did not affect reducing power. Inhibition of peroxidation was also increased by boiling and frying, whereas, in contrast it was decreased by microwave cooking.

3.4 UV visible spectroscopy:

The UV Visible spectra of three different spinach samples were shown in figure 7

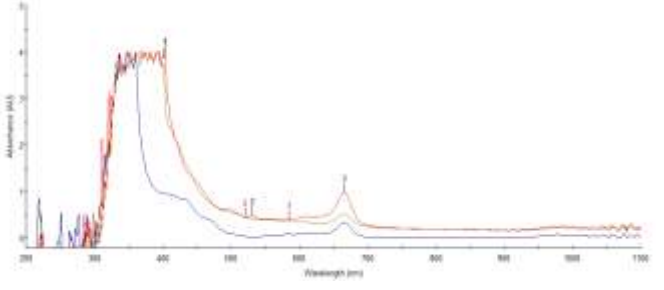

Figure 7: Shows the UV visible spectra of spinach samples.

The UV visible spectra of raw, boiled and freeze spinach samples described the presence of different phytochemicals which are responsible for their specific functions. The identification of compounds was determined.

1 Raw spinach

2 Freeze spinach

3 Boiled spinach

From the examined values of absorbance in raw, boiled and freeze spinach samples shows the presence of different pigments as shown in table 3.

Table 3: Shows the spectral data of three sample of spinach.

Absorbance	Constituents	Raw spinach	Freeze spinach	Boiled spinach
401-403 nm	Chlorophyll d	+	-	-
430 nm	Chlorophyll a	-	-	+
450 nm	Chlorophyll b	-	-	+
480 nm	Zeaxanthin	-	-	-
400-500 nm	Carotene	-	+	+
425-475 nm	Xanthophyll	-	+	+
435 nm	Lutein and vioxanthin	+	-	-
510 nm	Lycopene	-	+	+
600-700 nm	Chlorophyll a and b	+	+	+

+ positive, - negative

Boiled spinach sample contains more plant pigments than freeze and raw spinach. Thus, boiled spinach plays vital role in the daily operation of the body.

Conclusion

In the present study showed metabolic changes in boiled and freeze spinach extracts and compared to raw spinach extracts. Column chromatography used to separate the spinach pigments. These pigments are examined by thin layer chromatography (TLC) on the basis of R_f value. The antioxidant activity determined by the DPPH (2,2 diphenyl-1-picrylhydrazyl) and Dot blot assay. It was found that boiled spinach is highly beneficial as compared to freeze and raw spinach due to presence of pigments like (chlorophyll a, chlorophyll b, carotene, xanthophyll, lycopene), large antioxidants as compared to raw and freeze extract. Spinach contains natural antioxidants to suppress oxidative stress and cure many diseases. Spinach is a good source of minerals (iron, copper, phosphorous, zinc, selenium), vitamin-B complex (niacin and folic acid, ascorbic acid, carotenoids (β-carotene, lutein, zeaxanthin), phenols (flavonoids, p-coumaric acid), apocynin and omega -3-fatty acids. The whole plant is medicinally important due to the presence

of various phytochemicals which helps to prevent chronic health problems such as cancer and heart disease.

References

Baillie JK, Thompson AAR, Irving JB, Bates MGD, Sutherland AI, Mac Nee W, Maxwell SRJ and Webb DJ. 2009. Oral antioxidant supplementation does not prevent acute mountain sickness: double blind randomized placebo-controlled trial. *International Journal of Medicine* **102:**341-8.

Gross J. 1991. Pigments in Vegetables: Chlorophylls and Carotenoids. Van Nostrand Reinhold New York 1–351.

Helmut S. 1997. Oxidative stress: Oxidants and antioxidants *Experimental physiology* **82:** 291-295.

Kidmose U, Edelenbos M, Norbeak R and Christensen LP. 2002. Colour stability in vegetables. In Colour in Food: Improving Quality. D.B. MacDougall, Ed. CRC Press, Wood Head Publishing Limited, Cambridge, U.K **43:**179–232.

Lajolo F and Lanfer UM. 2003. Chlorophyll degradation in spinach system at low and intermediate water activities. *Journal of Food Science* **47:**1995–98.

Osborne TB and Mendel LB. 1999. The relation of growth to the chemical constituents of the diet. Journal of Biological Chemistry **15:**311-326.

Poppel GV. 1996.Epidemiological evidence of β-carotene in prevention of cancer and cardiovascular disease. *European Journal Clinical Nutrition* **50:** 57–61.

Quach HT, Steeper RL and Griffin GW. 1999. Introduction to Organic Laboratory Techniques: A Microscale Approach 3rd Edition Saunders College Publishing: New York. *Journal of Chemical Education* 81**:** 385-387**.**

Robert L, McLaughlin R, Masters K, and William G. 2004. Extraction and Thin-Layer Chromatography of Chlorophyll A and B from Spinach. *Journal of Chemical Education* **81:**385-387.

Salomon TG, Bahl J, Ben- Rais L, Allpha MJ, Centrel C and Dubacq C. 1991. Heat stress and changes of lipid and carotenoid composition in spinach, a temperate plant and jojoba, a desert plant, *Plant Physiology and Biochemistry* **29:**667-79.

Srinivasan R, Chandrasekar MJN and Najan B. 2007. Free Radical Scavenging activity of *Ipomoea obscura* (L.) Ker-Gawl. *Journal of Natural Remedies* **7:**184-188.

Solar RC, Espin JC and Wichers HJ. 2000. An easy and fast test to compare total free radical scavenger capacity of foodstuffs. *Phytochemicals Analysis* **11:**330–338.

Sultana B, Anwar F and Iqbal S. 2008. Effect of different cooking methods on the antioxidant activity of some vegetables from Pakistan. *International Journal of Food Science Technology* **43:**560-567.

Shon MY, Choi SD, Kahng GG, Nam SH and Sung NJ. 2004. Antimutagenic, antioxidant and free radical scavenging activity of ethyl acetate extracts from white, yellow and red onions. *Food chemistry Toxicology* **42:**659-66.

Zvalo V, Owen J, Kalt W, Shi J and Fillmore SAE. 2008. Diversely colored sweet potatoes (*Ipomoea batatas*) grown under Maritime conditions, McGill University, Montreal, Quebec City, Canada **8:** 13-16.