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CHARACTERIZATION OF ANTHOCYANINS BY GCMS

R. V. SARPATE*, T. K. DEORE, M. V. PATIL and S. V. TUPKARI

Smt. Sharadchandrika Suresh Patil College of Pharmacy, CHOPDA - 425107, Jalgaon (M.S.) INDIA

ABSTRACT

Anthocyanins (ACN) are part of a large and wide spread group of plant constituents, known collectively as flavanoids. Anthocyanins can be used as natural colorant. GCMS is the most powerful technique for determination of the molecular structure of anthocyanins. This technique provides molecular weight and mass spectral pattern for the exact structural characterization of the anthocyanins. In the present study, the colour pigments anthocyanins are extracted from fruits of *Ficus racemosa*. Family: Moraceae, using acidified methanol (1% HCl in methanol). The extracted anthocyanins were isolated and purified using the Amberllite–XAD_4 resin. The anthocyanins isolated and purified by column chromatography were eluted in methanol and were directly injected in the GCMS system. The spectral pattern was recorded and from the m/z ratio of the daughter and the parent fragment ions, the type of anthocyanins along with its structure was identified and characterized**.** In the present study, anthocyanins were characterized by means of chromatographic and spectral data obtained from GCMS, TLC and UV spectroscopy. Two major pigments identified were peonidin-3-glucoside and pelargonidin-3-glucoside. Peaks for the anthocyanins, which are present in very low amount, and in less concentration, can be verified by means of commercially available external standards. The objective of the present work was to isolate and characterize the anthocyanin pigments present in the fruits of *Ficus racemosa* by chromatographic and spectral data.

Keywords: Anthocyanins, GCMS, *Ficus racemosa* L.

INTRODUCTION

Anthocyanins (ACN) are part of a large and wide spread group of plant constituents, known collectively as flavanoids. They are mainly distributed among flowers, fruits and vegetables and are responsible for bright colours such as red, purple, magenta, orange[1]. Anthocyanins can be used as natural colorants[2]. As a potential major component of our daily diet, more and more research has concentrated on their biological activities and possible health benefits in protecting against some chronic diseases such as cancer, cardio,

*Author for correspondence; E-mail: rupali_sarpate@yahoo.co.in

cerebrovascular, atherosclerosis and diabetes[3]. Some of these biological activities and protective functions are attributable to their high antioxidant capacities[4]. One possible source of anthocyanin pigments is the fruit of *Ficus racemosa*, a highly productive plant, which provides fruits during long period of the year and it is a large deciduous tree distributed all over India mostly in the evergreen forest. Due to the high prevailing importance of flavanoids, many techniques have been used to identify and quantify these compounds. In the early 1990's, HPLC, with photodiode array detection was used to isolate and quantify the anthocyanins. ^{1}H and ^{13}CNMR spectroscopy and Tandem mass spectroscopy is also used to identify the anthocyanins[5]. GCMS is the most powerful technique for determination of the molecular structure of anthocyanins. This technique provides molecular weight and mass spectral pattern for the exact structural elucidation of the anthocyanins[6]. The interest in identifying sources of anthocyanin pigments, to be used in food, pharmaceutical and cosmetic industries is based on the consumer demand for natural colorants and on the nutraceutical properties, reported for flavanoids[7,8]. The anthocyanin pigments were previously isolated and identified from *Ficus racemosa* stem bark by Agarwal and Mishra[9]. The anthocyanins pigments isolated from *Ficus racemosa* bark were major ones being cyanidin-3-O-β-D glucopyranoside and pelargonodin-3-O-α-L-raomnopyranoside. This was the major incentive to isolate the anthocyanins from the fruits of *Ficus racemosa.* The objective of the present work was to isolate and characterize the anthocyanin pigments present in the fruits of *Ficus racemosa* by chromatographic and spectral data.

EXPERIMENTAL

Fruit

Ficus racemosa fruits were chosen with intensive red colour. The fruits were harvested during May at ideal stage of ripeness from Hindustan Antibiotics Colony Plantation in Pune city (India). After harvesting, the fruits were washed, packed in the polyethylene bags and stored in the dark at -15^{0} C for 24 hours, before the extraction of anthocyanins.

Chemicals

Peonidin-3-glucoside chloride, pelargonidin-3-glucoside chloride, cyanidin-3-glucoside chloride and maldivin-3-glucoside chloride were obtained as a gift sample from NCL, Pune, (India). The solvents used were of HPLC grade obtained from Merck (India). Amberllite XAD_4 resin was obtained from Thermax Chemical Ltd. (India). All other chemicals used were of analytical grade.

GCMS Equipment

GC-MS analysis was carried out on a Hewlett-Packard 6890 gas chromatograph fitted with a fused silica HP-5MS capillary column (30 m, 0.25 mm; film thickness 0.25 m). The oven temperature was programmed from 60-280º C at 4º C/min. Helium was used as carrier gas at a flow rate of 2 mL/min. The gas chromatograph was coupled to a Hewlett-Packard 6890 mass selective detector. The MS operating parameters were adjusted to ionization voltage, 70 eV; and ion source temperature, 200º C.

Extraction, isolation and purification of anthocyanins

The extraction was done using methanolic HCl[10,11] (1% HCl in methanol). The extracted anthocyanins were placed on a Buchi rotavapor at 35^0 C (25-30 min) until all residual methanol was evaporated. The methanolic extract containing high concentration of anthocyanins was passed through chromatographic column (25 x 0.5 cm) initially loaded with Amberllite XAD_4 resin. The adsorbed anthocyanins were then eluted with 0.1% HCl in methanol. The methanolic extract, containing high concentration of isolated anthocyanin pigments were then concentrated using a Buchi rotavapor at 35^0 C and stored at -10^0 C in cold conditions[12]

GCMS Analysis

Sample preparation for GCMS analysis

The anthocyanins isolated and purified by column chromatography were eluted in methanol and were directly injected in the GCMS system The spectral pattern was recorded and from the m/z ratio of the daughter and the parent fragment ions, the type of anthocyanins along with its structure was identified and characterized.

Characterization of anthocyanin

The anthocyanin characterization was carried out based on several physiochemical information such as separation by thin layer chromatography, spectroscopic characteristics and spectral shifts. Thin layer chromatography is performed by using n-butanol-acetic acid–water (4 : 1 : 2) as solvent system[13]**.** UV-Vis spectroscopy was obtained by scanning between 200-600 nm[14]**.** The absorption was registered before and after the addition of 3 drops of solution of aluminium chloride salt (5% m/v) in methanol[15]**.** The position of the sugar in the anthocyanin molecule was assigned based on the spectral shifts by calculating the ratio of absorption at 440 nm to the specific absorption maximum (λ_{max}) for each isolated pigments[16,17].

RESULTS AND DISCUSSION

The main objective of the present study was to isolate and characterize the anthocyanin pigments. The structures of the compound isolated and identified are shown in Fig 1. The structure shows the glycosidic linkage at the third position.

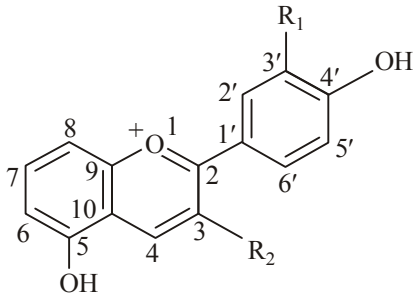

Fig. 1: Chemical structures of the anthocyanins identified in fruits of *Ficus racemosa*

(i) Peonide-3-glucoside ($R^1 = OCH_3$, R^2 = glucose)

(ii) Pelargonide-3-glucoside ($R^1 = H$, R^3 = glucose).

GCMS Analysis

The GCMS spectra provided information regarding the structural identification of anthocyanin pigments. The m/z ratio of the daughter and parents ions, gave the confirmation of the identity of the anthocyanins present.

Table 1: GCMS profile of isolated anthocyanin pigments

Peak	R. Time	Area	Area %	Height %
1.	14.700	578791	20.86	36.68
2.	25.300	493523	17.79	27.44

Table 2: Mass spectrum profile of the isolated anthocyanin pigments

Peak	R. Time	Daughter fragment	Parent fragment	Peak identification
1.	14.700	301.2	463.1	Peonidin-3-glucoside
2.	25.300	271.0	433.0	Pelargonidin-3-glucoside

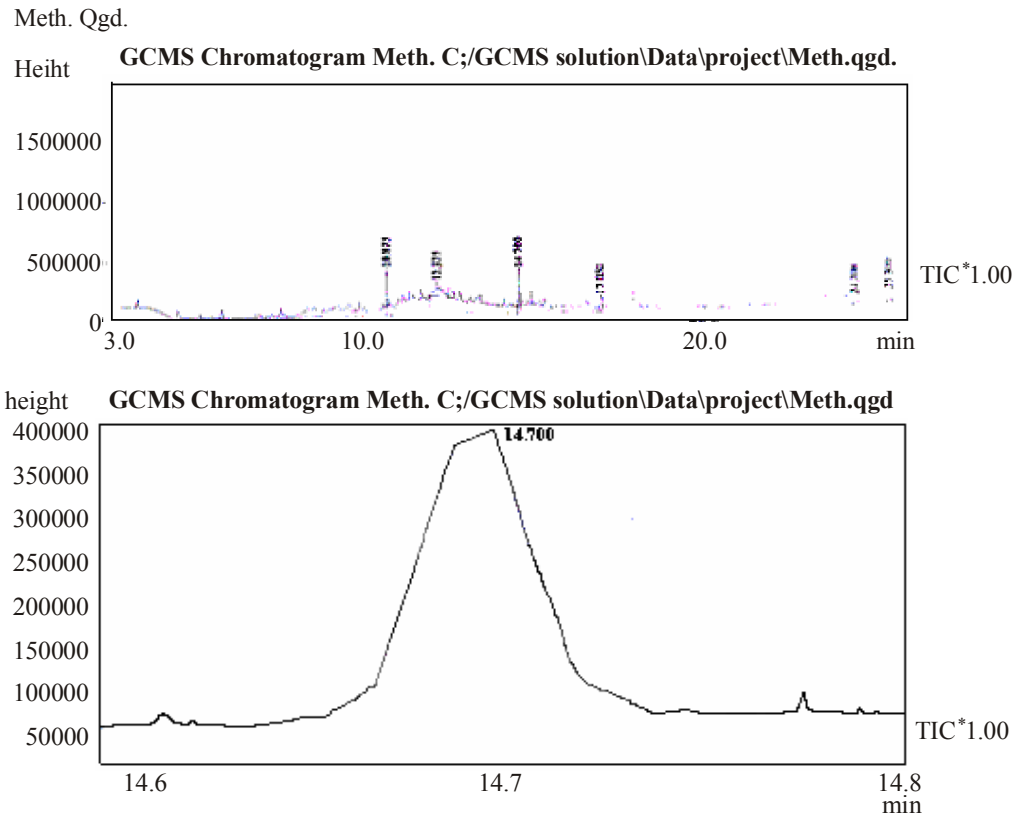

Fig. 2: The enlarged chromatogram of Peonidin at retention time 14.700

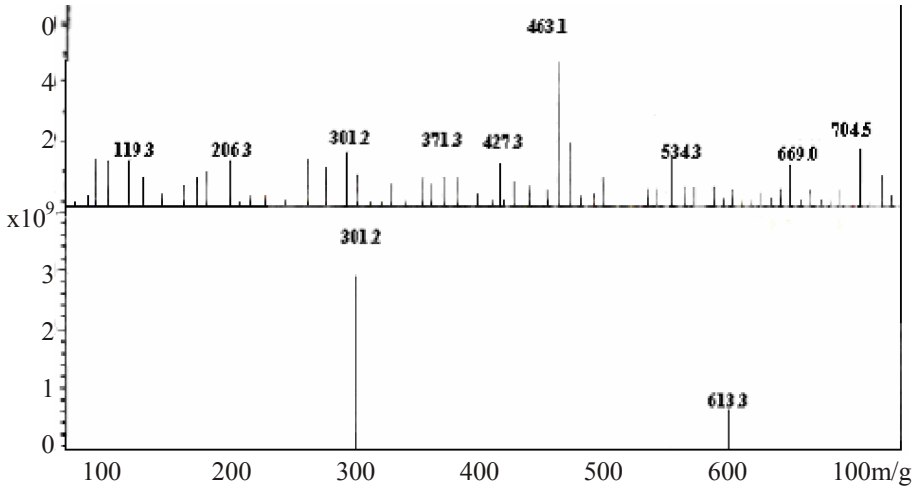

Fig. 3: The mass spectral pattern of the enlarged chromatogram of peonidin at retention time 14.700

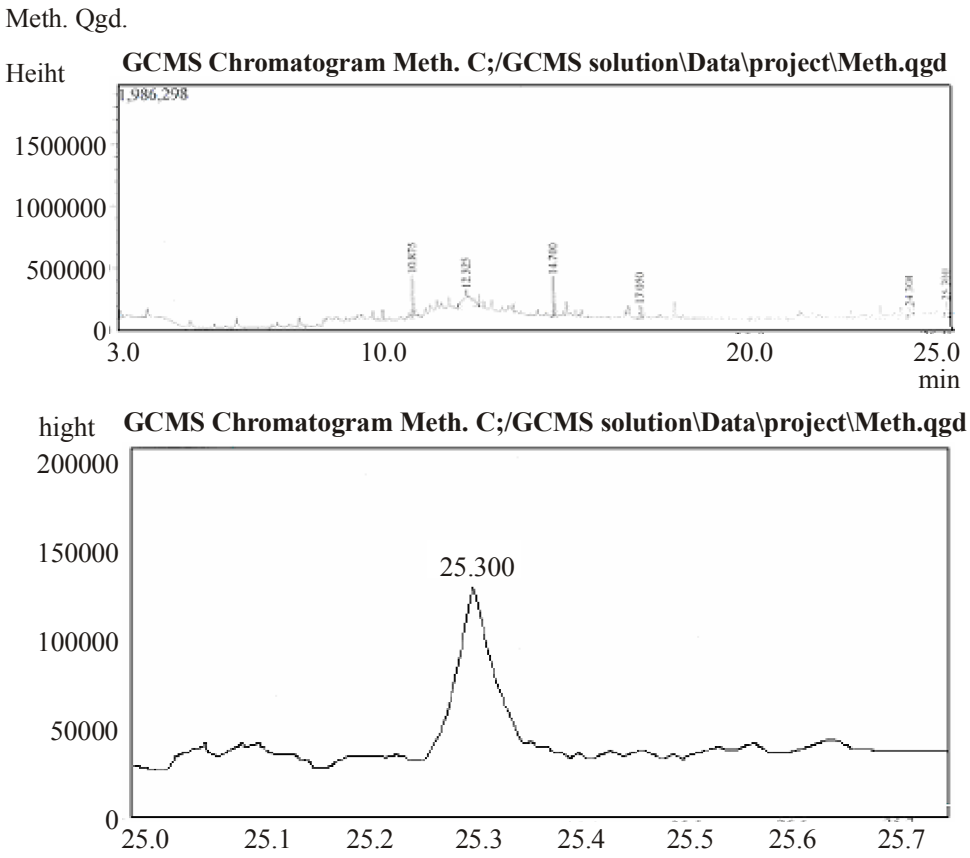

Fig. 4: The enlarged chromatogram of Pelargonidin at retention time 25.300

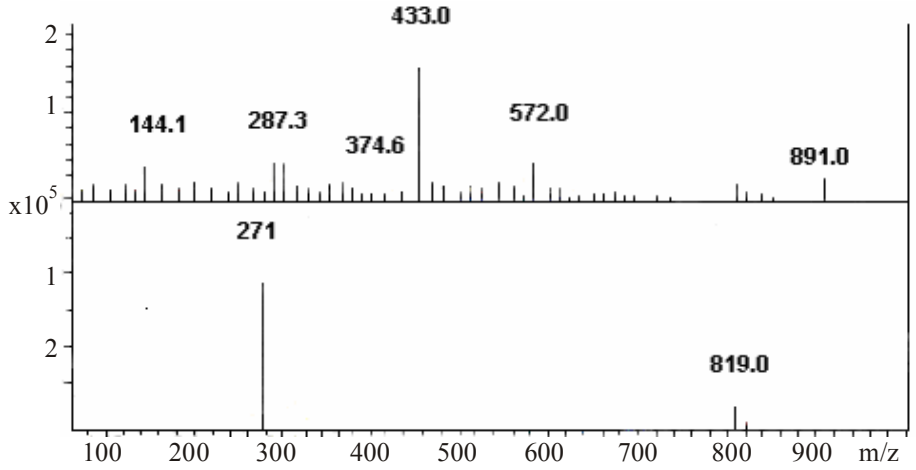

Fig. 5: The mass spectral pattern of the enlarged chromatogram of pelargonidin at retention time 25.300

From GCMS spectral data, an enlarged peak was focused in the chromatogram with the retention time of 14.7 min as shown in the Fig. 2. From mass spectral data (Fig. 3) the m/z ratio is 463.1; its daughter fragment ion had an m/z ratio of 301.2. Comparing the spectra, literature data and the mass spectral pattern, this peak was identified as peonidin-3-glucoside. Similarly, other peak appears with the retention time of 25.300 min as shown in the Fig 4. From the mass spectral data (Fig. 5), it has the m/z ratio of 433 and the daughter fragment ion had an m/z ratio of 271.0. Comparing spectra, literature data and the mass spectral pattern, this peak was identified as pelargonidin-3-glucoside. The chromatogram obtained and the mass spectral data identifies, the two compounds, along with the m/z ratio as peonidin-3-glucoside and pelargonidin-3- glucoside.

Thin layer chromatography

Thin layer chromatography revealed two bands with pink and magenta colours.

Table 3: Chromatographic data of TLC with BAW as a solvent system

S. No	Anthocyanin	R_f value	Colour
1.	Pelargonidin	0.36	Pink
2.	Peonidin	0.38	Magenta

The pink band corresponds to pelargonidin (R_f 0.36) and magenta (purple) band corresponds to peonidin (R_f 0.38). As per the literature data, the R_f values of the bands matches with the standard R_f values of peonidin and pelargonidin in BAW (n-butanol-acetic acid-water) (4 : 1 : 2) solvent system. The chromatographic data and shown in Table 3.

Spectral characteristics and spectral shifts

Table 4: Chromatographic and spectral data of the isolated anthocyanin pigments

Band No.	R_f obtained from TLC	λmax (nm)	$AlCl_3$ shift	$Abs_{\lambda 440}$ $Abs_{\lambda vismax}$	Peak assignment
1	0.38	281; 536	-	22	Peonidin -3-glucoside
2	0.36	263; 520	-	24	Pelargonidin-3-glucoside

Qualitative information was obtained with aid of spectral characteristics of TLC bands, which were scrapped and eluted in methanol. The spectral data presented in Table 4, shows λmax for band 1 (R_f value 0.38) as 281, 536 nm and λmax for band 2 (R_f value 0.36) as 263, 520 nm. These data correspond to the UV spectral data of peonidin and pelargonidin, as notified in the literature. No change in colour was obtained after addition of aluminium chloride in the anthocyanin fractions obtained by TLC. No change of shift (absorption in maxima) was observed for both the fractions corresponding to band 1 and band 2. This is an indication that isolated pigments from band 1 and band 2 with R_f value 0.38 and 0.36 are the ortho-dihydroxy anthocyanins, belonging to the groups of peonidin and pelargonidin[18]. This test enables to make a broad differentiation between the derivatives of cyanidin, petunidin and delphinidin groups, which show a positive colour change and those of pelargonidin and peonidin, which does not show any colour change. These results further support the assignment of the band 1 and band 2, as peonidin and pelargonidin and indicate that the band does not belong to the delphinidin, cyanidin or petunidin groups. The ratio of $Abs_{440}/Abs_{\lambda max}$ calculated for the anthocyanin fractions corresponding to band 1 and band 2 was 22 and 24 respectively. The ratio of absorption at 440 nm to the maximum is useful in distinguishing anthocyanins whether it is glycosilated at the 3rd or 5th position. The $Abs_{440}/Abs_{\lambda max}$ ratio is usually about 22 for peonidin and 24 for pelargonidin indicating the substitution at 3rd position. The $Abs_{440}/Abs_{\lambda max}$ between 10-20 corresponds to 3, 5 diglycosides, between 20-30 corresponds to 3-glycosides, above 30 confirms that the compound is not glycosilated at any of the position[14,16,17]. On this basis, the anthocyanin pigments corresponding to band 1 and band 2, showing $Abs_{440}/Abs_{\lambda max}$ as 22 and 24 were of peonidin and pelargonidin having the glycosidic linkage at 3rd position. Thus, the spectral shifts and spectral data confirm the identity of peonidin-3-glucoside and pelargonidin-3-glucoside.

CONCLUSION

Anthocyanin pigments were identified in fruits of *Ficus racemosa*. GCMS proved to be the most powerful technique and provided molecular weight and mass spectral pattern for structural characterization of anthocyanins. GCMS, UV-Visible spectral analysis, spectral shifts and thin layer chromatography, confirm the identity of the anthocyanin pigments isolated from the *Ficus racemosa* fruit as peonidin-3-glucoside and pelargonidin-3-glucoside.

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