Chapter 2

Analysis of anthocyanin in *Brassica oleracea* L. by Thin layer chromatography, High performance liquid chromatography and Gas chromatography and mass spectroscopy

2.1 INTRODUCTION

Red cabbage (RC; Brassica oleracea L. var. capitata) is a rich and relatively cheap source of anthocyanin then the other source of anthocyanin. Also RC contains higher vitamin C (24.38 mg/100 g), DL-α-tocopherol (0.261 mg/100 g) and phenolic content (101.30 mg/100 g) then white and savoy cabbage. RC anthocyanin are found to be thermostable at < 80ºC; showed less photodegradation quantum at pH-7 and are colored at wide range of pH (pH-3 Pink; pH-5 Violet; pH-7 Blue) than anthocyanin from grape skin, black current and elder berry, thus can be a natural alternative to synthetic hue (Bridle & Timberlake, 1997; Giusti & Wrolstad, 2003; Markakis, 1982; Stintzing & Carle, 2004)

Anthocyanin composition is specific for vegetables and fruits; which are often used as food additives in fruit jams, fruit juice and to detect adulteration in red wine (Stój et al., 2006; Wrolstad et al., 1982; Wrolstad et al., 1995). Chemically, anthocyanin is glycoside of monomeric anthocyanidin viz cyanidin (cn), delphinidin (dp), pelargonidin (pg), malvidin (mv), peonidin (pn), and petunidin (pt); that differ by their degree of hydroxylation and methoxylation. Also, some anthocyanins are found to be acylated with one or more molecules of acyl acids viz sinapyl, p-coumaryl, ferulyl (Andersen & Markham, 2005). However, the qualitative and quantitative chemical analysis of extract can help to determine the anthocyanin content, sources of extraction and method of extraction to improve the purity and stability at industrial level, according to the latest opinion of the European Food Safety Authority (EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS), 2013).

Color characteristic of RC anthocyanin was studied based on visible spectra and molar absorption (ε) which shows the tinctorial strength of anthocyanin at different pH. RC pigment showed high ε and λ_{max} in methanol than buffered solution at pH 1 (Volden et al., 2008). According to the Piccaglia and team, RC is more preferable then berries due to its higher yield, more stable acylated anthocyanins and color (Piccaglia et al., 2002). The concept of determining the amount of anthocyanin present in a extract by measuring the change in absorbance at two different pH values (3.4 and 2.0) was first introduced by Sondheimer & Kertesz, (1948) in 1948. Since then, researchers have been using pH (1.0 and 4.5) differential method to determine the concentration of monomeric anthocyanin using molecular weight of cyanidin-3-glucoide as standard (Fuleki & Francis, 1968; Wrolstad, 2001). Monomeric anthocyanin undergo a reversible structural transformation as a function of pH (colored oxonium form at pH 1.0 and colorless hemiketal form at pH 4.5; Figure 1), thus the difference in absorbance at the $\lambda_{vis\text{-}max}$ 520 nm is proportional to the concentration of pigment. HPLC analysis of RC extract showed the presence of cyanidin-3-diglucoside-5-glucoside and cyanidin-3-glucoside with acetylated group or groups attached to glucoside or diglucoside (Wiczkowski et al., 2013).

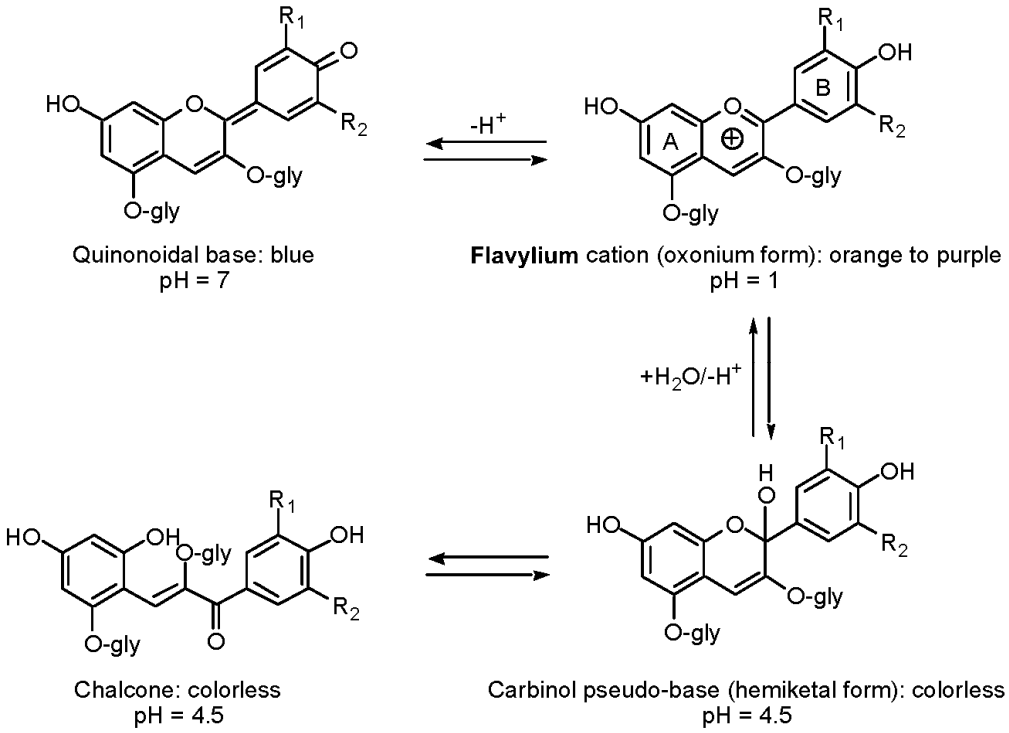

Figure 2.1: Transition in structural forms of anthocyanin at different pH (Lee et al., 2005)**.**

Several chromatographic techniques were used to identify the anthocyanin from different source of functional food. Farina et al., (1995) had evidenced the presence and type of anthocyanin from *Malva Silvestris L.* and showed HPTLC to be more sensitive then HPLC-DAD. Also, 3-glucoside of cyanidin, delphinidin, malvidin and peonidin in powdered berry extracts of bilberry, blueberry, chokeberry, açai berry and cranberry were identified using HPTLC (Cretu & Morlock, 2014). Pelagornidin-3-glucoside and peonidin-3-glucoside were identified from *Ficus racemosa* fruits using GC-MS (Sarpate et al., 2010).

In this study ARCE was characterized with regard to their anthocyanin content using thin layer chromatography (TLC), gas chromatography and mass spectroscopy (GC-MS) and high-performance thin-layer chromatography (HPTLC) methods.

Aim: *To prepare anthocyanin rich red cabbage extract (ARCE) and to characterize anthocyanin by TLC, HPTLC and GC-MS.*

2.2 MATERIALS AND METHODS

Preparation of extract

Red cabbage (*Brassica oleracea* L. var. Capitata f. rubra DC.) was procured from Spencer's mall, Vadodara, Gujarat, India. Sample was identified and authenticated by Dr. Vinay Raole, Department of Botany and voucher specimen (accession no. 213) was submitted to departmental herbarium (BARO), The M. S. University of Baroda, Vadodara, Gujarat. Fresh red cabbage was washed, chopped into small pieces and extracted using methanol:water:HCl (50:50:1) solvent system. Extract was filtered using muslin cloth, dried in rotatory evaporator at 40 °C, cooled at room temperature and stored in 4 °C for further analysis. Total yield was 7.1% W/W.

Characterization of Anthocyanin

pH differential method: Total monomeric anthocyanin content was measured spectrophotometrically using molar extinction coefficient of cyanidin-3-glucoside (26,900 M^{-1} cm^{-1}). Briefly, two dilution of extract was prepared in pH 1.0 (0.025 M KCl; pH adjusted with HCl) and in pH 4.5 (0.4 M Sodium acetate; pH adjusted with HCl) and the absorbance of both dilutions were measured at 520 and 700 nm. pH differential method is a rapid and simple spectrophotometric method based on the anthocyanin structural transformation that occurs with a change in pH (colored at pH 1.0 and colorless at pH 4.5). Anthocyanin content (mg/L) was measured by the formula as given below:

$$\frac{\boldsymbol{A \times Molecular\ weight \times Diliution\ factor \times 10^3}}{\boldsymbol{\varepsilon \times l}}$$

Where, $A = (A_{520nm} - A_{700nm})pH1.0 - (A_{520nm} - A_{700nm})pH4.5$
$\varepsilon = 26{,}900\ L \times mol^{-1} \times cm^{-1}(molar\ extinction\ coeficient)$
$l = Path\ length\ in\ cm$
$Molecualr\ weight = 449.2\ {}^{g}/_{mol}\ for\ Cyanidin-3-glucoside$
$10^3 = factor\ for\ conversion\ from\ g\ to\ mg$

Thin Layer Chromatography of ARCE: 40 mg of ARCE was properly dissolved in 1 ml methanol and 5 µl sample was spotted on TLC plate (Silica gel 60 F_{254}; Merck Millipore, USA). Sample loaded plate was air dried and kept in chamber saturated with ethyl acetate : glacial acetic acid : formic acid : H_2O (10 : 1.1 : 1.1 : 2.6). Later plates were air dried and distance travelled was measured from the origin to calculate Rf value using the formula given below:

$$\boldsymbol{Rf = \frac{Distance\ travelled\ by\ Solute}{Distance\ travelled\ by\ Solvent}}$$

Gas liquid chromatography and mass spectroscopy (GC-MS) of ARCE: GC-MS was performed with crude ARCE or samples separated and isolated from preparative TLC. Preparative TLC was performed to collect more amount of sample from each band using the similar conditions mentioned above. Briefly, methanolic solution of ARCE (40 mg/ml) was subjected to TLC (Silica gel 60 F254) and developed in a pre-saturated chamber of ethyl acetate: glacial acetic acid: formic acid: H_2O (10: 1.1: 1.1: 2.6). Plates were dried at room temperature and bands were scraped using a clean scalpel. The contents were dissolved in 5 ml methanol and filtered (Whatman Filter paper No. 1). Filtrates were dried in rotatory evaporator (40ºC), cooled to room temperature and stored at 4ºC till further analysis. Sample (2.5 mg/ml methanol) was injected through pre-filter unit using Helium gas (99.9% gas carrier) with a flow rate

of 1ml/min. The column (30m PE-5ms) temperature was held at 60ºC for 5min and then increased up to 280ºC at the rate of 10ºC/min. Mass spectra were scanned from 10 to 610 u.

High performance thin layer chromatography of ARCE: Chromatography was performed on analytical HPTLC plates (10 cm x 10 cm) pre-coated with Silica Gel 60 of 0.2 mm thickness (Merck Millipore, USA). 40 mg extract was dissolved in 100% methanol in 10 ml volumetric flask. Samples were filtered (Whatman Filter paper No. 1) and applied on the plates (2, 4 and 6 µl) as 8 mm bands by means of a Linomat V applicator (Camag, Muttenz, Switzerland) and the rate of delivery was 6 s μl^{-1}. Plates were developed for about 8 cm from the baseline (development time: about 30 min) in saturated horizontal developing chambers (Camag, Muttenz, Switzerland) with ethyl acetate : glacial acetic acid : formic acid : H_2O (10 : 1.1 : 1.1 : 2.6 v/v/v/v). Developed plates were air dried and analyzed by the means of a Camag TLC Scanner-III (Muttenz, Switerzland) linked with wincats 4.02 integration software. Densitometry scanning was performed in absorbance/reflectance mode at 520 nm with 2.0 mm s^{-1} scanning speed and slit dimension 1-0.1 mm.

2.3 RESULTS

Total monomeric anthocyanin concentration and TLC analysis of ARCE

Total anthocyanin content in ARCE was found to be 86.004 ± 3.103 mg/100gm. Presence of red to purple color band on TLC Plates confirms the presence of anthocyanins in ARCE samples. TLC of ARCE revealed two bands with Rf value 0.26 and 0.31 respectively. These values were in the Rf value range of 0.2-0.35, which indicates the presence of monoglucosides, delphinidin-3-glucoside and cyanidin-3-glucoside (Figure 2.2).

GC-MS analysis of ARCE

The GC-MS spectra provided information regarding the structural identification of anthocyanin pigments. The m/z ratio of the daughter and parents ions, confirmed the presence of anthocyanin. Analysis of crude extract showed presence of cyanidin-3-glucoside (449 m/z) and delphinidin-3-glucoside (465 m/z) (Figure 2.3; Table 2.1). Whereas, analysis of bands obtained from TLC showed presence of daughter ions of (epi) gallocatechin delphinidin (303 and 481 m/z), (epi) gallocatechin peonidin glucoside (605 m/z), peonidin glucoside (463 m/z) in the first band (Figure 2.4; Table 2.2) and cyanidin (287 m/z), cyanidin-3(6“-acetyl glucoside) (491 m/z), cyanidindioxalyl Glucoside (593 m/z), delphinidin-3(6“-acetyl glucoside) (507 m/z) and delphinidin-3-glucoside (465 m/z) in second band (Figure 2.5; Table 2.3).

HPTLC analysis of ARCE

Rf value of sample dissolved in 100 % methanol recorded two bands. Peak with maximum height was recorded at Rf 0.16 and another at 0.23. Rf value in the range of 0.15-0.2 indicates the presence of delphinidin-3-glucosyl-xyloside and cyanidin-3-

glucosyl-xyloside and in the range of 0.2-0.35 indicates the presence of delphinidin and cyanidin monoglucoside (Figure 2.6; Table 2.4).

2.4 FIGURES

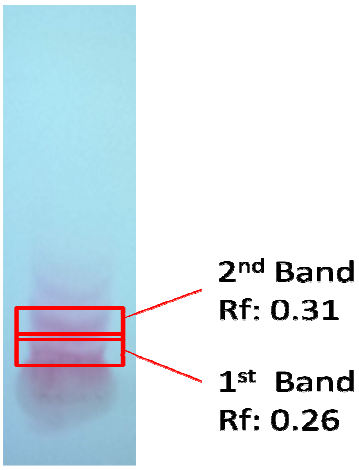

Figure 2.2: TLC chromatogram of ARCE. TLC chromatogram of ARCE separated on aluminum silica gel. Reddish to purple band represents the presence of cyanidin and delphinidin based anthocyanin. (Rf: Retention factor)

A.

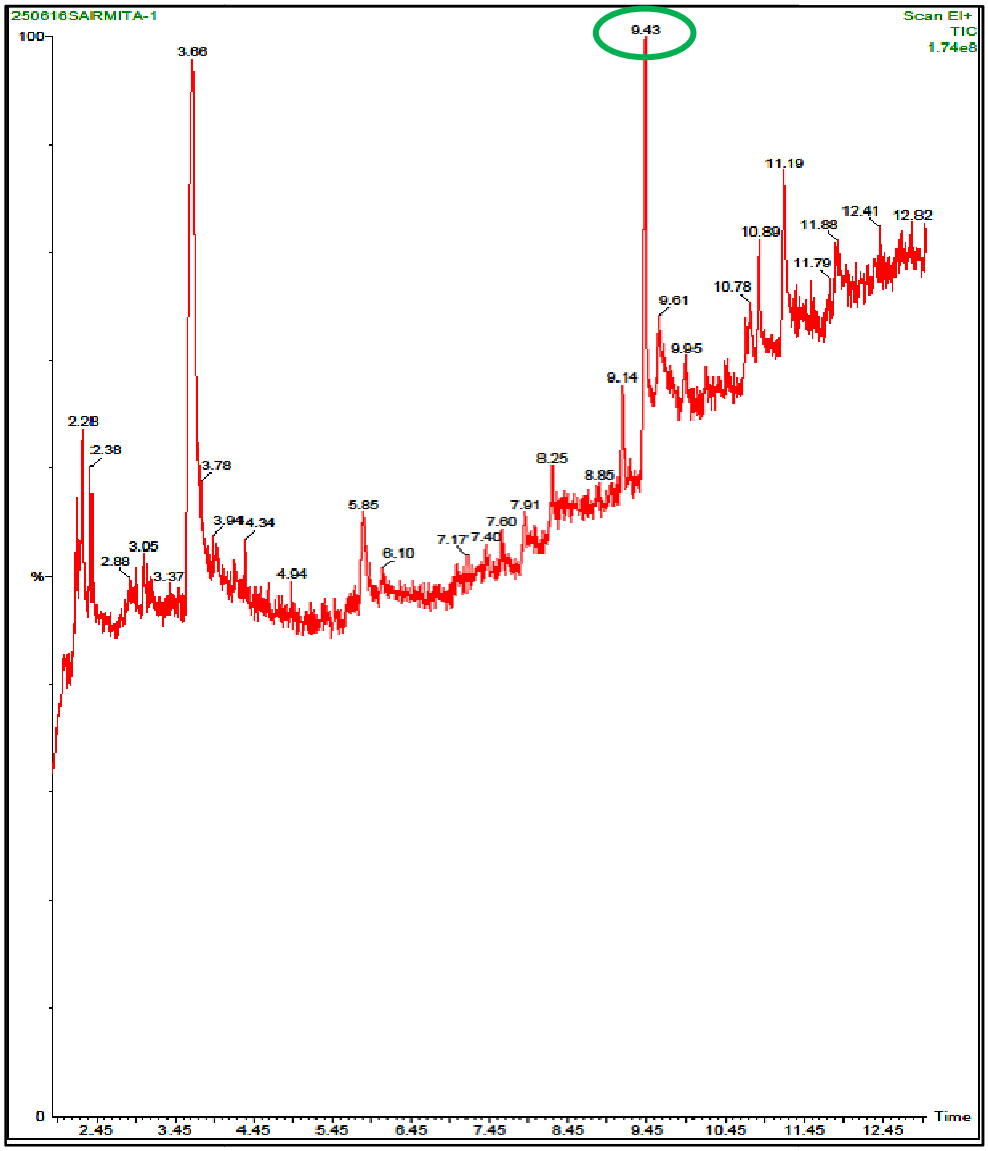

Figure 2.3: GC-MS analysis of crude extract. (A) GC chromatogram – Peak represents the GC profile of crude extract. Peak marked in green were further subjected to characterization by MS.

B.

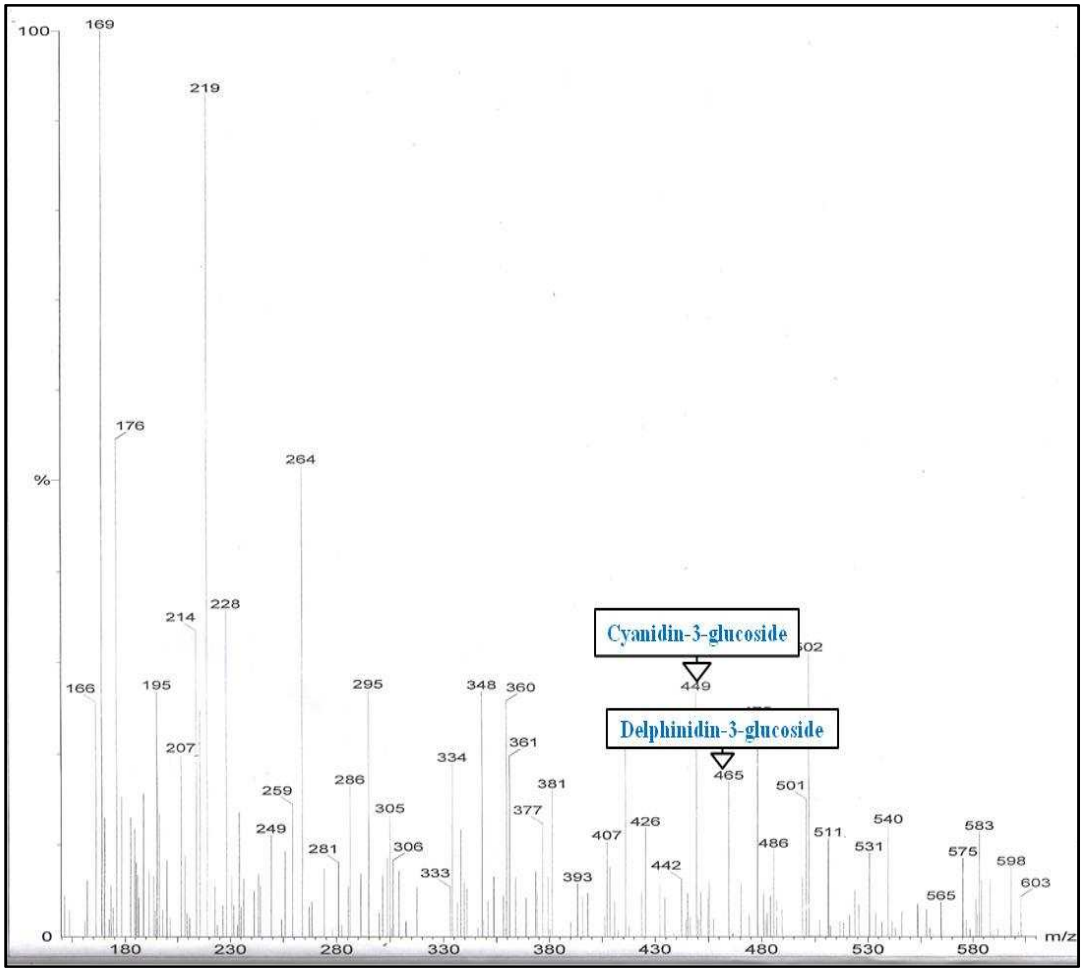

Figure 2.3: GC-MS analysis of crude extract. (B) Mass spectrometry – Spectra represents GC-MS profile of selected peak from the GC chromatogram of crude extract.

Table 2.1: GC-MS profile of crude extract

Peak	**Retention Time**	**Area**	**Height**	**Area %**
1	9.432	2183807.0	47,715,000	12.32
Mass Spectrum Profile				
Peak	**Retention Time**	**Daughter fragment**	**Parent fragment**	**Peak identification**
1	9.432	-	449	Cyanidin-3-glucoside
			465	Delphinidin-3-glucoside

A.

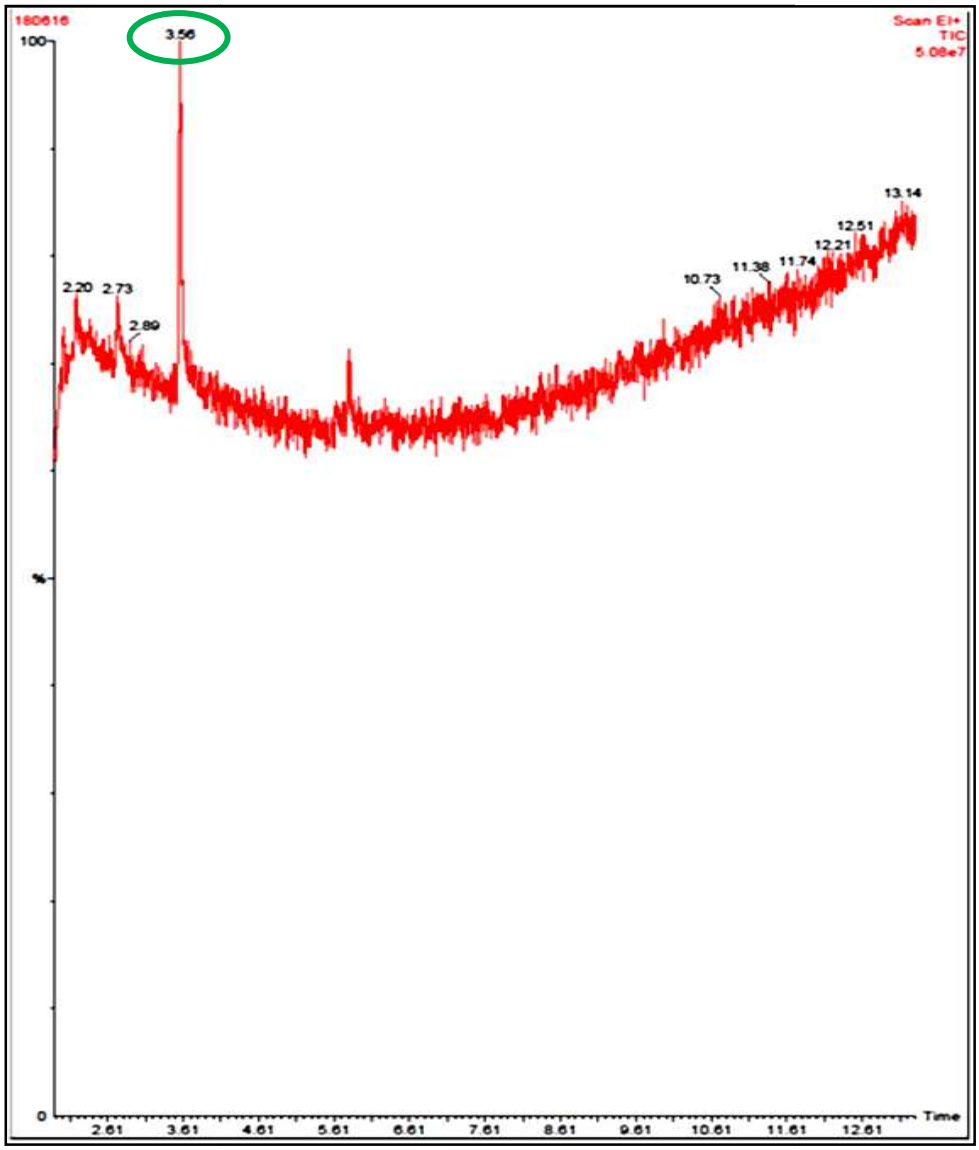

Figure 2.4: GC-MS analysis of 1st band separated on TLC. (A) GC chromatogram – Peak represents the GC profile of 1st band separated on TLC. Peak marked in green were further subjected to characterization by MS.

B.

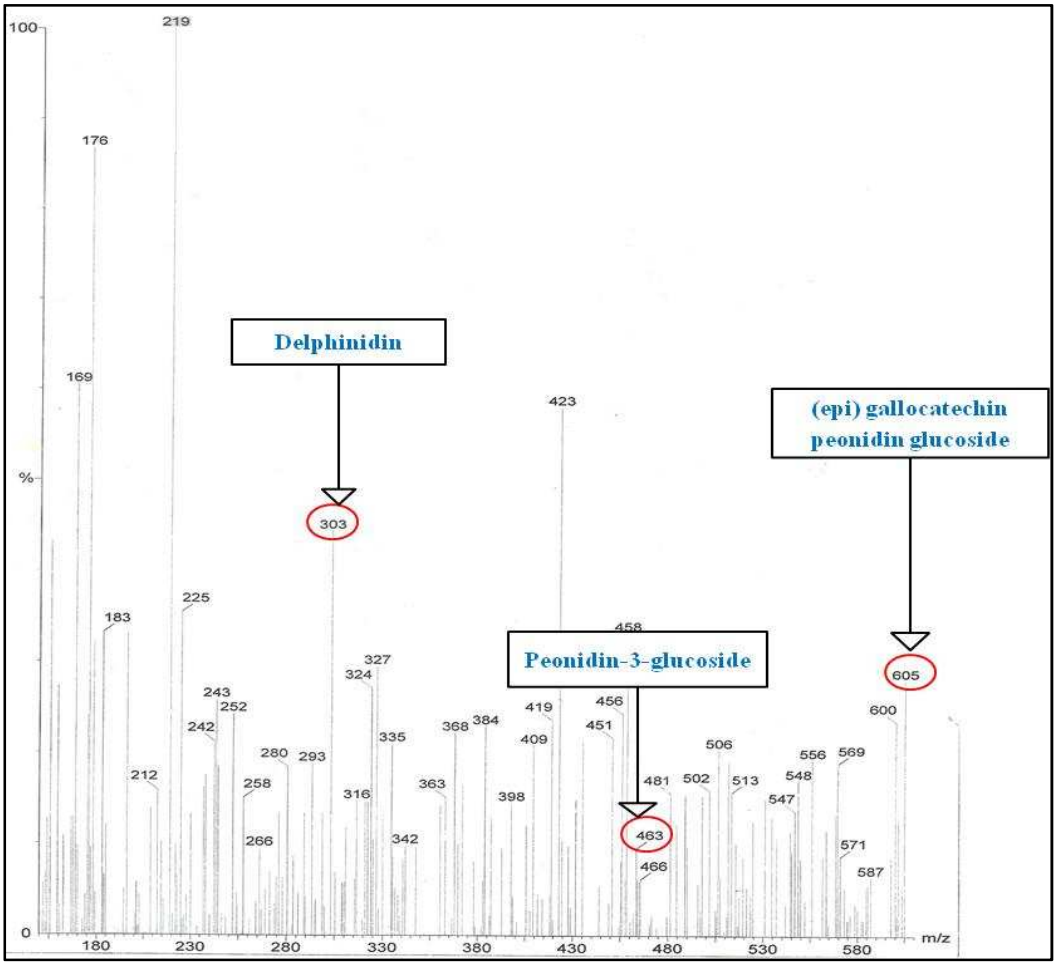

Figure 2.4: GC-MS analysis of 1st band separated on TLC (B) Mass spectrometry – Spectra represents GC-MS profile of selected peak from the GC chromatogram of 1st band.

Table 2.2: GC-MS profile of 1st band

Peak	Retention Time	Area	Height	Area %
1	3.569	706473.9	12,952,837	61.56
Mass spectrum profile				
Peak	**Retention time**	**Daughter fragment**	**Parent fragment**	**Peak identification**
1	3.569	463	605	(epi) gallocatechin peonidin glucoside
2	3.569	481, 303	-	(epi) gallocatechin delphinidin

A.

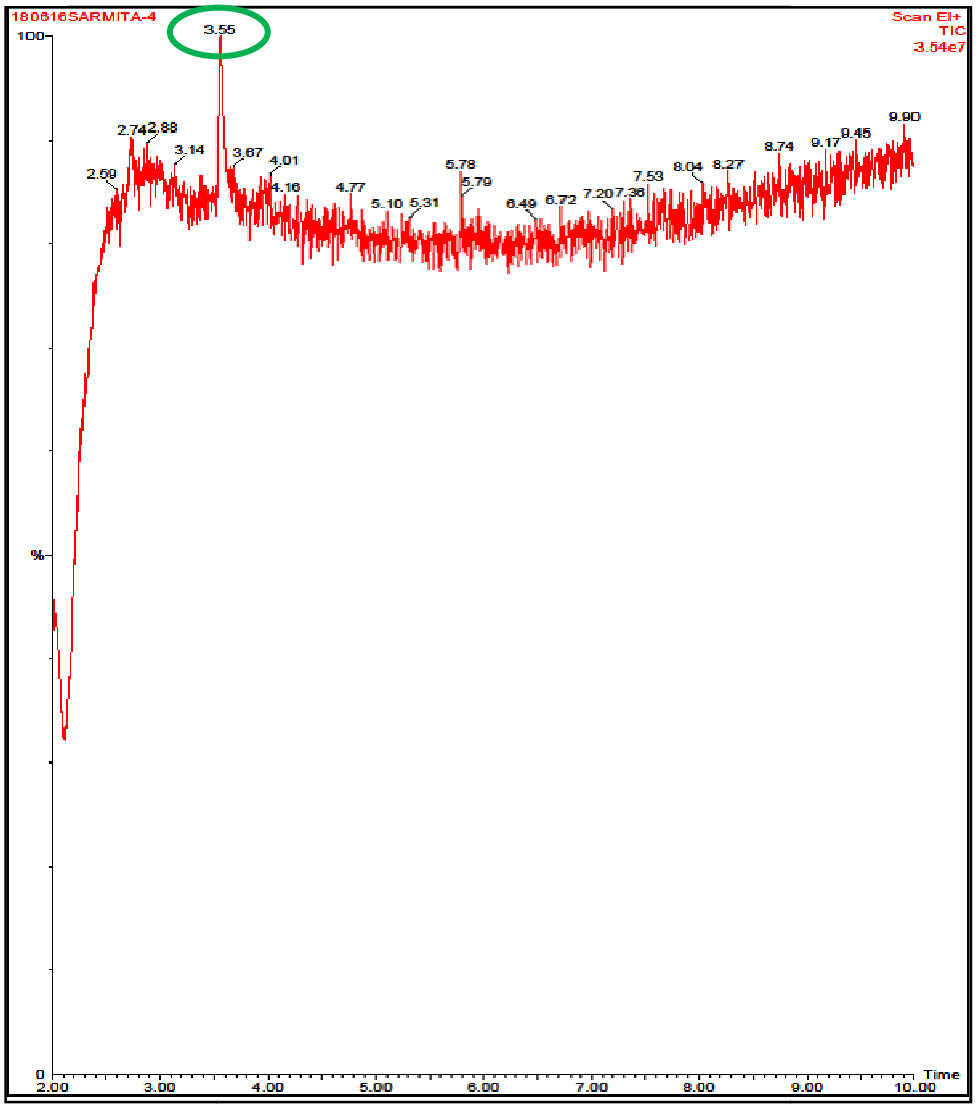

Figure 2.5: GC-MS analysis of 2nd band separated on TLC. (A) Chromatogram – Peak represents the GC profile of 2nd band separated on TLC. Peak marked in green were further subjected to characterization by MS.

B.

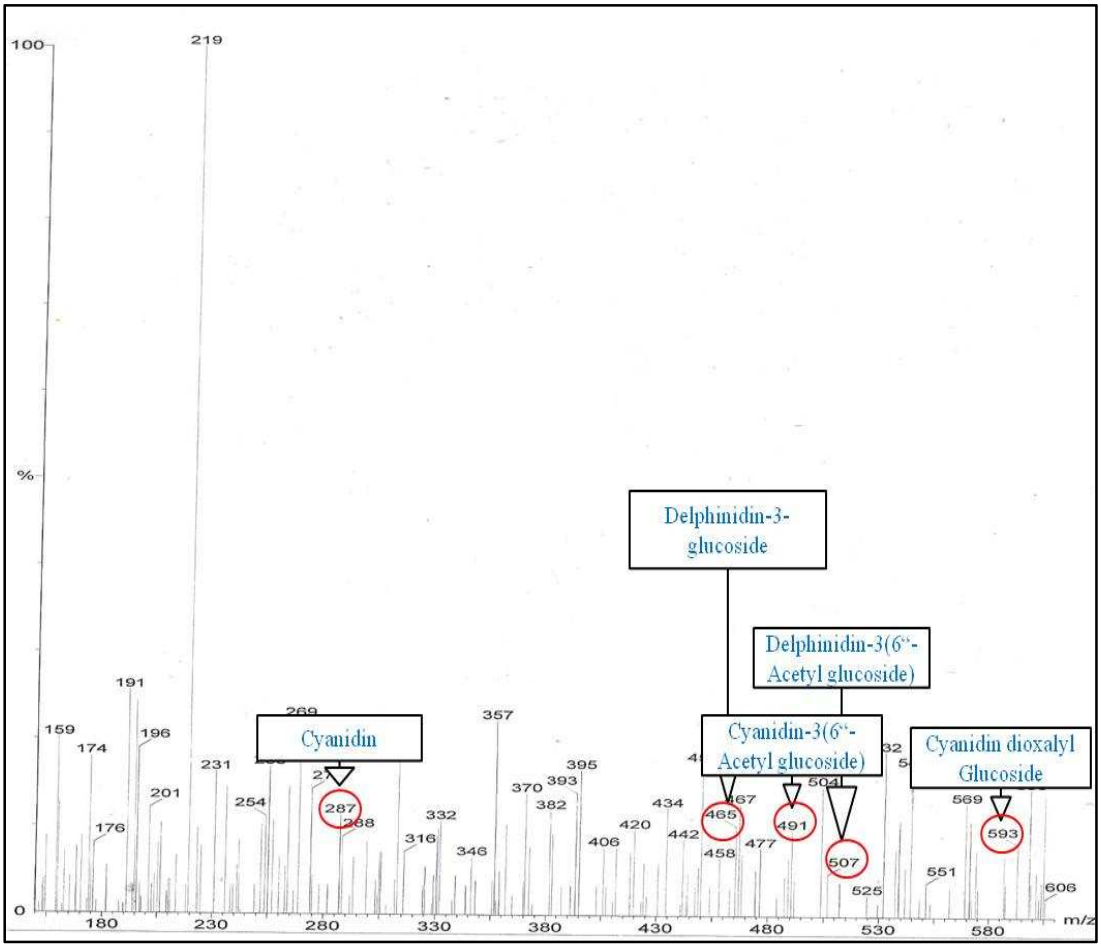

Figure 2.5: GC-MS analysis of 2nd band separated on TLC. (B) Mass spectrometry – Spectra represents GC-MS profile of selected peak from the GC chromatogram of 2nd band.

Table 2.3: GC-MS profile of 2nd band

Peak	**Retention Time**	**Area**	**Height**	**Area %**
1	3.55	475339.5	8,855,368	41.59
Mass spectrum profile				
Peak	**Retention Time**	**Daughter fragment**	**Parent fragment**	**Peak identification**
1	3.55	287	491	Cyanidin-3(6"-acetyl glucoside)
			593	Cyanidin dioxalyl glucoside
2	3.55	465	507	Delphinidin-3(6"-acetyl glucoside)

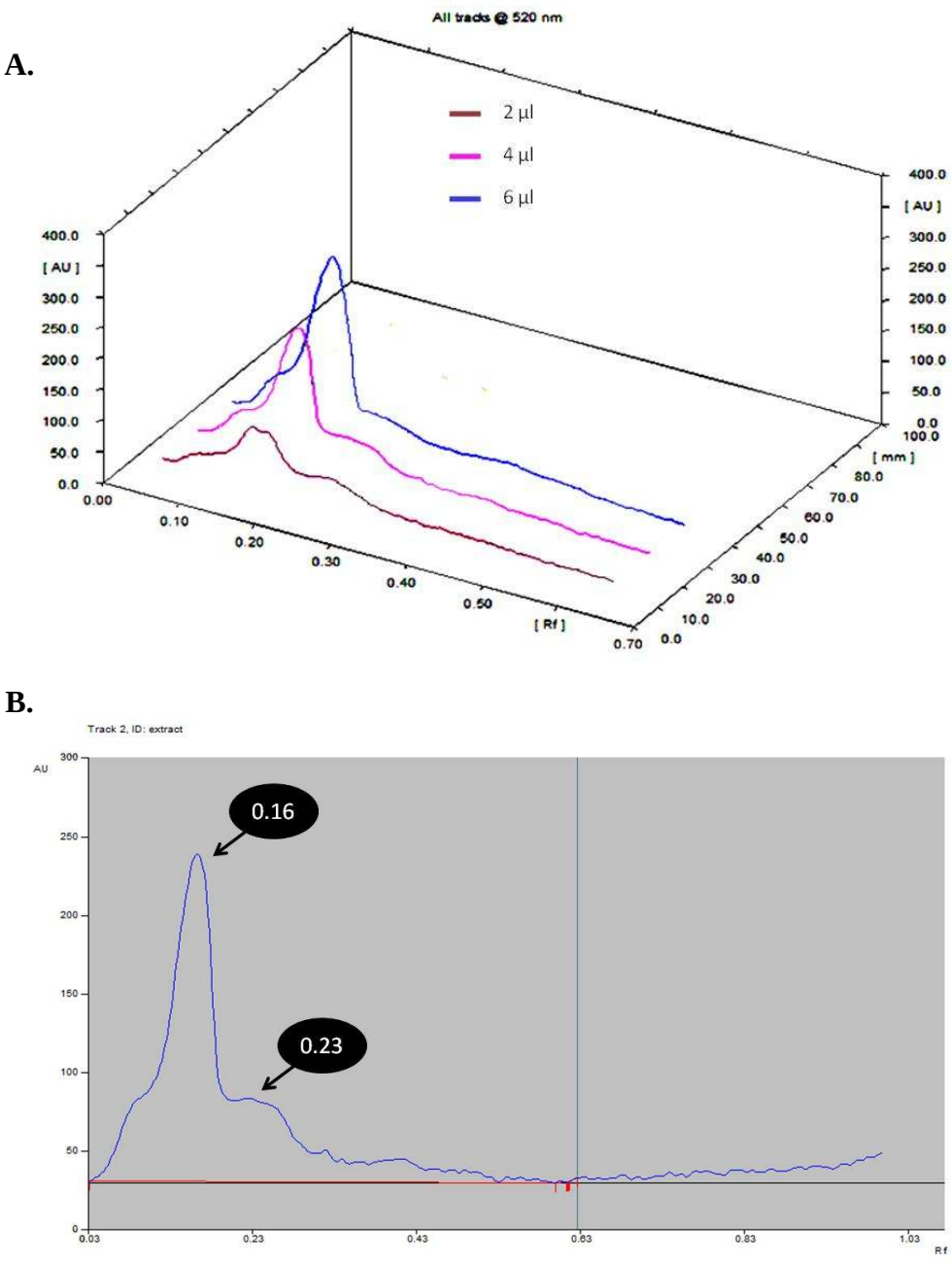

Figure 2.6: HPTLC chromatogram of ARCE. (A) Peak represents chromatogram of 2 µl, 4 µl and 6 µl of ARCE loaded on 10X10 cm aluminum silica gel. **(B)** Peak represents the chromatogram of 4 µl of ARCE. Peaks are marked with respective Rf.

Table 2.4: HPTLC profile of ARCE

Peak	Retention factor	Area	Height	Area %
1	0.16	9664.1	208.3	79.04
2	0.23	2562.6	53.1	20.96

2.4 DISCUSSION

Red cabbage has been extensively investigated for its nutrient composition, bioavailibility and its therapeutics. Previous reports on RC had shown to be rich in color which can be used for the coloration of soft drinks due to excellent thermal stability at < 80ºC, low sensitivity to photodegradation and broad range of colors at different pH. Thermal stability of RC anthocyanin is attributed to the formation of sandwich like stacking complexes between flavylium system, the π-electron rich co-pigment moieties and glucose moeities. Also, low sensitivity of photodegradation are due to aromatic acids attached to the anthocyanin which absorbs 313 nm light and provide filter like property to the anthocyanin (Dyrby et al., 2001).

In the present study, RC anthocyanin was analyzed using pH differential method, TLC, HPTLC and GC-MS. pH differential method has been extensively used by food technologists and horticulturists to assess the concentration of anthocyanin in fresh or processed fruits and vegetables and for the quality control of anthocyanin-containing fruit juices, wines, natural colorants, and other beverages (Lee et al., 2005). Study performed by Chandrasekhar et al. (2012) to determine the best solvent for isolating high concentration of anthocyanin showed that total anthocyanin concentration calculated using pH differential method was more in acetone or acidified methanol extraction or acidified ethanol extraction then other solvent. Cruz et al. (2016) had shown that water extract of RC contains more phenolic content, flavonoids and antioxidant activity compared to hydromethanolic extract, wherein acidified hydromethanol was not involved. This explains the role of acidified hydromethanol for the extraction of anthocyanin rich extract. In our study, pH differential method recorded 86 mg/100 gm monomeric anthocyanin in ARCE using

molar extinction coefficient of cyanidin-3-glucoside. Change in color was observed in dilutions at both the pH representing the presence of stable anhtocyanins as degraded anthocyanin are resistant to color change with change in pH. Similarly, McDougall et al. (2007), Gachovska et al. (2010), Sankhari et al. (2012), Podsędek et al. (2014) and Tong et al. (2017) had obtained 25 to 495 mg/g FW of total anthocyanin, wherein variation in anthocyanin concentration is attributable to type of cultivar selected and method of extraction.

The Rf value obtained by us using TLC was in range of 0.2-0.35, which according to Wagner et al. (1996) is due to presence of cyanidin and delphinidin monoglucosides. While in HPTLC chromatogram major peak were in the range of Rf 0.15-0.2, showing the presence of delphinidin-3-glucosyl-xyloside and cyanidin-3-glucosyl-xyloside and minor peak in the range of Rf 0.2-0.35. Among the two peaks in HPTLC ARCE maximum height was obtained at Rf 0.16 with small peak at Rf 0.23 and similar pattern was observed for all three results. Also, GC-MS analysis of ARCE evidenced the presence of cyanidin-3-glucoside and delphinidin-3-glucoside in crude extract of ARCE. While GC-MS analysis of 1st and 2nd bands scraped from TLC plate showed the presence of (epi) gallocatechin delphinidin, cyanidin-3(6”-acetyl glucoside), cyanidin dioxalyl glucoside and delphinidin-3(6”-acetyl glucoside). Similar studies with RC anthocyanins by Tanchev & Timberlake (1969), Dyrby et al. (2001), Charron et al. (2007) and Wiczkowski et al. (2013) had shown the presence of only cyanidin based anthocyanins which are acylated with one or two equivalent sinapic acids. In our study, presence of delphinidin-3-glucoside along with cyanidin-3-glucoside was observed.

Our study shows the presence of cyanidin and delphinidin derivatives in ARCE. Based on the results obtained in phytochemical analysis of ARCE, the next study was planned to investigate *in vitro* potential of ARCE as a cardioprotective agent.

2.5 SUMMARY

This study evidenced that ARCE is a rich source of cyanidin-3-glucoside and delphinidin-3-glucoside. Presence of bands at Rf 0.15-0.2 and 0.2-0.35 on HPTLC and TLC is showing the presence of cyanidin-3-glucosyl-xyloside, delphinidin-3-glucosyl-xyloside, cyanidin and delphinidin monoglucosides. Mass spectra obtained by GC-MS confirmed the presence of delphinidin and cyanidin monoglusides in ARCE. Also, GC-MS of bands scraped from TLC showed the presence for derivatives of cyanidin and delphinidin monoglucosides. This study validates the phytochemical composition of the ARCE prepared in our lab via GC-MS and HPTLC methods.