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Isolation and Identification of Anthocyanins in the Fruit Peels of Starkrimson and Marx Red Bartlett Common Pear Cultivars and Their Bud Mutants

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Abstract: Anthocyanins and anthocyanidins were determined in 3 varieties of common pears (*Pyrus communis* L.), Starkrimson, Clapps Favourite and Marx red Bartlett. The pigments were extracted from the fruit peel with 0.5% HCl in methanol and the extracts were hydrolysed and then applied on 3 mm cellulose paper chromatograms. The chromatograms were developed subsequently in one direction, using formic acid, hydrochloric acid, water (5:2:3) v/v), or n-Butanol-Acetic acid-Water (BAW) (4:1:5 v/v). There was only one anthocyanidin present, namely cyanidin (Cy), which was identified according to their R_f values, UV-vis and IT-IR spectrum. The results show that the analysed common pear varieties contained cyanidin rhaminosyl, Starkrimson mutant and Starkrimson wild type and cyanidin xylosyl, Clapps Favourite and Marx Red Bartlett. R_f values and spectral characteristics that were determined were compared to standards. The results obtained justify the conclusion that the compound responsible for the red pigmentation in pear fruit of the varieties analysed is a cyandin-glycoside.

Key words: Starkrimson, Clapps favourite, Marx red Bartlett, anthocyanins, anthocyanidins, IT-IR spectrum, UV-Vis spectrum

INTRODUCTION

Anthocyanins are the largest group of water-soluble pigments in the plant kingdom (Piero *et al*., 2005; Hamauzu, 2006). They are responsible for most of the red, blue and purple colours of fruits, vegetables, flowers and other plant tissues or products (Harborne, 1988; Vendramini and Trugo, 2004).

The analysis of anthocyanins is complex as a result of their ability to undergo structural transformations and complexation reactions. In addition, they are difficult to measure independently of other flavonoids, as they have similar reactivity characteristics. Anthocyanins are generally extracted with weakly acidified alcohol-based solvents, followed by concentration (under vacuum) and purification of pigments (Harborne, 1988).

Paper and/or thin-layer chromatography and UV-vis spectroscopy have traditionally been used for the identification of anthocyanins. Capillary zone electrophoresis, a hybrid of chromatography and electrophoresis, is gaining popularity for the analysis of anthocyanins; however, Liquid Chromatography (LC) has become the standard method for identification and separation in most laboratories and may be used for both preparative and quantitative analysis. LC with Mass Spectrometry (MS) and Nuclear Magnetic Resonance (NMR) spectroscopy are possibly the most powerful methods for the structural elucidation of anthocyanins available, to date. At present the most

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satisfactory method for mixture analysis is the multistep method of separation, isolation and quantification by LC with peak identification by MS and high field NMR (Mazza *et al*., 2004; Mozetic *et al*., 2002). In this study paper and liquid chromatography, infrared and UV-vis spectroscopy were used to isolate and identify the anthocyanins/anthocyanidin in the pear fruit peel methanolic extracts.

The anthocyanins profile of fruits represents a very high quality marker which has been used successfully in the characterization of fruits (Vendramini and Trugo, 2004). The regulation of their biosynthesis in pears, however, appears as an unexplored research field. Colour differences were noted in 2004-2005 season, on the fruits of Starkrimson (red) and Marx red Bartlett (red) common pear trees at Nyanga Experiment Station. New green coloured pear bud mutants were identified on these trees. Starkrimson mutant had a red blush similar to Clapps favourite, a wild type of Starkrimson, as shown in Fig. 1.

The Starkrimson cultivar is a bud mutant of Clapps favourite which was discovered in 1948. There has not been any report of mutation on Starkrimson in Zimbabwe. The Starkrimson cultivar could be regarded as a stable cultivar, considering that it has been in cultivation for so long without mutating.

Mutants observed on Marx red Bartlett wild type had green colouring as shown in Fig. 2.

The red fruits in Fig. 2 are wild type Marx Red Bartlett fruits, whilst the green fruits with red blush are mutants. In the green fruits there seems to be a suppression of the expression of the substances responsible for colour. Only surfaces exposed to light showed red blush colouring in the

Fig. 1: Chimeric Starkrimson cultivar with red-greenish fruits from mutated branch

Fig. 2: Chimeric Marx Red Bartlett fruit tree with green fruits from the mutant branch

green fruits. The red fruits, however, were not affected by light as the colour expression is even. Two mutants were observed on the Marx Red Bartlett tree, mutant A was higher up the tree canopy than mutant B. The different colour types of the same plant fruit may be compared and the biochemical effects of the genes controlling anthocyanin synthesis noted.

The overall objective was to fingerprint the bud mutants using the anthocyanin profile for proper referencing of the germplasm. The specific objectives of this study were to isolate and identify the anthocyanin molecules responsible for colouring in the fruit peels of the wild types and their bud mutants.

MATERIALS AND METHODS

Pear Samples

The pears investigated in the study are described in Table 1. The fruit samples were harvested ripe from Rhodes Inyanga Experiment Station, February 2006. The collected fruits samples were stored at -20°C and analysed at the University of Zimbabwe's Department of Biochemistry, in February to August 2006. Peel and pulp portions of the fruit were used as fresh samples.

Chemicals

Polyoxyethylene sorbitan monopalmitate (Tween 80); Ethanol; lead acetate-water (1:10,v/v), pyridine, 4% diphenylamine in acetone, 4% aniline in acetone, 80% ortho-phosphoric acid, ethyl acetate, monohydrate manganese sulphate, methanol-water (1:1, v/v), butanol, acetic acid, acetic acid, acetonitrile, standards: glucose, fructose, mannose, glucose, xylose, rhamnose, arabinose, maltose, (Sigma H 5882: Sigma Co., St Louis, 2004). All the other solvents/chemicals used were of analytical grade and purchased from Sigma Aldrich.

Extraction and Hydrolysis for HPLC

Total phenolic compounds were extracted from the peel. The peel sample (2 g) was sornicated for 10 min and hydrolysed with 2 M HCl in boiling water for: 0, 30 and 60 min followed by extraction with 100% methanol containing 0.5% HCl. The hydrolysates were centrifuged at 3000 rpm for 10 min. The supernatant was filtered through filter and concentrated in vacuum then transferred into sample bottles for analysis.

Determination of UV-Visible Spectrum of Pigments

The spectrum of the pigments in the extracts was determined using a Shimadzu UV-1601, UV-Visible spectrometer. Samples (20 μL) were diluted with 3 mL (0.5% HCl in 100% methanol) and placed in quartz cuvettes providing a one centimetre diameter. Readings were taken after blanking the machine with 100% methanol containing 0.5% HCl.

After the first reading, three drops of 5% $AlCl_3$ were added to the mixture and mixed. The shift in spectrum was then determined by measuring absorbance in the UV-visible spectrometer.

Table 1: Pears investigated in the study

Origin	Cultivar	Fruit colour	Average fruit mass (g)
South Africa	Starkrimson	Deep red	268
NES, Zimbabwe	Starkrimson mutant	Green with red blush	270
South Africa	Clapp's Favourite	Green with red blush	265
South Africa	Marx red Bartlett	Light red	272
NES, Zimbabwe	Marx red Bartlett mutant A	Green with red blush	204
NES, Zimbabwe	Marx red Bartlett mutant B	Green with red blush	323

Analysis for Sugars

Thin layer chromatography was used for qualitative analysis of sugars in the extracts. Silica gel plates were used as immobile phase. Aliquots (5 μL) of the sample solutions were applied 2 cm from the bottom of the TLC plates with 10 μL-pipette. The sizes of the spots were kept small by adding very small amounts at a time The TLC plates were developed in a tank containing the solvent mixture consisting of, Ethyl acetate: Pyridine: H_2O (100:35:25). After developing the plates were dried and then sprayed with a solution consisting of, 4% diphenylamine in acetone: 4% aniline in acetone: 80% ortho-phosphoric acid (25:25:5, v/v). The plates where then dried at 110°C for 10 min. The sugars were identified against standards that were prepared by dissolving standard powder samples (0.02 g) in distilled water (10 mL).

Separation of Pigments with Paper Chromatography (PC)

Paper chromatography was used for the separation of pigments in methanol extracts of pear peels. The extracts were concentrated in vacuum and spotted on 3 mm cellulose paper 2 cm from the bottom and 2 cm apart. The chromatograms were then developed in BAW (4:5:1, n-butanol: Acetic acid: H_2O) in saturated chromatography tanks. A formic acid solvent system (Conc. HCl: formic acid: H_2O) was also used to separate the pigments. The chromatograms were then allowed to dry in the dark and the R_f values were calculated. These values were then compared to the standards (Harborne, 1988).

Recovering Pigments from PC Bands

Visible bands were cut off from the chromatogram and eluted with 100% methanol with 0.5% HCl. The spectra of the eluted pigments were determined using UV-visible spectrometer and/or further separated with HPLC. The prominent peaks from the HPLC separation were collected into epperndoff tubes by timing retention times and their spectrum determined using UV-visible spectrometer.

Infrared Spectrum Determination of Pear Peel Red Pigments

Bond type and functional groups constituting the red pigments were determined using an infrared spectrophotometer. Red PC bands were eluted and concentrated in vacuum to dryness. The pigments were used to coat sodium chloride discs under infrared lamp to drive water away. The disc was immediately mounted in to the spectrophotometer and the spectrum taken. The positions of the different bond stretching and vibration were identified by comparison to standard spectra (Williams and Fleming, 1980).

RESULTS

Tests for Anthocyanins

Pear peel extracts (100% methanol with 0.5% HCl) where tested for the presence of Athocyanins by reacting with sodium hydroxide. A blue-green colour indicates the presence of Athocyanins in the extracts. The concentration of the anthocyanins was indicated by the intensity of the blue-green colour, which was then measured at 550 nm wavelength using a Spectronic 20 Genesys spectrophotometer (Table 2).

The highest amount of anthocyanins was obtained in Starkrimson peel while its mutant was the least. The intensity of the blue-green color showed correlation with fruit color, thus the redder the peel the higher the amount of anthocyanins present.

Table 3 shows Marx red Bartlett fruit peel to be having the highest amount of anthocyanins than its mutants A and B. The differences concur with the colour differences of the fruit peels. For example, Marx red Bartlett has a red peel compared to the green colour of the mutants. The peel colour is correlated to the amount of anthocyanins in the peel.

Table 2: Effects of sodium hydroxide treatment of the 100% methanol-0.5% HCl peel extracts of the pear cultivars on the color of the extracts

Cultivars	Color of extract	Absorbance (550 nm)	Fruit color
Starkrimson peel	Blue-green	0.534±0.003	Red
Starkrimson mutant peel	Blue-yellowish green	0.020±0.002	Green with slight red blush
Clapps favorite peel	Blue-greenish	0.120±0.001	Green with red blush

Table 3: Effects of sodium hydroxide treatment of the 100% methanol-0.5% HCl peel extracts of the pear cultivars on the color of the extracts

Cultivars	Extract color	Visual score of change in color	Fruit color
Marx red Bartlett	Blue-green	0.398±0.001	Red
Mutant A	Light blue-green	0.059±0.001	Green
Mutant B	Light blue-green	0.063±0.003	Green

Determination of Sugar Moieties of Hydrolysed Glycosides

There are two types of glycosides found in flavonoids, O and C-glycosides. O-glycosides are easily hydrolysed into sugar moieties and aglycone molecules using acids. The sugar moieties constituting the anthocyanins of the respective genotypes were acid hydrolysed from the aglycones and detected using silica gel thin layer chromatography columns. The sample sugars were detected by comparing the R_f values of the sample-extracts with those of the standard sugars.

Two simple sugars were identified as possible components of the hydrolysed glycosides, as shown in Table 4. The hydrolysis of wild type Starkrimson and its mutant extracts yielded rhamnose sugars. Clapps favorite, wild type Marx red Bartlett and its mutants extracts yielded xylose. Mutation of wild type Marx red Bartlett and wild type Starkrimson seems not to have changed the composition of the glycosides since the composition remained the same across the mutants. However, the mutation of Clapps favorite which gave rise to wild type Starkrimson resulted in the concomitant change in glycoside composition as observed by the change from xylose to rhamnose in wild type Starkrimson.

Paper Chromatographic Separation of Pear Peel Extracts

The cellulose molecule constituting the chromatographic paper has hydroxyl groups, which form intermolecular forces with compounds spotted onto the paper. Their polarity determines the rate and extent of migration on the paper. The two solvents used in this study are BAW and Formic acid. More polar molecules will migrate further in Formic acid and less in BAW; the reverse is true for less polar molecules in both solvents, as shown in Table 5. Unhydrolysed extracts have anthocyanins, however, when these are acid hydrolysed the sugar moieties are removed, leaving a less polar aglycone (anthocyanidin). Thus, attached sugar moiety increases the polarity of the anthocyanin molecule, hence its migration in formic acid. In BAW more polar molecules are bound to the cellulose molecules firmer, reducing their mobility.

In BAW solvent, acid hydrolysis of the extract produced spots migrating closely with the solvent front compared to unhydrolysed. These fast migrating spots were red coloured and thought to be cyanidins. In a formic acid system the hydrolysed samples produced spots migrating slower than the unhydrolysed sample spots. Formic acid solvent system is a more polar system than the BAW system, as such; less polar pigments will travel further in BAW than in formic acid. There was only one pigment fraction formed on the chromatogram from the unhydrolysed samples. This suggests the existence of only one pigment in the extracts which is responsible for the observed colour in the fruits.

The R_f values of the pigments from the respective fruits peel extracts, corresponds to cyanidin-3-glycoside ($R_f = 0.32$) in BAW and ($R_f = 0.23$) in formic acid corresponding to cyanidin (Harborne, 1988). Visible colour of the pigments (red) corresponds to the protonatted flavilium ion of cyanidin (Harborne, 1988).

The presence of a second band at an R_f value of 0.34 in 30 and 60 min hydrolysed extracts of Starkrimson could be attributed to the high concentration of anthocyanin in the extract compared to

Table 4: Thin Layer Chromatograph (TLC) for simple sugars from polyphenolic glycosides hydrolysed with 2 M HCl for 30 min at 100°C

Fruit sample	R_f
Mrb Mb peel	0.645±0.004
Mrb Mb pulp	0.645±0.003
Mrb Ma peel	0.648±0.006
Mrb Ma pulp	0.649±0.006
Mrb Normal peel	0.650±0.005
Mrb Normal pulp	0.651±0.002
Clapp's peel	0.647±0.003
Clapp's pulp	0.647±0.002
Stark peel	0.806±0.005
Stark pulp	0.806±0.001
Stark mutant peel	0.806±0.004
Stark mutant pulp	0.806±0.002
Galactose	0.187±0.002
Maltose	0.103±0.002
Rhamnose	0.806±0.005
Arabinose	0.426±0.003
Glucose	0.239±0.004
Xylose	0.645±0.001
Fructose	0.361±0.005
Mannose	0.335±0.003

Table 5: Chromatographic characteristics of anthocyanins from common pear fruits peels

		Chromatography R_f values				
		BAW		Formic acid		
Source of pigment	Band No.	Unhydrolysed	Hydrolysed	Unhydrolysed	Hydrolysed	Visual colour
Starkrimson peel	1	0.32	0.33	0.54	0.23	Red
	2		0.90			Red
Clapps Favourite peel	1	0.32	0.94	0.55	0.23	Red
	2		0.93			Red
Marx Red Bartlett peel	1	0.30	0.91	0.56	0.23	Red
	2		0.90			Red
Del-3-glu		0.24				Purple
Cya-3-glu		0.32				Red

BAW = n Butanol-Acetic acid-water (4:1:5), Formic = Concentrated Hydrochloric acid-formic acid-water (2:5:3), Del-3-glu = Delphinidin-3-glucoside, Cya-3-glu = Cyanidin-3-glucoside

Clapp's favourite and Marx red Bartlett, such that some anthocyanins were not hydrolysed. Hydrolysates of Clapps favourite and Marx red Bartlett showed complete hydrolysis as indicated by the absence of the second band.

It can be noted in the formic acid chromatogram that there is a band migrating almost with the solvent front, with an R_f value of 0.94 in all the hydrolysates. The band is not observed in unhydrolysed extracts, suggesting that the compound responsible for the band exist naturally as a conjugate of the anthocyanidin.

Spectrophotometric Analysis of Pear Peel Extracts

Phenolic compounds absorb light at different wavelengths depending on the properties of the molecules and the moieties constituting the compounds. Thus it is possible to differentiate compounds using spectrophotometry.

Methanolic extracts from peels of six genotypes were subjected to spectral analysis in the wavelength range of 700 to 200 nm peaks in the range of 661 to 654 nm correspond to chlorophyll, while peaks in the 545 to 500 nm range correspond to flavonoids.

Table 6 shows that 50% methanolic extracts did not produce peaks in the 545 to 500 nm range for all the genotypes. There were peaks in the 545 to 500 nm range in Skw, Skm, CF and MrbW upon

Table 6: Spectral characteristics of anthocyanins from common pear fruits peels

		Spectral data (λmax nm)							
		Methanol (+0.5% HCl)			Methanol (+0.01% HCl)			(e)	
Peel extract	HPLC peak	50% methanol	50% methanol (+0.5% HCl)	100% methanol (+0.5% HCl)	100% methanol (+0.5% HCl)	**100% methanol (+2M HCl)	PC band	Colour shift with $AlCl_3$	Inference
Skw peel	522, 275, 208	661, 326, 283, 208	657, 522, 329, 283	654, 528, 419, 328, 272, 238	654, 527, 327, 283, 214	532, 293, 225	656, 530, 419, 210	546	Cyanidin-glycoside
Skm	356, 216	656, 336, 215	655, 513, 340, 215	654, 527, 340, 214			528, 352, 217		Cyanidin-glycoside
CF peel	656, 520, 350, 224	336, 238	656, 521, 337, 268	654, 528, 419, 335, 270, 217	654, 526, 372, 296, 227	532, 290, 214	528, 278, 218	546	Cyanidin-glycoside
Mrbw peel	526, 338, 223	660, 326, 283, 215	519, 327, 283, 217	654, 524, 419, 330	519, 329, 284, 222	288, 213	526, 280, 225	533	Cyanidin-glycoside
Mma peel	495, 216	327, 282, 215	329, 282, 211	655, 330, 283, 225			(a)		(a)
Mmb peel	(a)	329, 283, 224	331, 283, 214	330, 283, 239			(a)		(a)
* del-3-glu					535			572	
* cya-3-glu					525			545	
** cyanidin						535		562	
** pelargonodin						520			
** peonidin						532			

(a) Not detected; *del-3-glu; *cya-3-glu; **cyanidin, **pelargonodin, **peonidin standards obtained from J.B. Harborne, (1988). (e) 100% methanol (+ 0.5% hydrochloric acid) extracts were used in determining the $AlCl_3$ colour shifts in Methanol (+ 0.01% HCl). Measurements of the spectral data were done in a Shimadzu UV-vis spectrometer using quartzite cuvettes with lambda range of 700 to 200 nm. Skw, Starkrimson wild type; Skm, Starkrimson mutant; CF, Clapp's favourite; MMa, Mrb mutant A; MMb, Mrb mutant b; Mrbw, Marx Red Bartlett wild type

addition of HCl. The methanolic extracts to which HCl was added were separated using PC to produce red bands in Skw, CF and Mrbw. The red band in Skm was however very faint and the pigment could not be eluted. The red pigments were eluted and their spectra determined, producing peaks in the 545 to 500 nm range.

Methanolic extracts containing HCl were also separated using HPLC and the peaks collected and their spectra determined. A Table 6 shows that peaks were produced in the 526 to 520 nm range. Bathochromic shifts of the anthocyanins/anthocyanidins were determined by adding $AlCl_3$ in HCl. The red pigments eluted from PC showed bathochromic shift in all the three genotypes.

Lambda max (nm) in the visible range (700-400 nm) of the anthocyanins in the extracts, as shown in Table 6, is within the 525 to 527 nm range. This range corresponds to cyanidin-glycoside, as such the main anthocyanin in all the fruit extracts is a cyanidin-glycoside. Upon hydrolysis lambda max changed to about 535 nm, corresponding to cyanidin.

HPLC Separation of Pear Peel Extracts

All flavilium ion containing phenolic compounds produces peaks at 520 nm. However, because of different moieties that may be attached to the ion, the compounds have different retention times and thus can be separated and identified using HPLC. Peel extracts of six genotypes were analysed for anthocyanins at 520 nm. The extracts were separated by PC and the red pigments eluted and analysed using HPLC.

No peaks were produced from 50% methanolic extracts of Skm, CF, MMa and MMb peels. The addition of HCl resulted in peaks being produced in all extracts except MMa and MMb peel. Bright red bands were produced upon separating the extracts on a 3 mm cellulose paper, except on Skm, MMa and MMb peel extracts. Bands on Skm were very faint and colud not be eluted for analysis.

The results in Table 7 indicate the existence of one major peak in the entire acidified methanol extracts, with a retention time (R-min) of approximately 53±3 min. This peak had UV-vis spectra characteristics similar to those of a cyanidin-glycoside with a λmax of approximately 524±3.

Table 7: HPLC (520 nm) retention times of anthocyanins from common pear fruits peels

	HPLC peaks retention time (min) (R min)			
	λ 520 nm			
Peel extract	50% methanol	50% methanol (+ 0.5% HCl)	100% methanol (+ 0.5% HCl)	PC band
Skw peel	43.675, 51.107, 55.229, 59.236, 66.396	49.961, 56.907	53.424, 60.109	54.982, 62.909, 70.938
Skm peel	(a)	52.876	41.836, 53.755, 60.109	
CF peel	(a)	53.427	55.625, 62.251	55.971
Mrbw peel	55.829, 62.751	47.992, 51.442, 54.578	55.578	55.151
MMa peel		51.079		
MMb peel				

(a) Not detected, Skw, Starkrimson wild type; Skm, Starkrimson mutant; CF, Clapp's favourite; MMa, Mrb mutant A; MMb, Mrb mutant b; Mrbw, Marx Red Bartlett wild type

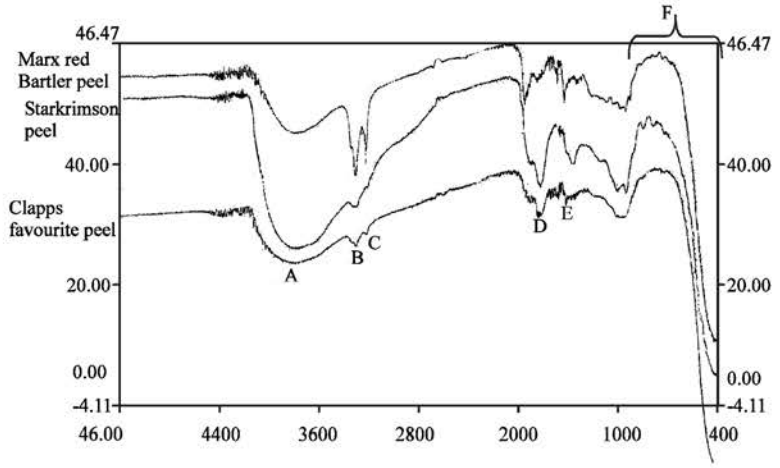

Fig. 3: Infrared spectra of red PC bands from common pear peel methanol extracts.

A: 3600-3000 cm^{-1} OH of water, Arly C-H stretching, H-Bonded O-H

B: 3000-2900 cm^{-1} CH_2, CH_3 strong response, CO_2H carboxylic acid (weak)

C: 2900-2800 cm^{-1} CH weak stretching

D: 1800-1600 cm^{-1} conjugated CO_2H, Benzene ring (medium)

E: 1508.5 cm^{-1} Benzene ring (medium response)

F: Fingerprint region

Infrared-Spectra Determination of Pear Peel Red Pigments

An infrared spectrum shows mainly the type of bonds and the functional groups constituting the compounds.

A preparative PC was used to develop the extracts. The red pigment formed a distinct band, which was eluted and concentrated under vacuum.

Figure 3 shows the infrared spectra of red pigments separated by PC from the common pear methanol extracts. Results from the analysis in Fig. 3 showed a similarity of the molecules responsible for the red pigmentation in the pear fruits peels. Their fingerprint region is almost the same regardless of the unresolved bands in MRB and Clapps Favourite spectra possibly due to noise. The bands between 15500 and 400 cm^{-1} cannot be assigned to any particular functional group with certainty due to overtones and combination of bands that characterise this region. Using this results, in particular the

fingerprint region, the compounds in the three extracts of MRB, Starkrimson and its green mutant have probably the same functional groups and types of bonds. The finger print region, however, is not enough to separate closely related molecules such as anthocyanidins, which may differ in only one hydroxyl group only.

DISCUSSION

The main objective of the present study was to isolate and identify the anthocyanin molecules responsible for colouring in the pear fruit peels of the wild types and their bud mutants. Anthocyanin pigments are usually found as glycosides in plants; however, when they are ingested as food the sugars are easily hydrolysed from the aglycones. Paper chromatography revealed one band with red colour (Table 5). The single red band (R_f = 0.32 in BAW and 0.23 in formic acid) observed in the paper chromatogram (Table 5) corresponded to the single peak (R = 53±3) observed in the HPLC chromatogram (Table 7). The different colours of anthocyanin pigments reflect the nature of their hydroxylation and methoxylation pattern. An increase in hydroxylation is accompanied by an increase in blue colour while methoxylation enhances the red colour (Jackman and Smith, 1996). This would indicate that the red coloured band may correspond to cyanidin (Harborne, 1988, 1967, 1989; Jackman and Smith, 1996; Rice *et al*., 1986; Rooyen and Bower, 2003; Price *et al*., 1999).

Additional qualitative information was obtained with the aid of the spectral characteristics of the chromatographic bands. The spectral data presented in Table 6 show the maximum absorptions for the chromatographic peaks to be in the range 527 to 525 nm before hydrolysis and 532 to 535 nm after hydrolysis. These data are corresponding to spectral data of cyaniding-glycoside and its aglycone, respectively (Harborne, 1989; Mazza and Velioglu, 1992; Francis, 1982, Harborne, 1958).

Evidence for the identity of anthocyanidins can be obtained from spectral measurements. The wavelength of maximum absorption of most anthocyanidins falls in the 530 to 550 nm ranges, so that little information can be gained from the maximum alone. However, this maximum coupled with the shift in the maximum caused by complexing the anthocyanidin with aluminium ion is quite diagnostic. Only anthocyanidins containing 3, 4 vicinal hydroxyls are capable of forming this complex (Harborne, 1989; Mozeitic *et al*., 2002). There was colour change observed after addition of aluminum chloride in the fractions corresponding to the red band (Table 6). The colour changed from red to blue as a consequence of a shift in the maximum absorption. The test enables to make a broad differentiation between the derivatives of the cyanidin, delphinidin and petunidin groups, which show a positive colour change and those of the pelargonidin, peonidin and malvidin groups, which show no colour change (Harborne, 1958). These results further support the assignment of the red coloured band as cyanidin. The positions of attachment to the aglycone by the sugars (Table 4) were however, not determined in this study. This can be calculated using the ratio $Abs_{440}/Abs_{\lambda max}$. Cyanidin derivatives would be expected to have a ration of about 24 (Guedes, 1993; Markakis, 1982) it is not unusual for cyanidin to be glycosilated to a rhamnose or xylose sugar, but non such anthocyanin have been isolated from pear peel (Harborne and Williams, 1998; Nortje and Koeppen, 1965).

Presence of absorption in the 308-328 nm regions of the spectra indicated that the red pigment in the wild type Starkrimson was acylated. The red pigment of wild type Marx red Bartlet, Clapps favourite and Starkrimson mutant, however, showed no absorption in the region indicating absence of acylation with aromatic organic acid (Dekazos, 1970).

The infrared spectra of the fruit peel extracts were similar. As would be expected, there is strong hydroxyl absorption at 3400 cm^{-1}. The aromatic bands are present at 1620 and 1520 cm^{-1}. It seems probable that the band at 2900 cm^{-1} can be attributed to aliphatic CH vibrations. Hydroxyl absorption in the 1300 to 1400 cm^{-1} region obscures the identification of other characteristic absorption bands for these compounds. The non crystalinity of these compounds may be responsible for the broadening

of the bands in the 650 to 1000 cm^{-1} region and the general lack of sharpness for the whole spectrum (Manson, 1959; Banwell, 1983; Montedoro *et al*., 1993; Williams and Fleming, 1980).

Phenolic compounds have been shown to vary with plant tissue, developmental changes and regulation of biosynthesis. Besides the ontogenetic variation in biosynthesis and accumulation, the stress-induced and pathogenesis-related changes are known to occur (Macheix *et al*., 1990; Graham, 1991; Awad, 2001).

It is important to note that Clapps favourite and the mutant of Starkrimson are green pear fruits with a reddish blush. The blushing is only pronounced on surfaces receiving high incidence of light. The red colouring, however, appears as spots on the red blushes. It would seem, therefore, that there is differential regulation of the genes responsible for the uniform expression of the red colour in Clapps favourite and Starkrimson mutant. This does not seem to be the case in Starkrimson, since the colouring is uniform. The genes responsible for full expression of colour in Starkrimson are possibly partially expressed in Clapps favourite and Starkrimson mutant. The blushing on Starkrimson mutant is less compared to Clapps favourite. This again points to the probable differential expression of the genes encoding for colour (Strack, 1997; Preez *et al*., 2004; Perez de la Vega, 1993; Oleszek *et al*., 1988).

The implications of colour differences in fruit peels constitute a biochemical marker, which could be used to differentiate cultivars. Low molecular weight markers, however, have a disadvantage in that they are greatly influenced by the environment in which fruits are grown. As such, the phenotypic characteristic may not be inherited in simple Mendelian fashion. It is invaluable then to link these biochemical differences in the pear fruits to the putative genetic differences in the anthocyanin biosynthetic pathway.

CONCLUSIONS

The reddish pigment in the extracts f the common pear fruits Starkrimson, Starkrimson mutant, Clapps Favourite and Marx red Bartlett has chromatographic and UV-vis and IR spectral characteristics similar to those of cyanidin-glycoside and cyanidin upon hydrolysis. The pigment has been identified as a cyanidin-rhamnosyl (in Starkrimson and its mutant and cyanidin-xylosyl (Clapps Favourite; Marx red Bartlett and its mutants). The information gathered in this study helps towards characterizing the pear cultivars and their mutants. This will assist in breeding towards, good sensory quality attributes and defense against ultraviolet radiation or aggression by pathogens (Hamauzu, 2006; Sapers *et al*., 1983). Characterization of anthocyanins is also important due to their biological activities particularly in relation to their antioxidant properties (Hamauzu, 2006).

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