ISOLATION OF NEW PIGMENT FROM IRAQI RED RADISH PEELS (RAPHANUS SATIVUS L.)[+]

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Abstract

A new pigment was isolated from aqueous extract of Iraqi red radish peels (Raphanus sativus L.) the pigment is characterized by conventional characterization methods i.e. UV, IR, TLC, PC, elemental analysis and molecular weight determination.

المستخلص

عزلت صبغة من المحتوى المائي لقشور درنات الفجل الأحمر العراقي Redradish (Raphanus sativus L.) وشملت الدراسة الحالية التعرف على المحتوى الكيميائي للمستخلص المائي لقشور هذا النبات من خلال تقنية كروموتوغرافي الصفائح الرقيقة TLC وبعض الاختبارات الكيميائية، وحدد التركيب الكيميائي لهذه الصبغة باستخدام طريقة تعيين الوزن الجزيئي وتقنيات الـ UV، IR TLC, PC.

Introduction

Color is important attribute of most fruits and vegetables as well as products, the identification of compounds responsible for color is of interest. The red and blue colors of plants are due to the presence of anthocyanin pigments [1] . anthocyanins major natural phenol compounds. They are water soluble glycosides and acyl glycosides of anthocyanidins, which are poly hydroxy and poly methoxy derivatives of 2- phenyl benzo pyrylium (flavylium cation). Anthocyanins belong to a large and widespread group of plant constituents collectively known as flavonoids. The most common naturally occurring anthocyanins are 3-0-glycosides or 3,5-di-0-glycosides [2].

The major importance comes from their potential application as natural colorants to replace synthetic foods dyes . Other important aspects of anthocyanins are medicinal because of their biological activities. Natural anthocyanins are prescribed as medicine in many countries. They have been reported to have positive effects in treating various microcirculation diseases resulting from capillary fragility such as preventing cholesterol – induced atheroselerosis, inhibiting platelets aggregation and improving visual function.

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Anthocyanins are also potent antioxidants in vitro against various reactive oxygen species [3, 4, 5].

Our objective was to isolate the pigments in the peels of red radish (Raphanus sativus L.) which is an easy vegetable to grow and it occur in a variety of shapes and colors . The anthocyanin (ACN)pigments are resoponsible for red and purple radishes and have been characterized by different researchers [6, 7, 1].

Materials and Methods

Plant material :

Red radishes (Raphanus Sativus L.) were obtained from Basrah market. They were cleaned and the peels were collected carefully and allowed to dry at room temperature. The dried peels were blended by using (Electrical millblender), the powder of the peels were kept until required.

Chemicals and Materials :

All chemicals were of purely analytical grades. Methyl alcohol, sulphuric acid (Analar), sodium hydroxide, 2,4-dinitro phenyl hydrazine, Ammonium hydroxide, magnessium turninges were purchased from Fluka; Iodine, Glucose, silica gel plates, silica gel powder, chloroform and ferric chloride were purchased from Merck, p-anisaldehyde, ninhydrine and sodium carbonate from RDH; phthalic acid, acetic acid, lead acetate, n-butanol, acetone, sodium bicarbonate, hydrochloric acid (analar), ribose, xylose, fructose and potassium alum were purchased from BDH; -naphthol from H and w; 95% ethanol from Baghdad factory for drugs and cosmetics; copper sulphate, sodium citrate and folin cioalteus were purchased from Ajax.

Extraction Method:

10gm of red peels powder were extracted by soaking in 400 ml of cold water for 5 hours, the extract was filtered through (Whatmann No. 541), filter paper. To the filtrate 2% aqueous lead acetate was added until the formation of flocculent and blue precipitate, the precipitate was separated by (Whatmann No. 540) filter paper. The red filtrate was concentrated to 200 ml by evaporation under vacuum; Lead acetate (2% methanolic solution) was added to the red solution until the formation of purple precipitate, the precipitate was separated through (Whatmann No. 540) and washed with water, methanol and ethyl acetate consecutively [6].

The product salt was converted into chloride by dissolving in (25 ml acetone and 5 ml 2N HCl) and filtered through (Whatmann No. 542). The filtrate was placed in petri dish at room temperature until dry. The weight of amorphous red powder formed was 0.600 gm.

Isolation of pigments:

Column chromatography was performed using glass column (43 x 2) cm. The column was packed firmly by silica gel slurry to 37 cm high. The dry red pigment (0.500 gm) was dissolved in eluent. The solution applied to the column, and the chromatogram developed with solvent system (n-butanol – acetic acid – water 4 : 1 : 5). the eluate was collected in (2 ml / min) fractions and the TLC and the optical density of each fraction at 366 nm was measured. The separated fractions with the same Rf values were collected together. The features appeared were two pigments, the greatest offort of this investigation was concentrated on pigment A which represented 8% of the total pigments.

Preliminary Qualitative Test:

Preliminary tests were carried out on the aqueous extract and on the isolated pigment as shown in Table (1).

Acid hydrolysis:

10 gm of the isolated pigment were placed in round bottom flask, 10 ml of 2N HCl was added and the solution was refluxed at 100 ^{o}C for one hour. After cooling the solution was poured into separating funnel[2]. Chloroform (10 ml) was added and the solution was shakn to separate the organic layer, the process was repeated three times with 10 ml chloroform and the organic layers were collected and condensed to 5 ml by rotary evaporator at 40 ^{o}C.

The aqueous layer was neutralized by adding 2M NaOH and evaporated to 5 ml by rotary evaporator at 60 ^{o}C.

Identification of sugar moieties:

Examination of the sugar components in aqueous layer was carried out . Preliminary test was made using Molisch's, Benedict, Barfoed's, Biel's and Seliwonoff's tests (Table 3). A further test was done on TLC by applying (6 drops) of the aqueous layer to silica gel plates (10 x 6 cm) and the plate was run with n-butanol – HOAC – H_2O (4 : 1 : 5) as eluant for 60 min. Standard sugars were applied as well at the same plate as shown in Table (4).

Test of organic layer (Aglycone component):

The aglycone component was examined after hydrolysis of the pigments with 2N HCl. The resultant hydrolysate was spotted directly on paper chromatography (Whatmann No. 3)

(3 x 12 cm). Two eluated systems were used, conc. HCl – HCO_2H – H_2O (2 : 5 : 3) and n-butanol – HOAC – H_2O (4 : 1 : 5) for 45 min the paper was dried and examined under uv at 366 nm. The results are shown in Table (5).

Examination of acyl groups [6]:

(0.030 gm) of the isolation pigment was mixed with 10 ml of 2N NaOH in a three neck round bottom flask under nitrogen in to the darkness at room temp. for 45 min. The resulting solution was neutralized by adding 2N HCl.

The resultant hydrolysate was spotted directly (5 drops) on paper chromatography (Whatmann No. 3) (12 x 4 cm) using three solvents system as mobile phase,n-butanol–HOAC–H_2O(4:1:5),n-butanol–2 MNH_4OH (1 : 1) and n-butanol – HOAC – H_2O (4 : 1 : 2.2) for 45 min. after drying the papers were examined under uv at 366 min wave length. The papers were treated with ammonia and further examination under uv Lamp were done, the results are shown in Table (6).

Test for raphanusin [6]:

Ascending PC (Whatmann No. 3) (12 x 4 cm) was carried out using four mobile phases HOAC – HCl – H_2O (5 : 1 : 5), isoAmOH – HCl – H_2O (4 : 5 : 4), n-butanol –HCl – H_2O (7 : 2 : 5) and n-butanol – HOAC – H_2O (4 : 1 : 5) for 45 min. And the four papers were tested under uv Lamp after drying. Results are shown in Table (7).

Infrared and uv – visible spectroscopy:

IR spectra using Pye – Unicam – 3 – 300S infrared – spectrophotometer and uv – vis. Spectra on JASCO uv – visible spectrophotometer were recorded . They are shown in Fig. (1, 2), (3) and table (8).

Results and Discussion :

Red pigment was separated by column chromatography using n-butanol – acetic acid – water (4 : 1 : 5) as mobile phase, the preliminary tests of aqueous extract of red radish (Raphanus Sative L.) and the isolated pigment show the presence of flavonoid as glycoside Table (1). The TLC shows the presence of anthocyanin pigments Table (2) which change their color by changing the pH values. The same results are obtained by other authors [6, 8, 9, 1, 10,11].

Table (1): Results of preliminary qualitative tests for pigment and aqueous extract

Tests	Flavono-	Carbohydr-	Glycosid-	Alkolid-	Amino	Saponin test

sample	id test	ate test	e test	e test	acid test	5% $HgCl_2$	Foam test
Aqueous extract	+	+	+	-	+	-	-
Pigment A	+	+	+	-	-	-	-

Table (2): TLC for pigment

Test / sample	uv Lamp	Iodine	Folin	Vanillin	HCl 10%	$FeCl_3$ + $K_3Fe(CN)_4$	p-anisaldehyd	Drangdroff
R_f of Pigment	0.46	0.46	0.46	0.46	0.46	0.46	0.46	-
Test sample	10% NH_4OH	40% H_2SO_4	Day light	Ninhydrin				
R_f of Pigment	0.46	0.46	0.46	-				

In the aqueous fraction (after acid hydrolysis) one type of sugar was identified, Table (3). The TLC of the aqueous fraction and the standard sugars showed a very high agreement between Rf of the aqueous layer and the glucose which indicates that the sugar is glucose **Table (4).** As illustrated by other authors, the major sugar which appears in the anthocyanin pigments of the red radish peels is the glucose [9, 6, 12, 13, 1].

Table (3): **Prelimiray test of aqueous fraction**

Seliwonoff's	Barfoed's test	Biel's test	Benedict test	Molisch's test
-	+	-	+	+

Table (4) TLC results :- aqueous fraction of pigment

Aniline hydrogen phthalate	Glycose	Aqueous fraction
Brown	RF = 0.30	RF = 0.31

In the organic layer, the a glycone was identified as pelargonidin, based on pc comparisons with anthentic anthocyanin, Table (5) [2].

Table (5) : PC results for organic layer

U.V.	Visible colour	Conc. HCl – HCO_2H-H_2O 2 : 5 : 3	n-butanol – HOAC – H_2O 4 : 1 : 5

Darkness	Red	RF = 0.33	RF = 0.78

Alkaline hydrolysis of the pigment showed shift in retention time using pc, the acyl was identified, Table (6), as p-coumaric acid, based on the value of Rf comparisons with authentic acyl group [2] Table (7) shows the comparison of Raphanusin (Perlagonidin-3-sophoreside-5-glucoside) with literature values [14, 1, 13].

Table (6) **: PC results for acyl group**

NH_3 gass + uv	**uv**	**n-butanol – ethanol – H_2O 4 : 1 : 2.2**	**n-butanol – HOAC – H_2O 4 : 1 : 5**	**n-butanol – 2M NH_4OH 1 : 1**
Mauve	None	RF = 0.87	RF = 0.93	RF = 0.17

Table (7) **: PC results for Raphanusin group**

HOAC – HCl-H_2O 5 : 1 : 5	**n-butanol – HCl – H_2O 7 : 2 : 5**	**n-butanol – HOAC – H_2O 4 : 1 : 5**	**Isoamoh-HCl-H_2O 21 : 5 : 4**
RF = 0.85	RF = 0.4	RF = 0.4	RF = 0.05

Fig. (1,2) shows the UV spectraum Fig. (3) and Table (8) show the full scan of IR spectrum of the pigments, the UV spectrum shows maximum absorption at 296 nm due to $\pi \rightarrow \pi^*$ transition which is the characteristic of unsaturated double bond and λ_{max} = 326 due to $n \rightarrow \pi^*$ transition due to non-bonding, the visible spectrum also shows max absorption at λ = 550 nm due to the transition of n π^* [15, 16].

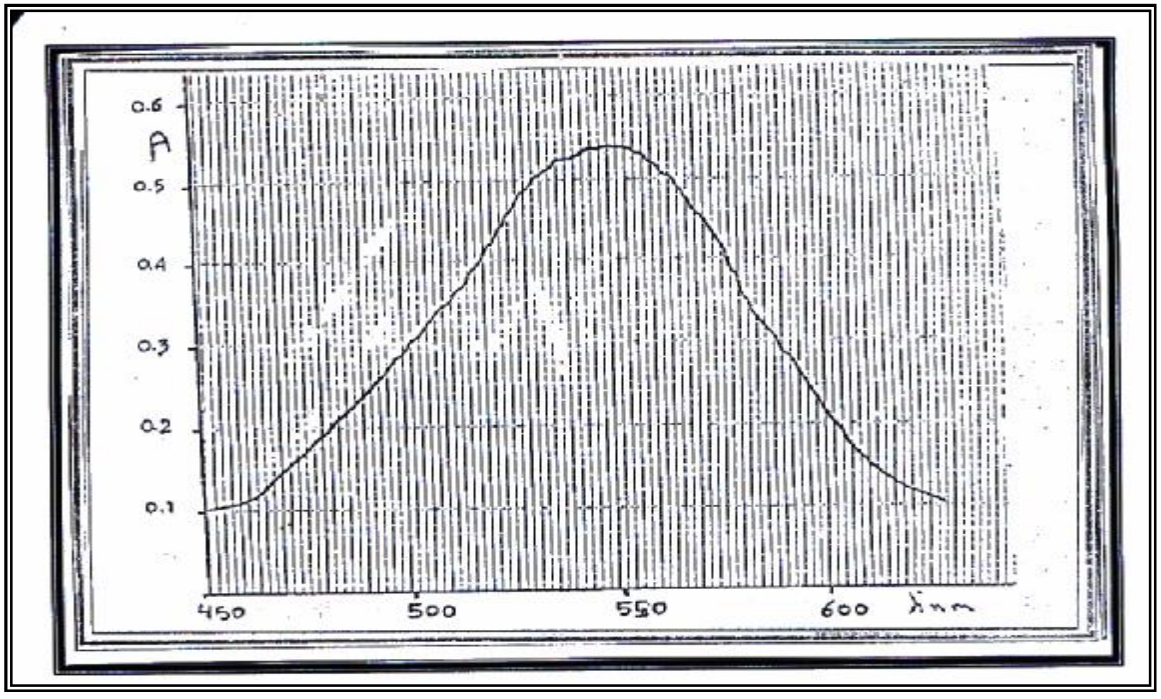

Fig. (1) **:**

Visible spectra of pigment

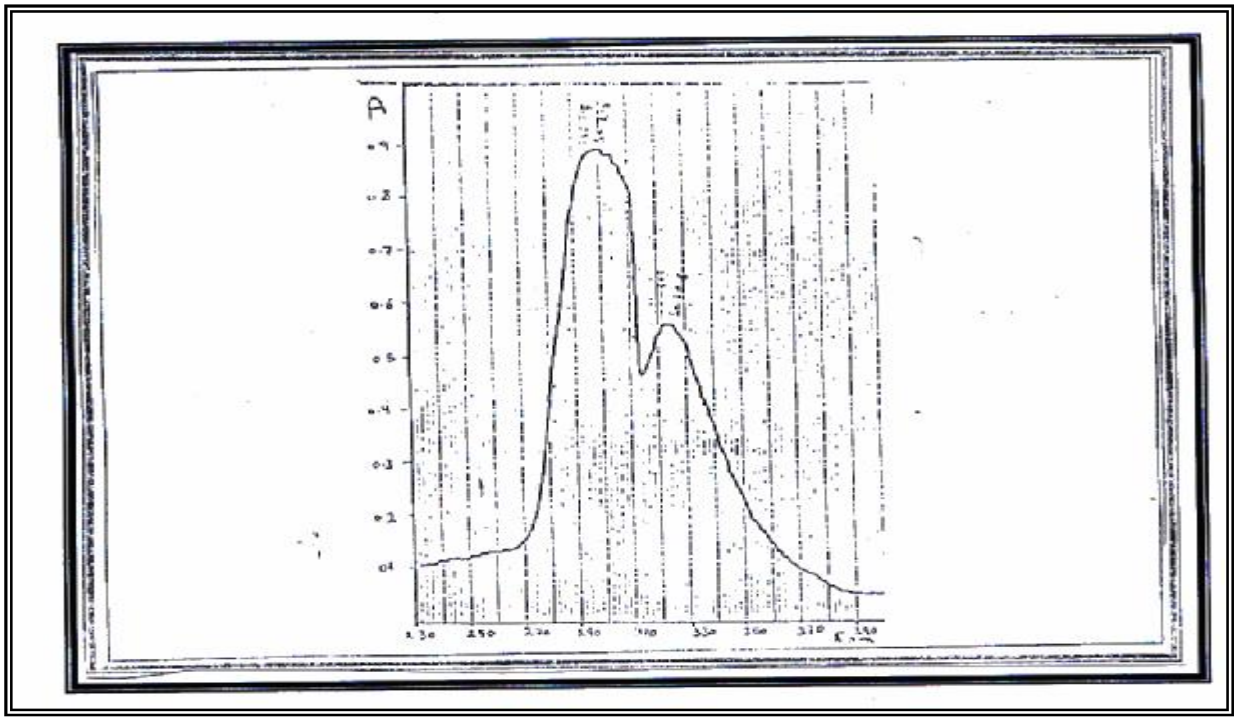

Fig. (2) : UV spectra of pigment

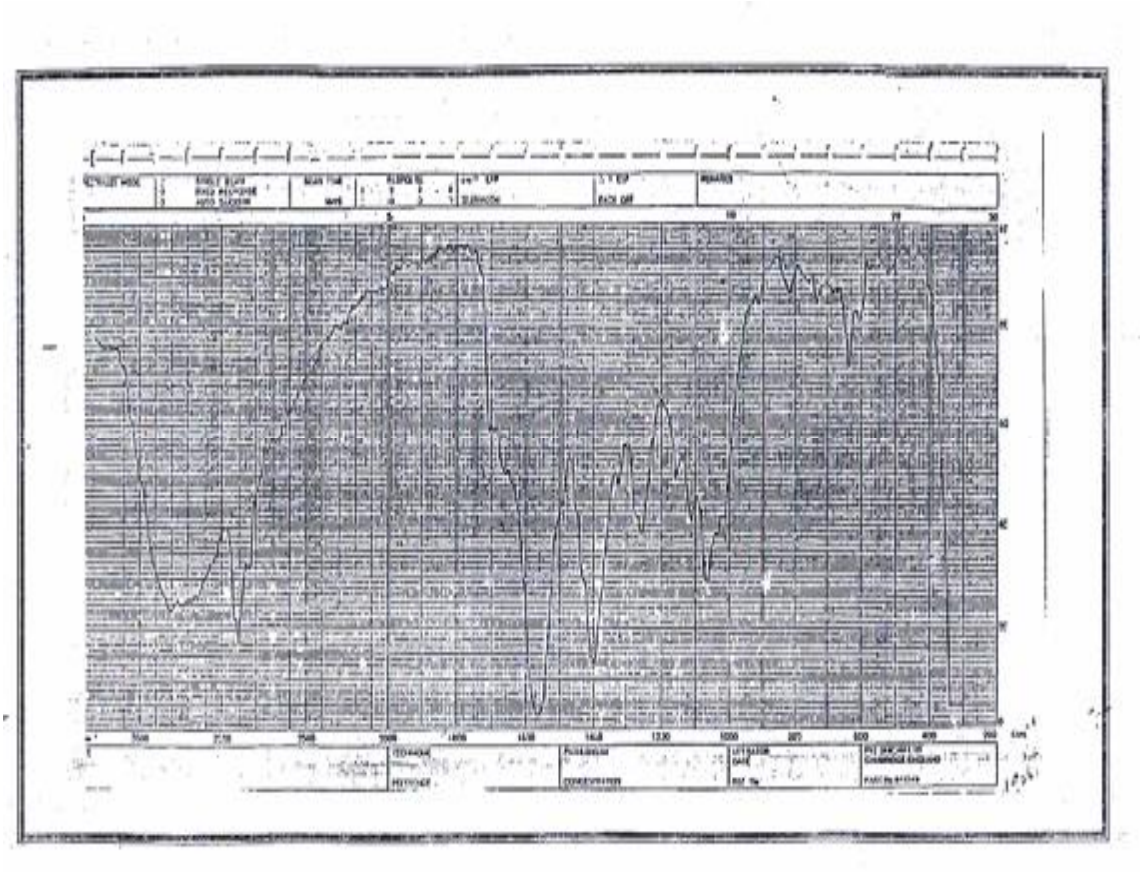

Fig.(3) IR Spectrum

Table (8) full scan of IR spectrum of the pigment

Band frequency cm^{-1}	Band shape	Bond	Function group
3400 – 3100	Br.	O-H	Alcoholic, phenolic
2840 – 2900	Sh.	C-H	Aliphatic
1700	Sh.	C=O	Carbonyl group
1660	W-W	C=C	Aromatic (benzene)
1580 – 1560	S	C-O-C	Glycosidic linkage
1400	S, Br.	Bending	Benzene ring
1260	M, Br.	**Ar – O – C-**	Alkyl aryl ether
1070	S, Br.	-OH	Alcoholic C-OH
1110 – 1170	M	Bending	Ethers –C-O-C-

S = Strong ; W = Weak; Br. = Broad ; Sh. = Sharp ; M = Medium

The range of the max. absorption of the anthocyanin pigment (296, 326 and 550 nm) due to the absorption of anthocyanin pigments containing acyl groups is the same as those found in the authentic literatures values[12, 17, 18].

Table (9) gives the rigments obtained by some chemical and physical test. The molecular weight of pigments was determined by grycosscopic method [19] and found to be 932.6 g/mol.

Table(9): chemical and physical test

Test	Pigment
Physical principle	Mauve amorphous
Melting point	160-170 decomp.
Ignition test	Black smoke with black carbon
Solubility test	The pigment soluble in water, DMSO, ethanol, acetone and insoluble ether, hexane and ethyl acetate
Acidity test	pH = 6.2

Table (10) shows the comparison of the pigment with natural pigments founds in the literature[2].

Table (10) **pc results for pigment**

Sample	HOAC –HCl-H_2O 15 : 3 : 82	n-butanol – 2NHCl 1 : 1	H_2O – conc. HCl 97 : 3	n-butanol – HOAC – H_2O 4 : 1 : 5
Pigment	0.75	0.34	0.49	0.35
Raphanusin A	0.73	0.34	0.49	0.34

From the information above it appears that the pigment contains an acyl group, the acid hydrolysis showed that the organic part is pelargonidin as compared with literature values [2] by pc. The sugar moieties are glucose and the acyl group is p-coumaric acid.

Based on the obtained spectroscopic and chromatography data, a proposed structure of the anthocyanin of Iraqi red radish peels (Raphanus Sativus L.) is suggested to be Raphanusin A (pelargonidin-3-p-coumaroyl sophoroside-5-glucoside). Fig. (4)suggests the chemical composition of the anthocyanin molecule of the pigment. Further work will focus on the second pigment isolated from the red radish peels (pigment B).

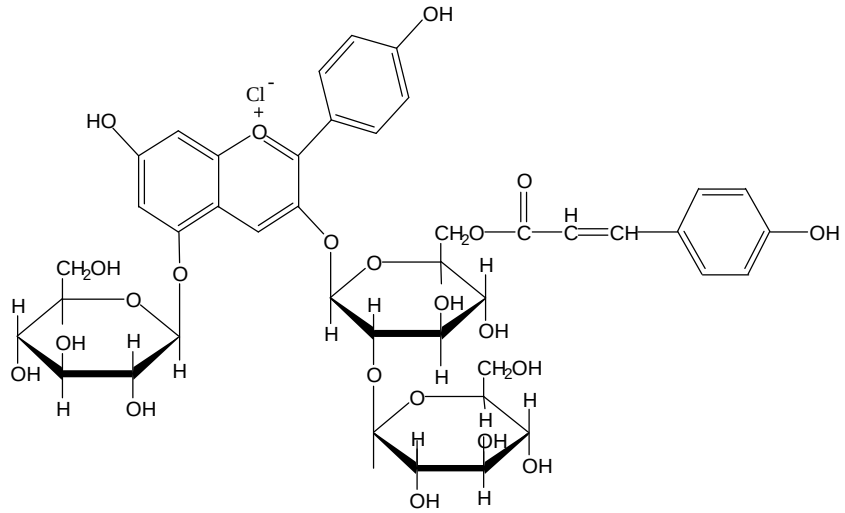

Fig (4) : Raphanusin A (pelargonidin-3-p-coumaroyl sophoroside – 5- glucoside

References :

1. Fuleki, T. "The Anthocynine of Strawberry Rhubarb, Radish and Onion." *J. of Food Science*, Vol. 34, pp. 365-369, 1969.
2. Harborne, J., *Phytochemical Methods, 2nd edition*, Chapman and Hall, New York, 1984.
3. Hammouri, M. : Al-Samdi, M; Bataineh, M. and Ou, B., "Separation and Characterization of an Anthocyanin, Cyaniding-3-O-Arabinosyl Gluoside from Petals of *Flowers of Pheasant's eye (Adonis aestivalist L.)". International J. of Biochromatography*, vol. 6, pp. 173-183,2001.
4. Yoshida, K.; Kameda, K. and Kondo, T., *"The chemistry and application of anthocyanins and flavones from perilia leaves" In "perilia", (Yu, H.; Kosuan, K. and Haga, M.),* Harwood academic publishers, The Netherland, 1997.
5. Giuisti, M. and Wrolstad, R. Characterization and Measurement of Anthocyanins by uv-visible Spectroscopy , *Current Protocols in Food Analytical Chemistry*, pp. 1-12, 2001.
6. Ishikura, N. and Hayashi, K. *Anthocyanin and other Flavonoids* , Natural Products Reports University of Reading, UK, 1962.
7. Harborne, J., *Plant polyphenols Ix. The Glycoside Pattern of Anthocyanin Pigment* Phytochemistry, vol. 2, 1963.
8. Xiaoling, Lu. "Prelimery Identification of Anthocyanins in Red radish", *Shipin Yu Fajiao Gongye*, Vol. 6, pp. 19-25 (chem.. Abs.) 104 : 106243n. 1985.
9. Harborne, J., *Comparative Biochemistry of the Flavonoids* Academic Press, London. 1967.
10. Gross, J., *pigment in fruits*, Academic press, London, 1987
11. Yositama, K.; Ishikura, N.; Fuleki, T. and Nakamura, S. "Effect of Anthocyanin Flavonol co. Pigmentation and pH on the Color of the Berries Ampelosis Breuipedum Culato", *J. Plant Physiol*, Vol. 139, pp. 513-518, 1992.
12. Wrolstad, R.; Giust, M.; Rodiguez-Saona, L. and Durst, R. *Anthocyanins From Radishes and Red Fleshed Potatoes. In (Chemistry and Physiology of Selected Food Colorants*). (Ames, J. and Hofmann, T.) chapter 5, Amer. Chem. Soc., Washigton, DC., 2001.
13. Giuisti, M. and Wrolstad, R. "Characterization of Red radish Anthocyanins", *J. Food. Sc*., Vol. 61, No. 2,pp. 322-326, 1996a.
14. Giusti., M.; Ghanadan, H. and Wrolstad, R. "Elucidation of the Structure and Conformation of Red Radish "Raphanus Sativus L." Anthocyanins using one and two

Dimensional Nuclear Magnetic Resonance Techniques", *J. Agric Food Chem.*, vol. 46, No. 12, pp. 4858-4863, 1998b.

15. Silverstone, R., Bassler, G. and Moril, T., *Spectrometric Identification of Organic Compounds, 4th* , John Wiley and Sons. Inc., USA. 1981.
16. Sherba, A. K.; Al-Rawi, J. M.; Iraqi, M. A. *Absorption Spectroscopy of orGanic Molecules* Printed in Mosuel University, Translated from "Absorption Spectroscopy of Organic Molecules"1985.
17. Rodriguez-saona, L. Giusti, M. and Wrolstad, R. "Molar absorptivity and color Characteristics of Acylated and Nonacylated Perlargonidin-Based Anthocyanins", *J. Agric Food, Chem.*, Vol. 47, No. 4631-4637, 1999b.
18. Pomilio, A. B. and Sproviero, J. F., *Acylated Anthocyanins From Ipomoeas Cairica* Phytochemistry, Vol. 11, 1972.
19. Saed, A. S.; Nahida, A. H.; Nuha S. M., *Practical Physical Chemistry*, Printed in Basrah University, Translated from "Practical Physical Chemistry"1985.