HPLC Analysis of Strawberry Anthocyanins at Partially Ripe and Ripe Levels

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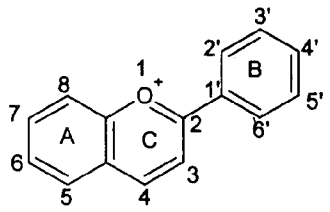
(Received 30th March, 2006, revised 21st April, 2007)

Summary: Distribution of anthocyanins has been investigated in strawberry fruit (*Fragaria ananassa*) at partially ripe and ripe levels. A fraction rich in anthocyanins was obtained by liquid-liquid extraction using acidic methanol (1: 99) followed by removal of lipids by *n*-hexane. Lipid free extract was subjected to column chromatography to separate sugars and acids; whereas carotenoids were isolated from anthocyanins using TLC. The anthocyanin content represents 50.0 mg/ kg and 300 mg / kg in partially ripe and ripe fruit respectively; which is in agreement with the literature data. HPLC analysis was used to characterize individual anthocyanin and their relative concentration. Information from HPLC retention data revealed that major anthocyanin in partially ripe strawberry was pelargonidin 3-glucoside (33 mg / kg; II); whereas, cyanidin 3-glucoside (152.6 mg/ kg, I), pelargonidin 3-glucoside (53.25 mg/ kg; II) and pelargonidin 3-rutinoside (26.4 mg/ kg; III), were predominant anthocyanins in ripe strawberry fruit.

Introduction

Anthocyanins are natural pigments belonging to flavonoid family of polyphenols. They are responsible for the red, blue and purple colors of many fruits, vegetables and flowers [1]. Fruits and berries are an ample source of anthocyanins in nature. Anthocyanins are mainly found in the peel and pulp of fruits. In Strawberries, the anthocyanin concentration correlates well with the darkness of the Strawberry; the darker the color of the fruit is, the higher is its anthocyanin contents. Strawberry has a simple anthocyanins profile with only a few major pigments. The total anthocyanins content in ripe Strawberry ranges from 200–600mg/ kg. The Strawberry anthocyanins are non-acylated [2].

The main structural unit of a anthocyanin is 2-phenylbenzopyrylium cation also called flavylium cation or aglycone. Flavylium cation contains conjugated double bonds responsible for absorption of light around 500 nm causing the pigments appear red to human eye. Anthocyanins are glycosylated polyhydroxy and polymethoxy derivatives of flavylium cation, which are usually penta- (3, 5, 7, 3′, 4′) or hexa-substituted (3, 5, 7, 3′, 4′, 5′). Different varieties of anthocyanins are known today but only six of them are significant and most common from food usage. The most important anthocyanins are pelargonidin, cyanidin, peonidin, delphinidin, malvidin and petunidin. These aglycones differ in the number of hydroxyl and methoxy groups in their B-ring of the flavylium cation [3 - 4]. Anthocyanins are highly unstable and easily susceptible to degradation. The stability of anthocyanins is affected by pH, storage temperature, light and oxygen [5], which are important in defining their antioxidant, anti-carcinogenic, anti-inflammatory and anti-angiogenic properties [6-11].



Flavylium cation

Results and Discussion

Separation and Purification

Two fractions were obtained when lipid-free extract was subjected to silica gel column chromatography using *n*-hexane: ethyl acetate:

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methanol mixture (60: 20: 20). Fraction 1 (colorless band) contained sugars and acids; it was therefore not further analyzed. Fraction 2 (colored band) containing anthocyanins and carotenoids was fractionated by thin-layer chromatography using a solvent system containing *n*-hexane and acetone (40:60). The compounds of lower polarity i.e. carotenoids moved along with the solvent mixture and were located at an R_f value 0.95, identified on the basis of R_f values reported in the literature [12 - 13].

The anthocyanins being more polar did not move from the baseline. The anthocyanin fraction was scratched from the baseline and extracted with methanol and quantified. The anthocyanins content thus obtained represents 50mg/ kg and 300 mg/ kg for partially ripe and ripe Strawberries respectively.

HPLC Analysis

The HPLC chromatograms display twenty-two peaks in the ripe Strawberry. However, only one major peak along with other minor peaks were observed in the partially ripe fruit (Fig. 1). The minor peaks having an area less than 1 % and those with poor resolution were not considered. The three major anthocyanins (I-III) were identified in these chromatograms (Fig. 2) on the basis of retention behavior and consultation with the literature data [2]. One major anthocyanin having retention characteristics similar to pelargonidin 3-glucoside (peak 7; II) was present in the partially ripe Strawberry.

The three anthocyanins identified in the ripe Strawberry were cyanidin 3-glucoside (peak 4; I), pelargonidin 3-glucoside (peak 7; II) and pelargonidin 3-rutinoside (peak 14; III). The concentration of pelargonidin 3-glucoside (II) in ripe fruit was comparatively higher than that in partially ripe fruits. Two other anthocyanins, cyanidin 3-glucoside (I) and pelargonidin 3-rutinoside (III) identified in the ripe Strawberries, were however below detection limits in the partially ripe sample. The structures and relative concentrations are shown in Fig. 2 and Table- 1.

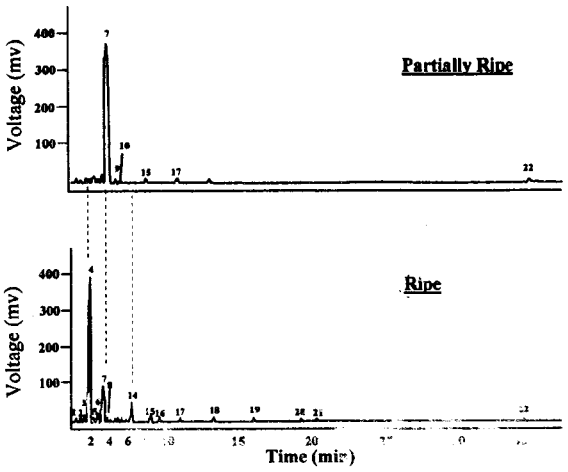


Fig. 1 HPLC chromatogram showing distribution of anthocyanins in strawberry fruit at partially ripe and ripe levels. Refer to Table- 1 for peak identity.

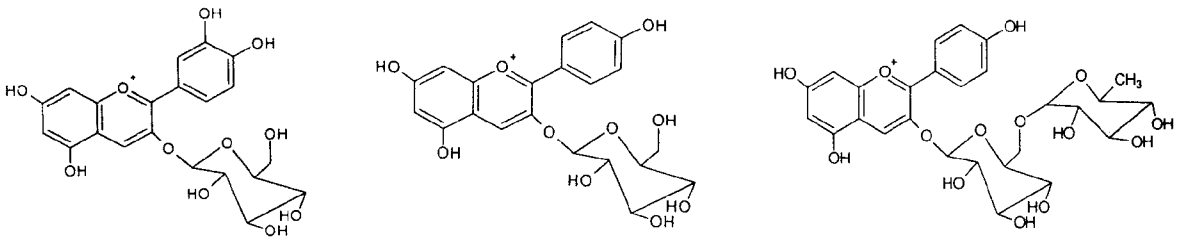
Quantification

Because anthocyanins are unstable compounds, exacting standards is difficult. Therefore, the absolute concentration could not be

Table- 1: Identification and relative concentration of anthocyanins in partially ripe Strawberries

Peak No.	RT (min)	Rel. Concentration # (mg/ kg) Partially Rip	Ripe	Peak Identity
3	1.748	-	-	-
4	1.973	-	152.6	Cyanidin 3-glucoside
6	2.857	3.1	11.3	-
7	3.432	33.0	53.2	Pelargonidin 3-glucoside
9	4.315	0.3	5.3	-
10	4.759	0.3	-	-
12	5.215	-	5.3	-
14	6.482	-	26.4	Pelargonidin 3-rutinoside
15	8.848	2.3	5.9	-
17	11.298	2.4	4.6	-
18	13.565	-	5.0	-

#: Rel conc. (mg/ kg) of desired compound = $\frac{\text{Peak area of desired compound}}{\text{Sum peak area of all compounds}}$ X Conc. (mg/ kg) of anthocyanin mixture injected



(I) (II) (III)

Fig. 2 Structures of identified anthocyanins (I) Cyanidin 3-glucoside; (II) Pelargonidin 3-glucoside; (III) Pelargonidin 3-rutinoside

calculated. The total anthocyanin content determined gravimetrically was 50.0 mg/ kg and 300.0 mg/ kg in partially ripe and ripe samples respectively. The relative concentrations were calculated by dividing the peak area of desired compound by the total area of all peaks and then multiplying with the total concentration of sample injected, as shown in Table- 1.

The relative concentration of pelargonidin 3- glucoside (II) calculated using above relation in partially ripe Strawberry is 33 mg/ kg. The relative concentrations of major anthocyanins in ripe Strawberry are: pelargonidin 3-glucoside (53.25mg/ kg; II), cyanidin 3-glucoside (152.6 mg/ kg; I) and pelargonidin 3-rutinoside (26.49 mg/ kg; III).

The partially ripe fruit contains a large amount of acids, which protect the fruit from the bacterial attack, and chlorophyll to absorb the light. As these compounds have not been degraded yet therefore anthocyanins are in lower relative amounts in contrast to the ripe fruit, where the concentration of acids has decreased and chlorophyll has been degraded resulting in an increase in the concentration of anthocyanins as the fruit ripens. The concentration of cyanidin 3-glucoside (peak 4; I) has been negligible in the partially ripe fruit. Similarly the pigment pelargonidin 3-rutinoside (peak 14, III) identified in the ripe fruit has not been formed in partially ripe fruit. These observations require further study on the mechanism of their formation or interconversion.

Experimental

Sample

The Strawberries (*Fragaria ananassa*) were obtained from Sukhian Pull, Lahore, in May 2006. The light pink color and fully-developed red color Strawberries, obtained from the same plant, represent partial or full ripeness of the fruit. The fruits were washed with water and stored at -10 °C in a freezer until analyzed.

Liquid-Liquid Extraction

Crushed Strawberries (10g) were soaked in methanol (30 ml) containing 0.1 % HCl (99: 1) for overnight at 5-7 °C in a refrigerator and then filtered through a Büchner funnel under vacuum. The residue was washed twice with methanol – HCl mixture.

The alcoholic extract was concentrated in a rotary evaporator at 30 ° C to obtain total extract. The extract was treated with *n*-hexane to remove lipids, and *n*-hexane soluble lipid fraction was wasted.

Fractionation by Column Chromatography

Lipid free extract was subjected to silica gel column chromatography. A glass column (40 cm x 1.2 cm) was dry packed with freshly activated silica gel (110 °C, 24 hrs, 3g). The lipid free extract of partially ripe Strawberries dissolved in the minimum amount of methanol was introduced onto

the column and eluted with *n*-hexane: ethyl acetate: methanol mixture (60: 20: 20). Fraction I (colorless band), comprising of mainly sugars and acids was collected using 30 ml of the eluent and was not analyzed further. Fraction II (colored band), containing anthocyanins and carotenoids, was obtained using 60 ml of eluent. The same procedure was adopted for ripe Strawberries. However, the volume of eluent was 55 ml and 90 ml for colorless and colored fractions respectively.

Thin-Layer Chromatography

Thin-layer chromatography was performed on a TLC plate (20 cm x 20 cm) coated with 0.5mm layer of silica gel. The extract containing anthocyanins and carotenoids was dissolved in acetone and applied in the form of a streak. The plate was developed using *n*-hexane: acetone mixture (40: 60). The compounds were visualized under short wavelength UV lamp (MODEL UVGL-58, multiband UV- 254/ 366nm). The band due to carotenoids was isolated from anthocyanins at an R_f value of 9.5. The fractions were recovered by methanol extraction and evaporation of solvent, and quantified.

HPLC Analysis

HPLC analysis was carried out using a Varian HPLC Polaris series isocratic system equipped with UV-VIS detector (range 190-900 nm). Chromatographic separation was achieved on a Particil Octadodecylsilane (C_{18}) column (250 mm x 4.6mm) with particle size 5 μm at 35 °C. The mobile phase was composed of 0.1 % TFA in water (solvent A) and HPLC- grade acetonitrile (solvent B), establishing the gradient 85 % (solvent A) and 15% (solvent B) using a flow rate of 0.5 ml/ min. Detection was carried at 480 nm wavelength.

Conclusions

Three major anthocyanins were identified in the Strawberry fruit. Pelargonidin 3-glucoside is the major anthocyanin in partially ripe fruit, whereas cyanidin 3-glucoside, pelargonidin 3-glucoside and pelargonidin 3-rutinoside were predominant anthocyanins of ripe Strawberry.

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