ANTHOCYANINS IN SELECTED *PROTEACEAE*

BY

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DECLARATION

I, the undersigned, herby declare that the work contained in this thesis is my own, original work and has not previously, in its entirety or in part, been submitted at any university for a degree.

SUMMARY

Trials were conducted to follow colour development during the growth season of the commercial *Leucadendron* cultivar 'Silvan' Red', as well as the *Protea* cultivars 'Ivy', 'Carnival' and 'Sylvia'. HPLC analysis showed that pigments responsible for red colour in *Leucadendron* and *Protea* cultivars are anthocyanins.

Initial extraction of anthocyanins from freeze-dried and finely ground *Leucadendron* leaves, using 5% formic acid in methanol, resulted in the precipitation of gel-like compounds, making the extracts impossible to filter for HPLC. Trials were conducted to determine the optimum extraction solvent and extraction time for anthocyanin quantification in *Leucadendron*, using 'Safari Sunset' (*Leucadendron salignum x L. laureolum)* as reference material. Acetone, 80% acetone, 5% formic acid in water and 5% formic acid in methanol were added to freeze dried leaf samples and allowed to extract for one hour. Extracts were analysed by reverse-phase HPLC. Extractions with methanol and water yielded extracts with a high content of pectin-like compounds, making them difficult to purify for HPLC. 80% acetone proved to be the most efficient extraction solvent, yielding the highest anthocyanin concentration, and showing the least amount of insoluble compounds. Optimal extraction time was determined for 80% acetone by testing 1, 6 and 24-hour extractions respectively. Although 6 hour extraction showed a slightly higher yield, a 1-hour extraction should suffice for quantification of anthocyanin changes over time, as trends will clearly be evident.

'Silvan Red' shoots meeting export quality can generally be harvested from mid February to mid April (late summer to autumn). The harvesting period ends as the colour of the distal leaves forming the flower head change from red to green. Anthocyanin, chlorophyll and carotenoid fluctuations were investigated in 'Silvan Red' during the 2000 growth season. The observed red colour loss is the result of the net degradation of anthocyanins and appeared to be correlated to the phenological development of the shoot. Although a positive correlation was noted between anthocyanin content and average daily temperature and mean daily

sunlight hours, it appears unlikely that they are the causal factors for the observed colour change. Anthocyanin degradation started during conditions of long exposure to high light intensities and continued during a period of low temperature (autumn), both which are known to favour anthocyanin synthesis rather than its degradation. Therefore it appears that colour development in 'Silvan Red' is developmentally regulated.

The *Leucadendron* cultivar 'Safari Sunset' with the same parentage as 'Silvan Red', is noted for its more intense red-purple colouration, which it does not lose during its development. The more intense colouration of 'Safari Sunset' has been ascribed to the higher total anthocyanin concentration, which was almost double that encountered in 'Silvan Red'. 'Safari Sunset' was shown to contain the same major types of anthocyanins, and hence the purple colouration (not seen in 'Silvan Red') can not be explained in terms of different types of anthocyanins being present. However, the ratio between the two major anthocyanins present in both 'Safari Sunset' and 'Silvan Red' were significantly different in that 'Safari Sunset' showed a considerably higher concentration of peak 1, which is most likely responsible for the observed purple colouration. High anthocyanin concentrations have been noted to buffer against visual changes in colour, which is the most probable reason that a colour loss is not observed in 'Safari Sunset'.

Colour development of the innermost involucral bracts of three *Protea* cultivars was followed from an initially selected inflorescence size to commercial harvest (when flowers have opened slightly). 'Ivy', 'Carnival' and 'Sylvia' show a light pink, dark pink and red colouration respectively, which has been ascribed to differences in total anthocyanin concentration.

Colour development in 'Carnival' showed a quadratic trend with time, with the highest rate of anthocyanin synthesis occurring a week prior to harvest. Colour development in 'Ivy' and 'Sylvia' were shown to be linear with time.

As inflorescences are closed during their development and anthocyanin synthesis in the innermost involucral bracts occurred in darkness, light does not appear to have a major influence on colour development. Temperature did not appear to

have a significant effect on flower colour, as flowers developing later in the season, when ambient temperatures were lower, showed no significant differences in anthocyanin concentration to those harvested earlier. Hence, it appears that colour development in all three cultivars is developmentally regulated.

OPSOMMING

Hierdie studie is onderneem om kleurontwikkeling van die *Leucadendron* kultivar 'Silvan Red' en die *Protea* kultivars 'Ivy', 'Carnival' en 'Sylvia' te ondersoek. Die rooi kleur van distale 'Silvan Red' blare en *Protea* omwindselblare is te wyte aan antosianiene.

Eerstens is gepoog om die ektraksie van antosianiene vir kwantifisering deur hoë druk vloeistof chromatografie (HPLC) te verfyn. Aanvanklik is 5% mieresuur in metanol gebruik om antosianien vanuit gefriesdroogde en fyngemaalde *Leucadendron* blare te ekstraheer. 'n Gelagtige neerslag het dit egter onmoontlik gemaak om die ekstrak te filtreer. Vervolgens is eksperimente uitgevoer om die geskiktheid van asetoon, 80% asetoon, 5% mieresuur in water en 5% mieresuur in metanol as alternatiewe vir 5% mieresuur in metanol te bepaal. Antosianiene is deur middel van HPLC gekwantifiseer na 'n uurlange ekstraksie vanuit 'Safari Sunset' (*Leucadendron salignum* x *L. laureolum*) blaarmonsters. 'n Onoplosbare neerslag van onsuiwerhede na ekstraksie met metanol en water het filtrasie bemoeilik. 80% asetoon was die doeltreffendste ekstraheermiddel deurdat dit beide die hoogste opbrengs van antosianiene en die minste onsuiwerhede gelewer het. Vervolgens is die optimale duur van antosianienekstraksie met 80% asetoon bepaal deur vir een, ses en 24 uur te ekstraheer. Resultate het getoon dat, alhoewel ses uur ekstraksie 'n effense hoër antosianienkonsentrasie lewer, 'n uur voldoende behoort te wees vir kwantifisering van antosianien.

Uitvoergehalte 'Silvan Red' lote word gewoonlik vanaf middel Februarie tot middel April (laat somer tot herfs) geoes. Die oestyd eindig met 'n verandering in die kleur van distale blare van rooi na groen wat die bemarkbaarheid van lote verlaag. Ten einde hierdie kleurverandering te kwantifiseer is veranderinge in die konsentrasies van antosianien, chlorofiel en karotenoide in distale 'Silvan Red' blare gedurende die 2000 groeiseisoen gemeet en in verband gebring met omgewingstoestande en ontwikkelingstadiums. Rooi kleurverlies van distale 'Silvan Red' blare hou klaarblyklik verband met 'n geleidelike afname in hul antosianienkonsentrasies vanweë netto degradasie. Veranderinge in die kleur en antosianienkonsentrasie van blare het saamgeval met spesifieke ontwikkelingsperiodes. Kleurveranderinge

in 'Silvan Red' kon nie met omgewingstoestande verbind word nie. Kleuronwikkeling word klaarblyklik intern gereguleer om saam te val met spesifieke ontwikkelingstadia.

Die *Leucadendron* kultivars 'Safari Sunset' en 'Silvan Red' het dieselfde ouers. Eersgenoemde kultivar het egter 'n intenser rooi-pers kleur wat ook stabiel bly oor die hele groeiseisoen. Die intenser kleur kan toegeskryf word aan die bykans twee keer hoër antosianienkonsentrasies wat 'Safari Sunset' blare oor die groeisoen handhaaf. Hierdie hoër antosianienkonsentrasie is moontlik ook die rede vir die oëskynlik groter kleurstabiliteit van 'Safari Sunset' aangesien hoë pigmentvlakke kleur buffer teen veranderinge in pigmentkonsentrasie. 'Safari Sunset' se pers skynsel is moontlik te wyte aan die relatief groter bydrae van piek 1 op die chromatogram tot die totale antosianienkonsentrasie. Andersins het die twee kultivars 'n soortgelyke antosianienprofiel.

Ten einde die regulering van kleurontwikkeling in *Protea* te ondersoek, is antosianienkonsentrasies in die binneste bloeiwyse-omwindelsblare van die *Protea* kultivars 'Ivy', 'Carnival' en 'Sylvia' gemeet vanaf 'n pre-geselekteerde blomgrootte tot en met kommersiële oestyd wanneer die blomme begin oopgaan. Die drie kultivars wissel onderskeidelik in kleur van lig pienk en donker pienk tot rooi. Hierdie kleurverskille was te wyte aan verskille in antosianienkonsentrasie. Antosianienkonsentrasies in 'Ivy' en 'Sylvia' het lineêr toegeneem oor tyd, terwyl in die geval van 'Carnival', maksimum antosianienvlakke reeds 'n week voor oes bereik is. Blomme wat by laer temperature later in die seisoen of vroeër by hoër temperature ontwikkel het, het nie betekenisvol verskil in antosianienkonsentrasie nie. Gevolglik is lig en temperatuur klaarblyklik van mindere belang vir *Protea* kleurontwikkeling. Soos met *Leucadendron* die geval was, word kleurontwikkeling in *Protea* dus ook intern gereguleer.

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CONTENTS

1. General Objectives

Thus far, no studies have been conducted on colour in the genera *Leucadendron* and *Protea*. Colouration is an important quality aspect of cut flowers on international markets. The general objectives of this study were:

The optimisation of the anthocyanin extraction methodology from freeze-dried and finely ground *Leucadendron* leaves, by testing for the most efficient extraction solvent, as well as for determining an optimal extraction time. Initial extractions with 5% formic acid in methanol resulted in the precipitation of gel-like substances, making it impossible to filter the extracts for HPLC, hence other extraction solvents had to be looked at.

To investigate pigment fluctuations in 'Silvan Red' (*Leucadendron salignum* x *L. laureolum*), to determine the pigments responsible for the observed colour loss from red to green, which considerably shortens the marketability of this commodity. Furthermore the pigmentation of 'Silvan Red' was compared to that of 'Safari Sunset', to determine why 'Safari Sunset' shows a far more intense colouration and does not show a colour loss during the growth season.

To conduct the first colour development study in *Protea* from an initial inflorescence size to commercial harvest, when the flowers have opened slightly. and determine whether a possible link to environmental conditions exists.

2 LITERATURE REVIEW: THE CHEMICAL DYNAMICS OF ANTHOCYANIN COLOUR EXPRESSION IN PLANTS

2.1 Introduction

Anthocyanins are water soluble pigments, which essentially accumulate in vacuoles of epidermal cells of flowers and fruit, but are also frequently encountered in other plant parts such as roots, stems, leaves and bracts (Brouillard, 1983; Dong et al., 1995; Strack and Wray, 1994). Here they strongly absorb light in the visible light range, giving rise to a vast array of colours ranging from pink, red and violet right through to blue.

Colour expression by anthocyanins is complex and determined by their structure, structural modifications, as well as the physiochemical conditions they exist in (Brouillard, 1988). The number of hydroxyl groups, degree of methylation of these hydroxyl groups, type and number of sugars attached, as well as the position of attachment on the anthocyanin molecule, are all primary structural aspects which determine the colour or colour stability of anthocyanins (Brouillard, 1982; Mazza and Brouillard, 1987; Mazza and Brouillard, 1990). Physiochemical factors, such as copigmentation and pH generally exert their influence by modifying or stabilising the primary structure of anthocyanins. A structural change of a pigment can result in a shift of the wavelength absorbed, which may change the colour we perceive. Colour changes may include an increase in colour intensity (as measured by absorbance) and/or a drastic colour change, evident from the shift in the maximum visible wavelength absorbed. The majority of these studies have been conducted *in vitro* in acidic aqueous solutions to determine the effects of individual factors influencing colour. The observed effects on colour in aqueous solutions serve as models to predict colour expression in plant tissues.

This review aims at giving a general report on colour expression of anthocyanins as influenced by structural variations and physiochemical conditions.

2.2 The primary structure of anthocyanins and colour expression

2.2.1 Anthocyanidins, the structural chromophore

The structural pigment part (chromophore) of an anthocyanin molecule is known as the anthocyanidin, also commonly referred to as the aglycone. There are about 20 naturally occurring anthocyanidins, of which only six are the most common constituents of anthocyanins in higher plants (Harborne, 1993; Timberlake and Bridle, 1975). Anthocyanidins are based on a 3-ring structure (flavylium cation) and vary in degree, type and position of substitution by hydroxyl or methyl groups on these ring structures (Delgado-Vargas et al., 2000; Viljoen and Huysamer, 1995). The three most basic anthocyanidins are pelargonidin, cyanidin and delphinidin, which differ in the number of hydroxyl groups on the B-ring (Fig. 1) and result in orange, red, magenta and mauve pigmentation (Asen, 1976; Harborne, 1993). Methylation of one or more of these free hydroxyl groups on the B-ring results in the simple methylated derivatives, of which peonidin, petunidin and malvidin are fairly common chromophores in higher plants (Fig 1). The remainder of the 20 anthocyanidins vary in occurrence and some may be restricted to be involved with pigmentation of only a few plant species (Harborne, 1993).

2.2.2 Anthocyanins

Anthocyanidins in their pure chemical form generally do not accumulate in plant vacuoles, due to their instability (Mazza and Miniati, 1993). According to Harborne (1993) all anthocyanidins *in vivo* occur as glycosides, which are then referred to as

anthocyanins, i.e. anthocyanins are glycosidic derivatives of the respective anthocyanidin chromophore involved. They may be structurally modified further by copigmentation and pH, resulting in so called secondary structures. In very acidic aqueous solutions the majority of anthocyanins exist in their primary forms (flavylium cations), with hydroxylation and methylation patterns inherited from the respective anthocyanidin chromophore. Hence it is important to note, that the following noted changes in colour expression, as a result of differences in commonly encountered primary structures, have been the result of studies conducted with pure, non-acylated anthocyanins (mono- and diglycosides) in buffered, strongly acidic aqueous solutions (pH <2), where they can be studied without interference form other possible copigments and pH related modifications, as will be discussed later.

2.2.3 Hydroxylation and methylation patterns of anthocyanins

The three most basic anthocyanins are pelargonidin-, cyanidin- and delphinidin-glycosides (single sugar attached to the C3 position), which differ only in the number of hydroxyl groups on the B-ring (Fig. 1) (Asen, 1976). This difference leads to the expression of different colours, as a shift towards longer visible wavelength absorbed occurs, resulting in a blueing of colour (bathochromic shift). Hence, the greater the number of hydroxyl groups on the B-ring, the bluer the colour perceived (Asen, 1976; Mazza and Brouillard, 1987; Torskangerpoll et al., 1998). The λ_{max} of the three main anthocyanins in a strong acidic solution are: pelargonidin 520 nm (orange), cyanidin 535 nm (orange-red) and delphinidin 545 nm (bluish-red) (Goto, 1987). Hence, delphinidin-3-glycosides exhibit a bluer colour than the respective cyanidin- and pelargonidin-glycosides (Asen, 1976).

Methylation of one or more of the free hydroxyl groups on the B-ring of the three basic anthocyanins above, results in the simple methylated derivatives peonidin-, petunidin- and malvidin-glycoside respectively. Methylation has a slight reddening

effect, but is probably more important in improving the stability of the anthocyanin (Harborne, 1993; Mazza and Brouillard, 1987). Cyanidin and delphinidin glycosides are slightly bluer than their corresponding methyl ethers, peonidin and petunidin (Asen, 1976).

2.2.4 Glycosylation

Sugars commonly involved with glycosylation have been identified as glucose, rhamnose, xylose, galactose, arabinose and fructose (Delgado-Vargas et al., 2000). Di- and trisaccharides of these sugars may also be involved in the glycosylation of some anthocyanins (Mazza and Brouillard, 1987). The most common anthocyanin glycosides encountered in plants are: 3-monosides, 3 biosides and 3,7 diglycosides (Mazza and Brouillard, 1987; Timberlake and Bridle, 1975). There is always a sugar attached at the 3 position, with the exception of 3-deoxyanthocyanins, such as apigeninidin, luteolinidin and tricitinidin (Brouillard, 1982; Francis, 1989; Timberlake, 1980). If more than one sugar is present, they may be attached to the 3, 5, 7, 3', and 5' and sometimes even 4' hydroxyl groups of the A and B ring (Fig. 2) (Brouillard, 1983).

As anthocyanidins are unstable in aqueous solution and less soluble than the respective anthocyanin derivative, addition of sugars appear to have a stabilising effect on these pigments, as well as increasing their solubility (Mazza and Miniati, 1993). Studies conducted by Iacobucci and Sweeny (1983) showed that cyanidin-3-rutinoside in a citric acid solution of pH 2.8 had a half life of about 65 days, whereas the corresponding anthocyanidin (obtained by hydrolysis) had a half life of 12 only hours.

Glycosylation was said to have little effect on colour, as it appears to be the rule, rather than the exception (Asen, 1976). Asen (1976), however, also states that

3,5-diglycosides are slightly bluer than the corresponding 3-glycoside. If sugar attachment occurs on the B-ring hydroxyls (which is rare) a small shift in λ_{max} towards shorter wavelength has been shown (hyspochromic shift) i.e. a slight reddening effect, for example cyanidin 3,5,3'-triglucoside (Harborne, 1993). Furthermore, the hydroxyl group at the 3 position is particularly significant, as the majority of anthocyanins (3-glycosylated forms) show a red colouration, whereas the 3-deoxyanthocyanidins are yellow (Mazza, 1987).

2.3 Structural instability of anthocyanins - The water and pH paradox

One of the major factors influencing the colour of anthocyanins is the acidity or alkalinity of the solution in which they are dissolved. The three species of water H^+, OH and H_2O are highly reactive towards anthocyanins, modifying the primary structure, leading to numerous secondary structures that influence colour expression (Brouillard, 1982; Mazza and Miniati, 1993). Secondary structures are derived from acid-base, hydration and tautomeric reactions when anthocyanins are dissolved in aqueous solutions and can result in a variety of coloured and non-coloured species. However, the coloured secondary structures are said to be unstable and the resultant colour fades fairly rapidly. The mechanism proposed by Brouillard (1983) for these reversible structural transformations in aqueous solutions appears to be the generally accepted explanation. As most current reviews and articles dealing with the pH factor appear to cite Brouillard, the pH effect will be discussed mainly according to that given by Brouillard (1983, 1988).

The following studies on the effect of pH changes in aqueous solutions have mainly been conducted with relatively simple and pure anthocyanins *in vitro*, used to model the behaviour of anthocyanins in plant vacuoles (*in vivo*). Here they co-exist with numerous other compounds, which may stabilise anthocyanins against structural changes, as will be explained later.

2.3.1 Acid-base reaction

Anthocyanins, dissolved in strongly acidic solutions at pH values of 1 and below, mainly exist in the orange to red coloured primary flavylium cation form (AH^+) (Fig. 3). As the pH is raised, from slightly acidic to slightly alkaline solutions (pH 4-5), a fast acid-base equilibrium reaction occurs, as one of the hydroxyl groups on positions 5, 7 or 4' can lose a proton, resulting in neutral quinonoidal bases (also referred to as anhydrobases), which are generally blue-purple in colour. Three species of neutral quinonoidal bases can exist, depending on the position that the proton transfer occurs (A_5, A_7, A_4'). Within the pH range of 6 to 8, if further free hydroxyl groups exist on the quinonoidal molecule, another proton can be lost which leads to a stabilised quinonoidal anion, of which there are also 3 structural forms ($A^-_{54'}$, $A^-_{75'}$, $A^-_{4'7}$) (Brouillard, 1983). According to Brouillard (1988) ionised quinonoidal bases can give rise to larger bathochromic and hyperchromic shifts, i.e. result in blue or reddish colours respectively.

All natural anthocyanins have at least one free hydroxyl in the 4', 5 or 7 position, indicating the importance of quinonoidal base formation in flower pigmentation. Their formation appears to be the rule, rather than the exception.

2.3.2 Hydration reaction

Flavylium cations (3-O-glycosides) are completely hydrated to colourless carbinol pseudobases (B) (Fig. 3) at pH values ranging from 3 to 6 (Brouillard, 1983). Hydration of the flavylium cation is said to be the key step for the formation of colourless species, causing rapid fading of colour in an aqueous medium (Brouillard and Dangles, 1994). Water addition takes place mainly at the 2 position (B_2). Although additions to the 4 position have been reported (B_4), this generally does not occur in measurable amounts. (Brouillard, 1983). All carbinol

pseudobases are colourless, with an absorption maximum of about 275 nm. Hence a solution, where most anthocyanins have been hydrated, shows only a poor colouration.

2.3.3 Tautomerisation

Carbinol pseudobases exist in an equilibrium with another structural form known as chalcone pseudobases (C), which are generally colourless, but may show a pale yellow colouration. They are the result of the opening of the pyran ring of the carbinol pseudobase, and exist as two isomeric forms C_E and C_Z (Fig. 3). In slightly acidic aqueous solutions, this equilibrium is only slowly attained, and commonly only small amounts of chalcone pseudobases have been detected (Brouillard, 1983; Brouillard and Dangles, 1994; Mazza and Miniati, 1993).

2.3.4 Colour expression of simple anthocyanins as a result of pH

The colour of an acidic aqueous solution depends on the equilibrium between the four main structures, the red flavylium cation, red or blue quinonoidal bases, colourless carbinol pseudobases and colourless chalcone pseudobases formed by the acid-base, hydration and tautomerisation reactions, depending on pH (Fig. 3) (Brouillard, 1988; Mazza and Brouillard, 1987).

In general, when a monomeric anthocyanin (non-acylated) is added to an aqueous solution (pH 5-6), the neutral quinonoidal and/or ionised quinonoidal bases are formed almost immediately by a fast acid-base reaction, showing the appearance of a bluish colouration. On standing further, the flavylium cation is hydrated to the colourless carbinol pseudobase, which in turn reaches a slow equilibrium with an open ring structure, the chalcone pseudobases. So the observed colour change of the solution will be red, to blue to almost completely colourless, depending on the

type of anthocyanin (Brouillard and Dangles, 1994; Mazza and Brouillard, 1987). The quinonoidal bases are unstable and said to evolve fairly rapidly to the more stable, colourless carbinol pseudobases. While Chen (1981) and others maintain that the loss of blue colour is the result of the hydration of the quinonoidal bases to the colourless form, kinetic and thermodynamic studies conducted by Brouillard show that this reaction is highly unlikely and that no chemical proof exists for the occurrence of this reaction (Brouillard and Dangles, 1994). They state that the majority of colour loss is the result of the hydration of the red flavylium cation, resulting in the formation of colourless carbinol pseudobase. However, an explanation of how the quinonoidal bases evolve, resulting in the loss of blue colour, is not given and one can only state, that it appears as if hydration of quinonoidal bases takes place, as apparent from the loss of blue colour.

Figure 4 shows a schematic example of the 'fate' of malvidin 3-glucoside at different pH levels. At pH of 0.5 and less, the majority exists in the flavylium cation form (AH^+). With increasing pH, the flavylium cation is hydrated to the colourless carbinol pseudobases, which exist in equal amounts at a pH of about 2.6. From a pH of about 2 upward small amounts of the blue quinonoidal bases (A) and colourless chalcones (C) are formed. Hence from a pH of 2 to about 4.5 the proportions of carbinol pseudobase, quinonoidal forms and chalcone increase with increasing pH, at the expense of the red flavylium cation form. At an pH above 5.5 the only coloured species left is the quinonoidal form, hence a solution of poor colouration (Mazza and Brouillard, 1987). The effect of pH on colour of pure non-acylated anthocyanins is evident from a change in absorption at $\lambda_{max,}$ which serves as an indication of colour loss and a change in λ_{max} and shows a change in colour expressed (Table 1). Cabrita (2000) studied the effect of pH on the six most common anthocyanin 3-glucosides (pelargonidin-, cyanidin-, delphinidin-, peonidin-, petunidin-, and malvidin glucoside), which confirmed the colour loss and colour shifts as discussed so far, within the pH range of 1 to 7. All anthocyanin 3-glucosides show a strong red colouration at a pH range of 1 to 3, with a decreasing colour intensity and a gradual shift towards bluer colours as the pH

increases towards neutrality (pH 5 to 7). However, in the alkaline region the properties of the anthocyanin 3-glucosides became evident. Pelargonidin-, peonidin-, and malvidin 3-glucosides, characterised by a single free hydroxyl group on the B-ring, showed a strongly blue coloured solution (pH 8 to 9). The 3-glucosides of cyanidin, delphinidin and petunidin (with 2 *ortho*-hydroxyl groups on the B-ring) showed a blue colouration in the pH range of about 6 to 8 and exhibited a strong hyspochromic shift (reddening) at pH 8.1, when the blue equilibrium forms turned into red quinonoidal anions.

Due to the strong hydration reaction, the simple mono- and diglycosidic anthocyanins alone can not explain the vast colour variation seen in plants. Anthocyanins responsible for most of the red to blue colours in plants are virtually colourless within the pH range of three to six (Asen, 1976; Chen and Hrazdina, 1981; Mazza and Miniati, 1993). Studies have shown, that the vacuolar pH of many plants fall within this range. (Lancaster et al., 1994) have shown that the pH of apple epidermal cells is in the range of 3.6 to 4.0. (Stewart et al., 1975) showed that the pH of many epidermal flower cells is between 2.5 and 7.5. Hence other mechanisms must be present *in vivo*, which protect the flavylium cation against the hydration reaction from occurring to a large extent, and thus allowing for the expression of colour at pH levels when they would normally be colourless.

2.4 Colour stabilisation mechanisms

The stabilisation effects of glycosylation and methylation and their respective effect on colour expression have already been mentioned (see Section 2), hence will not be discussed again, as they are not really regarded as being mechanisms that protect the flavylium cation against the drastic hydration reaction.

The colour stabilisation mechanisms are generally based on preventing and/or reducing the nucleophilic addition of water on the pyran ring of the flavylium cation, i.e. reduces the transformation of flavylium cations to the colourless carbinol pseudobases. Furthermore, the stabilisation of the quinonoidal bases has also been reported, hence the stabilisation of blue colour, commonly observed in flowers.

2.4.1 Copigmentation

The research on copigmentation has been vast in recent years and the reader is referred to the reviews by Brouillard (1994, 1988, 1983, 1982) as well as Osawa (1982) for a more detailed discussion on the precise mechanisms involved in copigmentation.

Anthocyanins accumulate in cell vacuoles along with numerous other compounds, with which they can interact (Mazza and Miniati, 1993). Generally, compounds that, when added to an anthocyanin containing solution, stabilise, enhance and even modify colour, are known as copigments. (Asen, 1976; Mazza and Brouillard, 1987; Mazza and Miniati, 1993).

Copigmentation has significant effect on colour stability and the magnitude of the effect is determined by the increase in absorption at pH levels where the non-copigmented parent molecule is almost colourless. Furthermore, copigments may significantly modify the colour as indicated by extent of the bathochromic shift of the visible λ_{max} (Mazza and Brouillard, 1987). Thus, copigmentation has generally been defined as the phenomenon which makes colour of anthocyanins bluer, brighter and more stable at pH of living plant tissues (Osawa, 1982).

Two main categories of copigmentation have thus far been defined. The first type is intramolecular copigmentation, which entails the strong bonding of organic acids to the anthocyanin molecule and is generally associated with colour stability. The second type referring to intermolecular copigmentation, where the copigment is only loosely associated with the anthocyanin molecule and results in stability of colour as well as a significant shift in the visible wavelength absorbed. (Brouillard, 1988; Osawa, 1982).

2.4.1.1 Intramolecular copigmentation

Intramolecular copigmentation generally refers to the acetylation process where aliphatic and/or aromatic organic acids bond covalently to the sugars on the anthocyanin molecule (Brouillard, 1982; Iacobucci and Sweeny, 1983). In nature, the most common are coumaric, caffeic, ferulic, *p*-hydroxy benzoic, synapic, malonic, acetic, succinic, oxalic and malic acyl groups (Francis, 1989; Harborne and Grayer, 1988; Jurd, 1972).

Two or more acyl groups must be bonded to the sugar moieties of the anthocyanin molecule for good colour stability in neutral solutions (Mazza and Brouillard, 1987). The stability of acylated anthocyanins has been attributed to the presence of two acyl moieties stacked below and above the pyrylium ring, resulting in a sandwich-type conformation, protecting the pyran ring against the nucleophilic attack of water at the 2 and 4 position. Monoacylated anthocyanins do not show great stability in neutral aqueous solutions, nor do some of the polyacylated anthocyanins, indicating that the structure of the acyl group, its position of attachment to the sugar, the type of sugar and position of attachment of the sugar residue to the anthocyanin molecule, are all important factors determining whether intramolecular stabilisation of colour will occur or not (Brouillard, 1988).

Studies have shown that acylated anthocyanins are more resistant to hydration and therefore possess a higher colour stability in slightly acidic to neutral solutions (Idaka et al., 1987; Saito et al., 1985). Fossen (1998) showed that the acylated anthocyanin petanin afforded a higher colour intensity and stability throughout the pH range 1 to 9 when compared to the non-acylated cyanidin 3-glucoside, which would make this anthocyanin a suitable food colourant.

Metal chelation has been noted to be a special kind of intramolecular copigmentation, which can only be formed with anthocyanins possessing a catechol group on their B-ring, i.e. only with three of the commonly occurring anthocyanidins and their glycosides. Strong blueing effects have been reported as a result of metal chelation of anthocyanins with trivalent metal ions such as aluminium and iron (Asen, 1976; Mazza and Miniati, 1993), however not with calcium, magnesium or potassium which are far more abundant in plant tissues. Other metals are not considered to be involved in complex formation as they are only present in trace amounts. However, unlike the original theory that metal chelation was the origin of blue colour, it has been shown that blue flowers in which metal chelation was reported also contained flavones which are excellent copigments and that often the blue colour was the result of anthocyanins without a catechol group on their B-ring. There still appears to be a certain degree of uncertainty in how exactly metal ions are involved in copigmentation (Brouillard, 1988).

2.4.1.2 Intermolecular copigmentation

Intermolecular copigmentation, i.e. the loose association of other compounds with anthocyanin molecules, is often seen as the classical copigment-effect. Many

compounds can act as copigments: polyphenols, flavonoids, nucleic acids and amino acids and even the anthocyanins themselves (Brouillard, 1983; Francis, 1989; Mazza and Brouillard, 1990).

This type of copigmentation has been noted to result in both colour stability (hyperchromic effect) and often a bathochromic shift of the visible λ_{max}, i.e. towards a blue colour (Asen et al., 1972; Asen, 1976; Brouillard and Dangles, 1994; Chen and Hrazdina, 1981; Mazza and Brouillard, 1987; Mazza and Brouillard, 1990). Flavonols and flavones were shown to give rise to the largest copigment effect and amino and benzoic acids the smallest (Asen, 1976). In intermolecular copigmentation the copigment appears to partly prevent the hydration of the flavylium cation, as evident from an increase in absorbance in the visible range, thus offering real protection against the nucleophilic attack (Brouillard, 1988). The copigment is also said to associate with the neutral quinonoidal bases, thus stabilising blue colour (Asen et al., 1972; Takeda et al., 1985).

The intensity of the copigmentation effect has been shown to be influenced by numerous other factors, which include type and concentration of anthocyanins, the type of copigment, the anthocyanin to copigment ratio, as well as the pH, temperature and solvent (Mazza and Brouillard, 1987). These influential factors have received much attention in recent years (Asen et al., 1972; Chen and Hrazdina, 1981; Mazza and Brouillard, 1987; Mazza and Brouillard, 1990; Takeda et al., 1985). The underlying chemistry is discussed extensively in a review by Brouillard (1994). Here we will only give a short example of the factors influencing copigmentation mainly according to the studies conducted by Mazza and Brouillard (1990).

When chlorogenic acid was added to solutions of cyanin and malvin, it enhanced colour at pH 1 to neutrality which indicated that both the flavylium cation and quinonoidal bases were stabilised, depending on pH. At constant pH a 3 fold increase of malvidin resulted in a 5.5 fold increase in absorption at λ_{max} , when the molar copigment/pigment ratio was 5 and showed an 8 fold increase when the molar copigment/pigment ratio was increased to 20. Unlike with malvidin, a 3 fold increase in cyanin resulted in a 3.7 fold increase in absorption with a molar copigment/pigment ratio 5 and only showed a 4.9 increase in absorption when the molar copigment/pigment ratio was raised to 20. Hence, malvidin shows a higher copigmentation efficiency than cyanin. Furthermore, by increasing the chlorogenic acid/anthocyanin ratio, the degree of blueing of the solution increases.

2.5 Conclusion

Colour expression by anthocyanins is complex and determined by their structure, structural modifications, as well as the physiochemical conditions they exist in. The capability of anthocyanins to undergo vast structural changes and associate with numerous other molecules co-existing in the vacuoles, explain how so few commonly encountered chromophores can result in a vast array of colours as seen in plants.

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Table 1. Effect of pH on λ_{max} and absorbance at visible λ_{max} of a 2.58 x 10^{-4} cyanidin 3,5-diglucoside solution (solvent: aqueous H_3PO_4- NaOAc buffer; ionic strength = 0.20 M; T = 20°) (adapted from Mazza, 1990)

pH		Absorbance	
	λ_{max}	λ_{max}	Δ Absorbance
2.74	509.2	1.625	-
3.64	510.8	0.329	-1.30
4.72	521.6	0.110	-0.22
5.74	528.8	0.097	-0.01

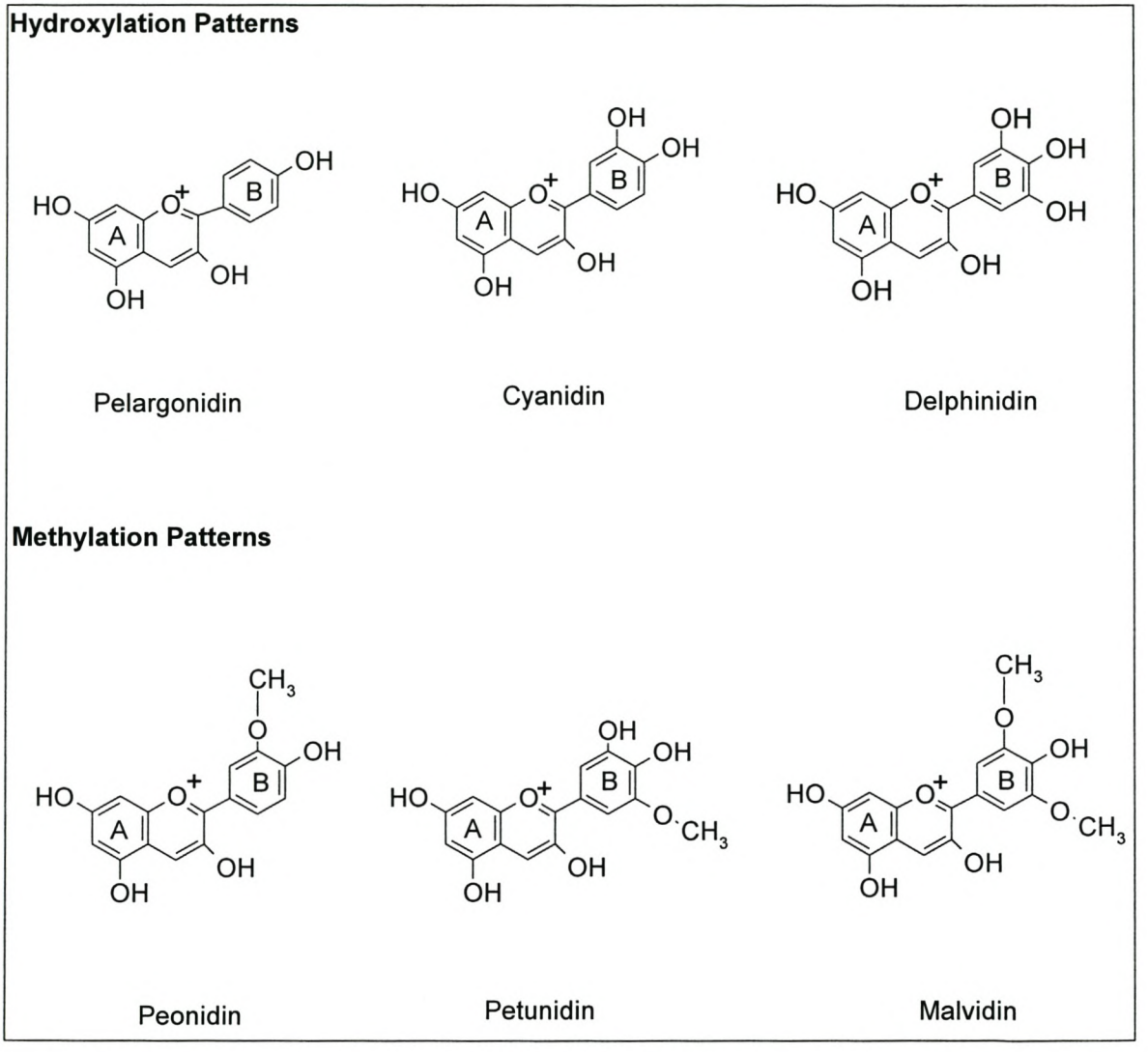

Fig. 1. The six most common anthocyanidin chromophores involved in pigmentation of higher plants, differing in hydroxylation and methylation patterns.

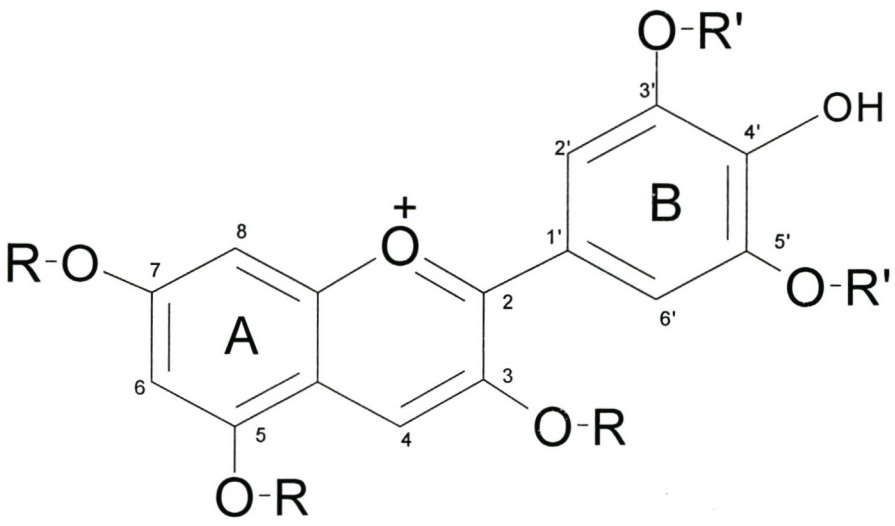

Fig. 2. A single anthocyanin molecule, showing the conventional structure numbering. R indicates any one of the common sugar residues involved in glycosylation of anthocyanins.

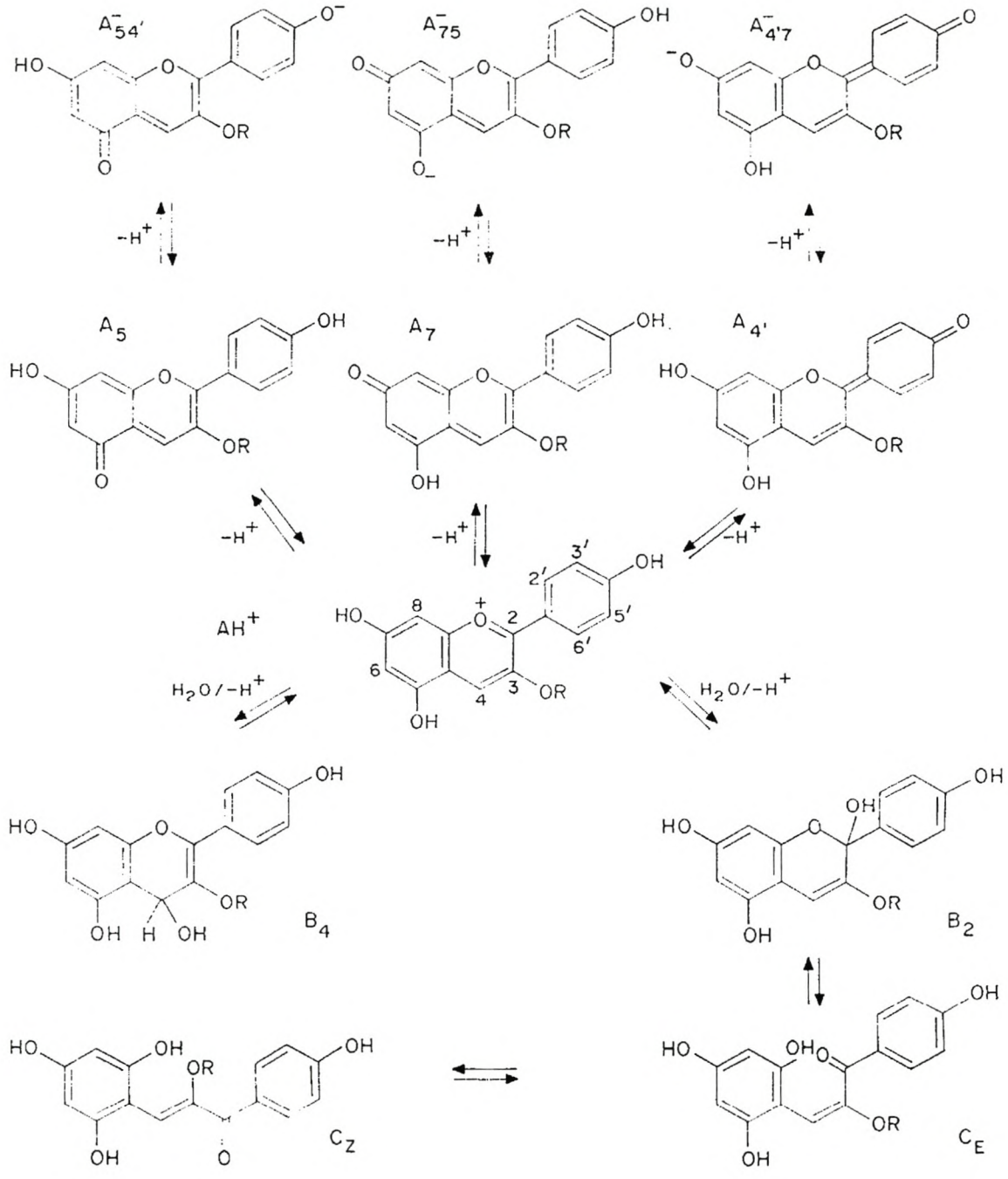

Fig. 3. Structural transformation of anthocyanins in aqueous solution (from Brouillard, 1983)

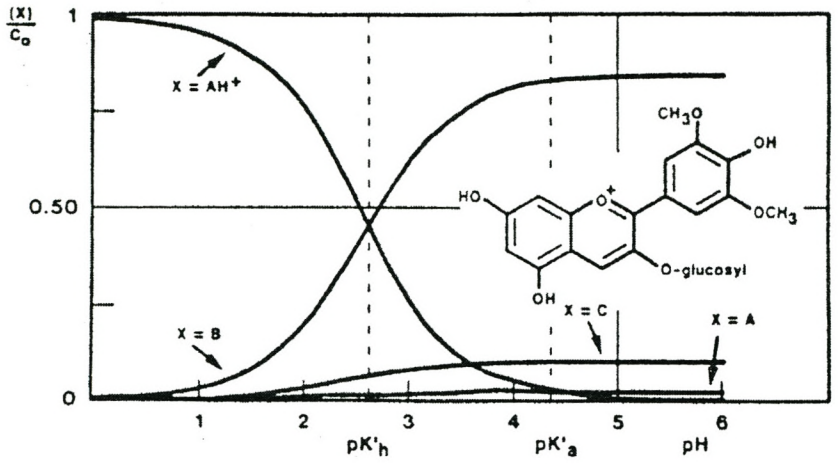

Fig. 4. Equilibrium distribution for AH^+, A, B and C with pH at 25°C for malvidin 3-glucoside (from Brouillard, 1982).

PAPER I: Extraction method optimisation for anthocyanin quantification in *Leucadendron* using reverse-phase high-performance liquid chromatography

Abstract

Extraction method optimisation is essential for identification, as well as quantitative analysis of anthocyanins. Trials were conducted to determine the optimum extraction solvent and extraction time for anthocyanin quantification in *Leucadendron*, using 'Safari Sunset' (*Leucadendron salignum x L. laureolum*) leaves as reference material. Acetone, 80% acetone, 5% formic acid in water and 5% formic acid in methanol were added to freeze dried sample respectively and allowed to extract for one hour. Extracts were analysed by reverse-phase HPLC. Extractions with methanol and water yielded extracts with a high content of pectin-like compounds, making them difficult to filter for HPLC. 80% acetone proved to be the most efficient extraction solvent, yielding the highest anthocyanin concentration, as well as showing the least amounts of insoluble compounds. Optimal extraction time was determined for 80% acetone by testing 1, 6 and 24-hour extractions. Although 6 hours extraction showed a slightly higher yield, a 1-hour extraction should suffice for quantification of anthocyanin changes during a growth season.

Many factors influence the stability of anthocyanins during and after the extraction procedure, such as type of extraction solvent, pH of solvent, temperature and ambient light conditions. According to Strack and Wray (1994) the extraction procedure should be optimised for a particular plant material, keeping the desired objective of the study in mind.

Anthocyanins consist of an aromatic ring with various polar substituents and may occur as even more complex forms via glycosylation and acylation (Mazza and Miniati, 1993). At low pH levels the anthocyanin molecule exists in a stabilised ionised form, resulting in an overall polar molecule (Brouillard, 1988). This characteristic makes a polar organic extraction solvent the natural choice, as anthocyanins are more soluble in polar than non-polar solvents (Delgado-Vargas

et al., 2000). Acidification of the solvent is required to prevent oxidation, as anthocyanins are unstable at neutral and alkaline pH (Brouillard, 1983).

The most common extraction solvent used for many years was methanol containing one percent hydrochloric acid. However, an increasing number of reports indicate that many highly acylated anthocyanins are labile and may be broken down by this strong mineral acid (Strack and Wray, 1994). Hence there is often a necessity to perform milder pigment extractions by using methanol containing weaker acids such as formic, citric or acetic acid (Durkee and Jones, 1969; Strack and Wray, 1994; Timberlake and Bridle, 1975). Furthermore, hydrochloric acid is fairly corrosive and may considerably shorten the lifespan of the analytical apparatus (Delgado-Vargas et al., 2000). Therefore we decided to conduct these trials using formic acid, rather than the more classical hydrochloric acid.

Initial anthocyanin extractions from *Leucadendron* leaves with methanol yielded extracts with high pectin-like compounds, which proved difficult to filter and concentrate for HPLC. Gil et al. (1997) encountered similar problems during phenolic extractions from fresh strawberries. Hence an acetone extraction was proposed for fruit with high pectin content (Chatterjee and Chatterjee, 1988; Garcia-Viguera et al., 1998). Furthermore, acetone is also commonly used for chlorophyll and carotenoid quantification (Lichtenthaler, 1987). This would enable us to combine the analysis of all pigments into a single procedure.

Hence, the objective of this study was to optimise the extraction methodology used for the quantification of anthocyanins in *Leucadendron* cultivars, by assessing the extraction efficiency of different solvent types and to determine the optimal extraction time of the most efficient solvent.

Materials and Methods

Plant material. Twenty 'Safari Sunset' shoots, uniform in colour, were obtained from a commercial farm in Grabouw situated in the Western Cape, South Africa

(34°10'S, 19°03'E). The shoots were placed in a bucket of water and immediately taken back to the laboratory for further preparations on the same day.

Eight of the most apical mature leaves surrounding the floral bud were removed for pigment analysis. Their fresh mass was determined before freezing in liquid nitrogen. In their frozen state, they were immediately placed in brown paper bags and stored at –80°C. Leaves were freeze-dried, milled to a fine powder and stored in a single large, lidded plastic vial, in a dry and dark cupboard.

Extraction solvent type. To test for the most efficient extraction medium, 10 ml of 100% acetone, 80% acetone, 5% formic acid (FA) in water and 5% FA in methanol was added to 200 mg sample in 50 ml polycarbonate centrifuge tubes respectively. Six replicates were used per treatment. The tubes were sealed and placed into a refrigerator at 4°C for 1 hour in the dark, whilst stirring. Extracts were centrifuged at 10000 x *g* for 10 minutes.

Optimal extraction time. The optimal extraction time was determined for 80% acetone only, as it proved to be the most suitable extraction medium for our quantification purpose. 10 ml of 80% acetone was added to 200 mg of freeze-dried sample and placed into a fridge at 4°C for 1 hour, 6 hours or 24 hours respectively. Samples were stirred continuously during the extraction process. Six replicates were used per treatment. Extracts were centrifuged at 10000 x *g* for 10 minutes.

Purification and pre-concentration of anthocyanins. Extracts were rotary evaporated on a Savant Speedvac Concentrator and re-dissolved in 5 ml 5% formic acid in water. Preparation of extracts for HPLC was done using a C18 Sep-Pak-T cartridge (Waters), activated with 5 ml of 100% methanol and washed with 5 ml of 5% formic acid in water. The extract was loaded and the cartridge rinsed three times with 5 ml of 5 % formic acid in water. Three ml of 5% formic acid in methanol was used for elution of anthocyanins and other phenolics. Extracts were filtered through a 0.45μm filter (Millex-HV, Millipore Corporation, Milford, MA) into reverse-phase high-performance liquid chromatography (HPLC) vials.

HPLC. Anthocyanins were separated and quantified using an HPLC system (HP 1100; Agilent Technologies, Palo Alto, CA). The analytical column was a 250 x 4.6 mm reverse-phase C_{18} Spherisorb (Phase Separations, Deeside, UK) column coupled with a Zorbax SB-C18 guard column (Agilent Technologies, Palo Alto, CA). Separation was performed with gradient elution. The mobile phase consisted of 5% formic acid in water (A) and 5% formic acid in methanol (B) with a linear gradient of 30 to 35.6% (B) for 21 minutes and then from 35.6 to 100% for the next 1 minute, followed by 4 minutes of 100% formic acid in methanol to clean the column between successive samples. Flow rate was 1 $ml \cdot min^{-1}$, with an injection volume of 15 µl. Two injections were done per vial for which an average was obtained. Scanning range was between 280 and 600 nm in steps of 2 nm. Hewlett Packard chromatic data analysis software (Chemstation, Revision A.06.03, 1998) was used to analyse detected signals and produce chromatograms at 520 nm for anthocyanins.

HPLC chromatograms indicated the presence of two major peaks and five or six minor peaks. A standard curve of cyanidin-3-galactoside (idaein-chloride) (Carl Roth, Karlsruhe, Germany) was used to calculate total anthocyanin concentration, and that of individual peaks. Concentrations are expressed as $g \cdot g^{-1}$ dry leaf mass.

Data analysis. The data was analysed with the General Linear Models (GLM) procedures of the SAS program (SAS release 6.12P, SAS Inst., Cary, NC).

Results and Discussion

The 80% acetone extraction was the most efficient of all solvents tested in terms of total anthocyanin yield, extracting almost 350 $g \cdot g^{-1}$ (16%) more than the classical methanol extraction (Fig. 1.). Furthermore, there was considerably less condensation of insoluble compounds, making the extracts far easier to filter for HPLC. The lower extraction efficiency of methanol and water could partly be ascribed to the high degree of insoluble compound formation, which appeared to

have a red tint, indicating possible enclosure of anthocyanins. Although 100% acetone has successfully been used for anthocyanin extraction in fruit peel, it proved unsuitable for freeze-dried *Leucadendron* leaves, indicated by the low yield and high insoluble compound formation. This can partly be ascribed to the fact that the majority of extractions using 100% acetone are done with fresh plant material, which, as a result of the water content, may dilute the acetone to an almost 80% acetone solution.

The analytical HPLC chromatograms of 'Safari Sunset' indicate that there were two major anthocyanin peaks (**1** and **2**) and five minor peaks (Fig. 2). There is a discrepancy between the different extraction media insofar both methanol and water, a late peak (**8**) with a retention time of ≈19 min is detected, which is absent in both acetone extractions. Detailed identification studies will have to be conducted to determine whether peak **8** is not a result of structural modifications and/or an aglycone of one of the major anthocyanins present. Furthermore, peak **8** could be a labile anthocyanin lost during the extraction procedure with acetone.

There was no significant difference in total anthocyanin yield between the 1, 6 and 24-hour 80% acetone extractions (Table 1). However, a significant difference was observed in the extraction quantity of the two major anthocyanins present. The 6-hour extraction proved to be optimal, yielding the highest concentration of both **1** and **2**. The 1-hour extraction yielded significantly less of peak **1** than the 24-hour extraction, yet extracted similar amounts of peak **2**. This seems to indicate that one hour was insufficient time to extract peak **1**, as the 6-hour and 24-hour extractions for peak **1** are not significantly different. The fact that there was no significant difference in quantities extracted for peak **2**, between the 1 and 24-hour extractions, indicates a possible degradation of peak **2** during the 24-hour extraction. Further identification and structure elucidation of individual anthocyanins present may provide an answer for this discrepancy.

The significant differences obtained with the various extraction solvents used, as well as in extraction time, highlight the importance of extraction method optimisation, however keeping the desired objective of the study in mind. To follow colour development of *Leucadendron* throughout a season an 80% acetone

extraction with a 1-hour extraction time should suffice, as trends will be clearly visible. Although a 5% formic acid in methanol could also be used to study pigment fluctuation trends over time, the acetone extraction had an advantage in that it allowed for simultaneous quantification of chlorophylls and carotenoids before purification for HPLC. However, if absolute anthocyanin concentrations are of importance, whether total or individual anthocyanins, an 80% acetone and an extraction time of six hours is recommended.

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Table 1. Comparison of anthocyanin extraction efficiency for 1, 6 and 24-hour 80% acetone extractions. Means separated by LSD (5%).

	Anthocyanins ($g \cdot g^{-1}$ DW) [z]		
Extraction time (hours)	Total	Peak 1	Peak 2
1	1968.6 a	766.8 b	848.5 b
6	2030.7 a	825.1 a	914.5 a
24	1973.8 a	804.4 a	830.1 b

z Means within each column followed by the same letter are not significantly different.

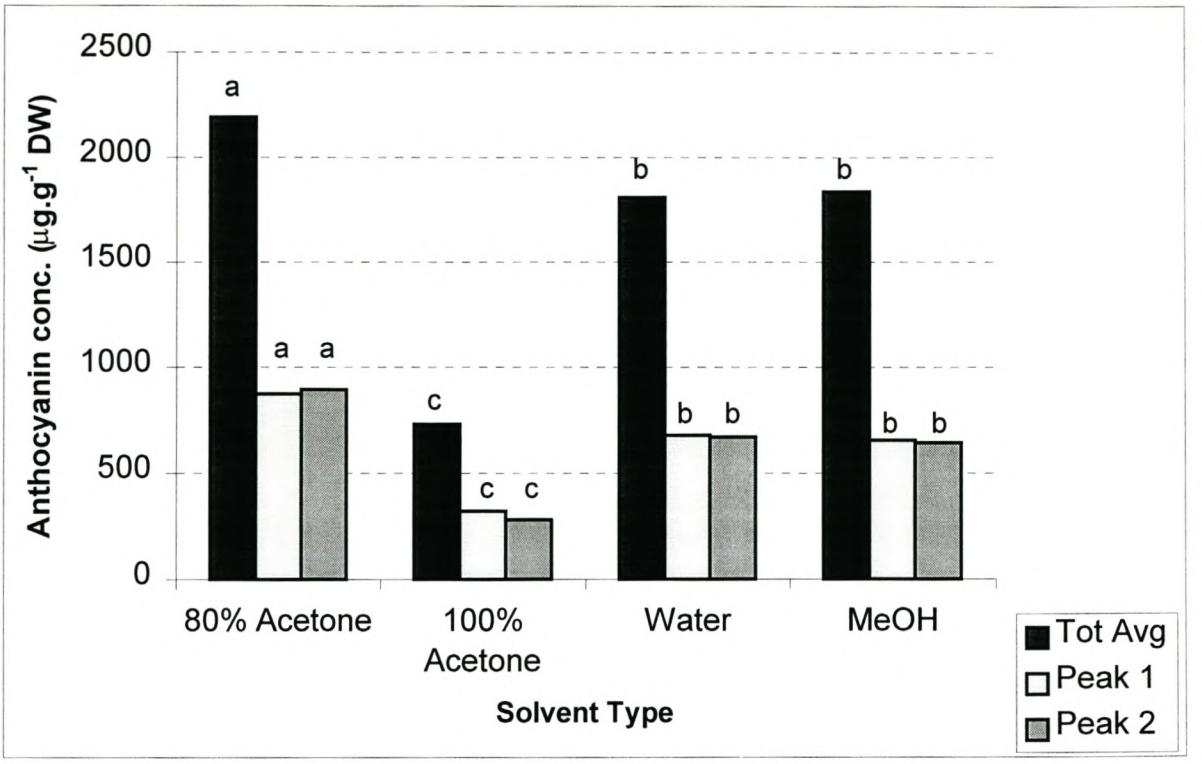

Fig 1. Comparison of anthocyanin extraction efficiency in *Leucadendron* cv. 'Safari Sunset', using different extraction solvents, with an extraction time of one hour. Mean separation between solvent types, within anthocyanin yields at 5% level LSD.

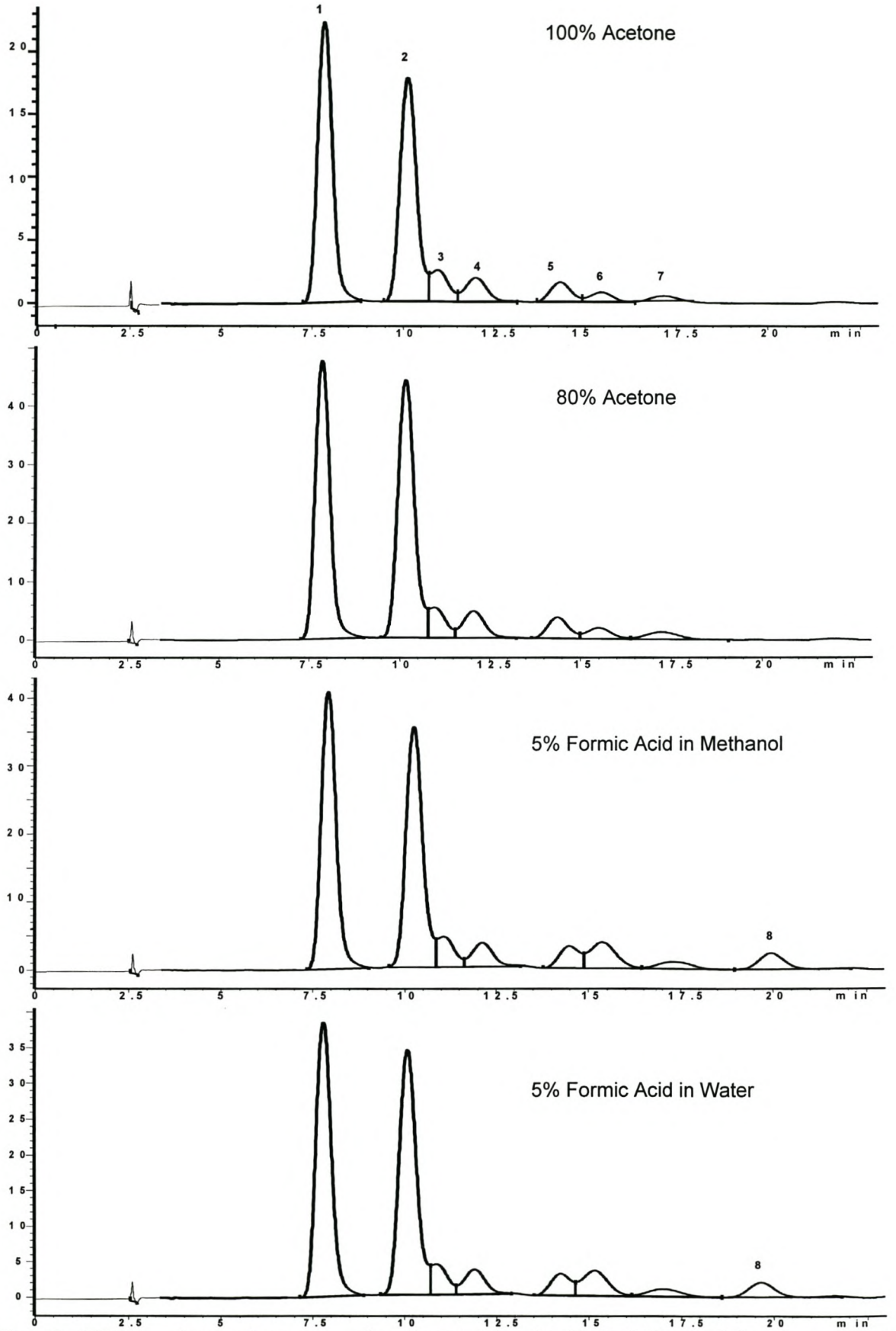

Fig. 2. HPLC chromatograms of anthocyanins in 'Safari Sunset' leaves, using different extraction solvents (detected at 520 nm).

PAPER II: RED COLOUR DEVELOPMENT IN *LEUCADENDRON* CULTIVARS 'SILVAN RED' AND 'SAFARI SUNSET'

Abstract

Anthocyanin, chlorophyll and carotenoid fluctuations were investigated in 'Silvan Red' (*Leucadendron laureolum* x *L. salignum*) throughout the season, since the marketability depends on red colour quality of the large 'flower head'. The observed colour loss is the result of anthocyanin degradation. There was no significant correlation between the fluctuation of anthocyanins and that of chlorophyll or carotenoids. Although a positive correlation was noted between anthocyanin content and existing environmental temperature and sunlight hours, it appears unlikely that they are the causal factors for the colour change. Anthocyanin degradation started during conditions of high light intensity and continued during a period of low temperature (autumn), both which are known to enhance anthocyanin synthesis. Therefore, it appears that colour development in 'Silvan Red' is developmentally controlled, but which may be influenced by environmental conditions. The fact that a colour loss is not reported for 'Safari Sunset' (*Leucadendron laureolum* x *L. salignum*), is most likely the result of a significantly higher anthocyanin concentration, which is a buffer against drastic visual colour changes apparent to the observer.

In South Africa, the genus *Leucadendron* contains about 60 species, collectively referred to as conebushes (Rebelo, 1995). Several species are cultivated commercially as cut flowers due to their decorative and often brightly coloured foliage. *Leucadendrons* are dioecious, where the female produces woody cones, not produced in the male counterpart (De Kock et al., 1994). Upon cessation of shoot growth a terminal inflorescence develops, enclosed by spirally arranged floral bracts. The floral bracts in the female are large, eventually forming a woody cone. Involucral bracts are small, brown and inconspicuous (Rebelo, 1995). The distal leaves (involucral leaves) around the inflorescence are enlarged, forming a showy 'flower head' which is often intensely coloured, generally red or yellow (Gerber, 2000). A few hybrid cultivars, such as 'Silvan Red' and 'Safari Sunset'

have been introduced to commercial markets in recent years and in addition to their desirable red colouration they are commercially viable due to their potential for very high yields (Barth et al., 1996).

Pigments responsible for red colour in 'Silvan Red' and 'Safari Sunset' are anthocyanins (Paper I) situated in the vacuoles of epidermal and hypodermal cells. Colour as we perceive it is based on complex interactions between total anthocyanin concentration, types of anthocyanins present and their relative concentration ratios as well as the blending effect of other pigments such as carotenoids and chlorophyll (Lancaster et al., 1994). Pigments *in vivo* are influenced greatly by external environmental factors, of which temperature and light are probably the most influential. Increased light intensity has been shown to have a promotive effect on anthocyanin synthesis in numerous fruit, vegetables and cut flowers (Ben-Tal and King, 1997; Biran and Halevy, 1974; Saure, 1990). High temperatures have been shown to prevent anthocyanin accumulation and enhance the degradation thereof (Deal et al., 1990; Marais et al., 2001; Oren-Shamir and Levi-Nissim, 1999).

The marketability of 'Silvan Red' largely depends on the red colour quality of the large 'flower head'. Shoots meeting export quality can generally be harvested from late February to early April (late summer to early autumn in the southern hemisphere). The harvest period is relatively short, as the involucral leaves turn from red to green and/or dull red/brown colour and do not meet the desired export quality standards. It would be desirable to extend or shift the harvesting period towards Mothers Day in May, when higher prices are obtained on international markets. The colour loss observed in 'Silvan Red' is not reported for 'Safari Sunset' and hence its marketing period stretches well beyond that of 'Silvan Red'.

The objective of this study was to determine the pigments responsible for the reported colour change in 'Silvan Red' by following anthocyanin, carotenoid and chlorophyll fluctuations throughout a growth season. Furthermore, differences in pigmentation between 'Safari Sunset' and 'Silvan Red' were investigated.

Materials and Methods

Plant material. 'Silvan Red' shoots were collected from the farm Protea Heights in Stellenbosch in the Western Cape, South Africa (33°58'S, 18°50'E). 'Safari Sunset' were obtained from a commercial farm in Grabouw also situated in the Western Cape (34°10'S, 19°03'E). Sample collection of 'Silvan Red' commenced on 8 February 2000 after cessation of shoot growth, once a measurable terminal bud had formed, and continued weekly until 19 July 2000. 'Safari Sunset' shoots were spot sampled between 7 June and 21 July 2001 and again from 16 February to 16 March 2001.

On the day that sampling commenced, 15 shoots were selected in the field for their uniformity in colour and apical bud dimensions (diameter and length), as well as similar shoot length. Colour was determined visually and apical bud dimensions measured with a calliper. A standard measuring tape was used to measure shoot length. The shoots were selected randomly from different rows and bushes, tagged and left as designated reference shoots, on which all subsequent sample collection was based.

On each subsequent sampling date, the reference shoots were again assessed visually for their colour, and apical bud dimensions were taken. Accordingly, 30 shoots similar to the reference shoots were picked and taken back to the laboratory for further processing. 'Silvan Red' shoots were divided into 3 repetitions with 10 shoots per replicate. 'Safari Sunset' shoots collected in 2001 were divided into 3 repetitions with 15 shoots per replicate.

The floral buds were removed from the shoots and their diameter and length determined with a calliper. To measure the length as accurately as possible, the points of the calliper were placed at the tip of the bud and at the base of the lowest floral bract respectively. An average bud size was obtained per replicate. The floral buds were dissected under a stereo-microscope.

Eight of the most apical mature leaves surrounding the floral bud were removed for pigment analysis. Their fresh mass was determined prior to freezing in liquid nitrogen. In their frozen state, they were placed in brown paper bags and stored at –80°C awaiting further analysis. Leaves were freeze-dried, milled to a fine powder and stored in a single large, lidded plastic vial and placed in a dry and dark cupboard.

Pigment Extraction. Anthocyanins, carotenoids and chlorophyll were extracted with 10 ml of 80% acetone, added to 200 mg sample in 50 ml polycarbonate centrifuge tubes. Tubes were sealed and placed in a refrigerator at 4°C for 1 hour in the dark, whilst stirring. Extracts where centrifuged at 10000 x *g* for 10 minutes.

Chlorophyll and carotenoid quantification. The supernatant was decanted into glass vials and the absorbance of carotenoids (A470 nm) and chlorophyll (A662 nm and A642 nm) read on a spectrophotometer (Beckman DU-64, California). Total carotenoid, chlorophyll a and b concentrations were calculated according to Lichtenthaler (1987).

Anthocyanin quantification. After spectrophotometer readings, the extracts were purified, pre-concentrated and analysed by reverse-phase HPLC according to Paper I.

Data analysis. The data was analysed with the General Linear Models (GLM) and Correlation (CORR) procedures of the SAS program (SAS release 6.12P, SAS Inst., Cary, NC).

Results and Discussion

The developmental period under investigation can be divided into 3 phases (Fig. 1). Phase **I** was characterised by a generally dome-shaped apical meristem and the production of spirally arranged floral bracts. From 8 March onwards, the

meristematic region became considerably more raised and showed the beginning of a central axis. As the apical meristem continued to produce new floral bracts, the older bracts shifted in a basipetal direction along a central elongated cone axis. The reproductive phase started at stage **A** (between 22 and 28 March) indicated by the formation of small florets in the axils of the floral bracts. Hence phase **II** was characterised as the floral development phase, which continued until the beginning of June. Stage **B** (about 19 June) shows the protrusion of the stigma between floral bracts, hence indicating the start of pollination, phase **III**. By 11 to 14 July the majority of stigmata had wilted, indicating the end of the pollination phase.

The harvesting period of 'Silvan Red' was relatively short as production of shoots meeting export quality in terms of adequate red colouration stretched from late February to early April, after which most shoots showed an undesirable green, and often reddish-brown colouration. The loss of red colour in 'Silvan Red' can be ascribed to the net degradation of anthocyanins during phases **I** and **II** (Fig.1). As the leaves had attained their maximum size at the beginning of the sampling period, the reduction in anthocyanin levels, was not the result of dilution. In 'Silvan Red', similar to many other higher plants possessing the capability to synthesise foliar anthocyanins, new growth flushes show an intensely bright red colouration. As leaves mature anthocyanins rapidly disappear, indicating a possible photo-protective function in young leaves. Anthocyanins have been noted to act as visible light screens, protecting the photosynthetic tissue against photoinhibition during periods of excess irradiation or physiological stages when the tissues are susceptible to photoinhibition, such as in young developing leaves (Smillie and Hetherington, 1999; Steyn et al., 2002). Upon cessation of shoot growth and subsequent development of the terminal inflorescence, the youngest distal leaves (involucral leaves) form the large red 'flower head'. Here again it appears that as these leaves mature, a fading of red colour occurs as indicated by the total net decrease in anthocyanin levels (Fig.1). Although it appears that continual anthocyanin degradation coincided almost precisely with the first morphological changes observed in the terminal bud (8 March – raised meristematic region), it should be noted that the gradual colour loss pattern appears similar to that

observed during the development of preceding basal leaves (personal observation). The chlorophyll accumulation at the beginning of phase **I** is probably related to involucral leaves reaching maturity. Anthocyanin levels in leaves commonly reach a maximum before maximum chlorophyll levels and disappear as leaves change from sink to source (Choinski and Wise, 1999; Kubasek et al., 1992). Accumulation and maintenance of anthocyanins is energy costly and may reduce light capture and ultimately reduce carbon assimilation (Steyn, 2002). Therefore, as chlorophyll levels increase and leaves acclimatise to irradiation levels, it is possibly an unnecessary waste of energy, trying to maintain a consistently high anthocyanin content during a phase they are no longer required.

Dissections of the cones revealed the presence of nectaries at the base of the ovaries and, according to Rebelo (1995), small flowered *Leucadendrons* with nectaries are pollinated by a number of different beetle, fly and wasp species. The sudden increase in red colour of the involucral leaves (phase **III**), after the emergence of the stigma, therefore most likely serves as an attractant for pollinators (Harborne, 1993).

There are two main anthocyanins in 'Silvan Red', which will be referred to as peak **1** and peak **2** respectively (Fig. 5). The reduction of total anthocyanin (which includes all peaks) was the result of the degradation of both major anthocyanins present (Fig. 2). However, the increase in total anthocyanin concentration during phase **III** appears to be due to the rapid synthesis of peak **2**, whereas that of peak **1** is far more gradual. Currently, there is no evidence to suggest a possible reason for this phenomenon.

The seasonal anthocyanin fluctuation appears, at least in terms of general functionality, fairly similar to that reported for apples. Apples show two distinct peaks of anthocyanin synthesis. The first peak occurs in young, developing fruit during the phase of active cell division, proposed for photoprotection. The second peak coinciding with ripening in red apple cultivars, proposed for attraction of seed dispersal agents (Saure, 1990). Statistically, there was no significant correlation

between anthocyanin fluctuations and that of chlorophyll or carotenoids throughout the season.

Although anthocyanin fluctuations, especially peak **1**, appeared to be positively correlated to temperature and sunlight hours, the relation is doubtful (Table 1). Low temperatures and high light intensity have been noted to induce and/or enhance anthocyanin synthesis in many crops, whereas high temperatures and low light intensities generally result in poor colouration (Mazza and Miniati, 1993; Reay, 1999; Viljoen and Huysamer, 1995). Hence, if temperature is to be considered a major influential factor in colour development in 'Silvan Red', the decreasing temperature during the sampling period should have favoured anthocyanin synthesis rather than its degradation (Fig. 2). Similarly, as the continual anthocyanin degradation already started at the beginning of March when leaves were still exposed to long periods of high irradiation levels (Fig. 3), a major functional role of light is not expected. Furthermore, irradiation levels, even in autumn are not expected to be limiting to anthocyanin synthesis. Hence it appears that colour development in 'Silvan Red' is developmentally regulated. Preliminary trials conducted to manipulate colour of 'Silvan Red' shoots did not result in any significant visual responses. Shoots were subjected to various temperature regimes (10 °C, 20 °C and 30 °C) at high light intensities, but no significant visual differences between treated and control shoots were observed. In 1999, a bagging trial was conducted in a commercial orchard, where shoots were bagged for three weeks. Subsequent anthocyanin analysis showed no significant difference between bagged and non-bagged shoots (data not presented). This further supported the theory that anthocyanin synthesis in 'Silvan Red' is developmentally regulated, as neither light nor temperature treatments elicited a visual response. These assumptions, however, do not negate the possibility of temperature or irradiance to influence the onset and rate of anthocyanin degradation indirectly by influencing the developmental rate of young leaves.

Although the intensity of red colour is directly related to anthocyanin content, the expression of colour in any plant part is based on complex interactions between anthocyanins and the blending effects of other pigments (mainly chlorophyll and

carotenoids) present in the same tissue (Brouillard, 1983; Lancaster et al., 1994). Colour and colour quality may change over time, as the ratios of relevant pigments change. However, there is insufficient evidence to speculate on the extent to which pigment fluctuations are apparent as colour differences to the observer (Lancaster et al., 1994). As anthocyanins degrade throughout the season, the green colour of chlorophyll becomes more apparent, since they are no longer masked. Carotenoid levels remained fairly constant throughout the sampling period (Fig. 1.) and probably contributed significantly to colour expression. In chrysanthemums, the presence of anthocyanins together with carotenoids was reported to modify the colour from orange-red to bronze (Teynor et al., 1989). Hence, the fading of red colour was the result of anthocyanin degradation, but the commonly observed green, dull reddish-brown colouration was most likely the result of the visual blending of all pigments present.

The above shown red colour loss of 'Silvan Red' is not reported for 'Safari Sunset' of the same parentage. Involucral leaves of 'Safari Sunset' have an intense red-purple colouration (not seen in 'Silvan Red'), which is generally retained throughout the season. From Table 2 it is evident that the total anthocyanin concentration of 'Safari Sunset' is almost double that of 'Silvan Red', which probably accounts for the intense colouration, keeping in mind that intensity of colour is directly related to anthocyanin concentration (Brouillard, 1983). Furthermore, if assumed that a similar rate of anthocyanin degradation occurs in 'Safari Sunset' as seen in 'Silvan Red', it would take far longer before a similar drastic colour loss would be observed. At the point when 'Silvan Red' shoots became unmarketable (beginning of April), about a 50% reduction in anthocyanin content had occurred, resulting in an anthocyanin concentration of 723 $\mu g.g^{-1}$. A 50% degradation in 'Safari Sunset' would result in a concentration of approximately 1300 $\mu g.g^{-1}$, which is almost as high as the highest anthocyanin content encountered in 'Silvan Red' throughout the season. High anthocyanin concentrations have been noted to buffer against changes in colour as we perceive it, i.e. a loss of, for example, 200 $\mu g.g^{-1}$ anthocyanin will be noted as a drastic colour change by the observer when the total anthocyanin concentration is low, compared to the same loss occurring when the anthocyanin concentration is

very high. Colour is also greatly influenced by the types of anthocyanins present, as well as their respective ratios. HPLC analysis showed that the same types of anthocyanins exist in both 'Silvan Red' and 'Safari Sunset', indicated by their similar retention times and λ max of 520 nm for peak **1** and 528 nm for peak **2** respectively (Fig. 5). Hence, the purple colour encountered in 'Safari Sunset' can not be explained in terms of a difference in the types of anthocyanins. There was, however, a significant difference in the ratio of the two major pigments present (Table 2). In 'Safari Sunset', peak **1** and peak **2** are found in almost equal ratios, whereas in 'Silvan Red' the concentration of peak **1** is almost half that of peak **2**. It appears that the higher concentration of peak **1** and probably the higher concentration of both peaks are responsible for the observed purple colour in 'Safari Sunset'. However, this can only be confirmed when peak **1** has been identified.

According to Harborne (1965) developmentally regulated accumulation of anthocyanin tends to be transient in nature. In 'Silvan Red' anthocyanins appear to be synthesised at different phenological stages as required and subsequently degraded: synthesis in young developing leaves (photoprotection); degradation during floral development in autumn (not required); synthesis during the pollination phase (attraction of pollinators). To be able to harvest 'Silvan Red' shoots of export colour quality in May (Mothers Day), we suggest further research in horticultural practices that manipulate the growth of *Leucadendron*. If cessation of shoot growth can be delayed, for example by pruning at different times of the year, the observed colour loss might shift accordingly.

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Table 1. Correlation between anthocyanin concentration, average daily temperature and average daily sunlight hours in the *Leucadendron* cultivar 'Silvan Red' during the 2000 growth season.

Anthocyanin concentration ($g \cdot g^{-1}$)	**Average daily temperature (°C)**	**Average daily sunlight hours (h)**
Total Anthocyanin	0.500[z]	0.564
Significance	0.0001[y]	<0.0001
Peak 1	0.712	0.728
Significance	<0.0001	<0.0001
Peak 2	0.237	0.325
Significance	0.083	0.016

[z] Pearson correlation coefficients

[y] Probability > R under Ho:Rho = 0

Table 2. Comparison of total anthocyanin concentration, peak 1 and peak 2, and respective peak ratios between the *Leucadendron* cultivars 'Silvan Red' and 'Safari Sunset' during the 2000/2001 season.

Safari Sunset					Silvan Red				
Date	Antho. ($g \cdot g^{-1}$)	Peak1 ($g \cdot g^{-1}$)	Peak 2 ($g \cdot g^{-1}$)	Ratio P1/P2	Date	Antho. ($g \cdot g^{-1}$)	Peak 1 ($g \cdot g^{-1}$)	Peak 2 ($g \cdot g^{-1}$)	Ratio P1/P2
00/06/07	2281.73	897.55	1046.82	0.86	00/06/05	318.09	53.05	228.27	0.23
00/06/23	2037.12	731.60	975.77	0.75	00/06/19	389.96	59.78	272.07	0.22
00/07/08	2049.10	420.57	1260.14	0.33	00/07/11	953.17	90.19	712.88	0.13
00/07/21	2146.88	546.79	1220.68	0.45	00/07/19	1111.89	109.14	829.62	0.13
01/02/16	1900.20	834.01	723.73	1.15	00/02/14	1447.13	421.30	721.15	0.58
01/03/02	2602.06	1075.87	1054.29	1.02	00/03/08	1288.75	304.11	720.24	0.42
01/03/16	2611.21	1056.77	1082.25	0.98	00/03/15	1081.87	243.17	623.33	0.39

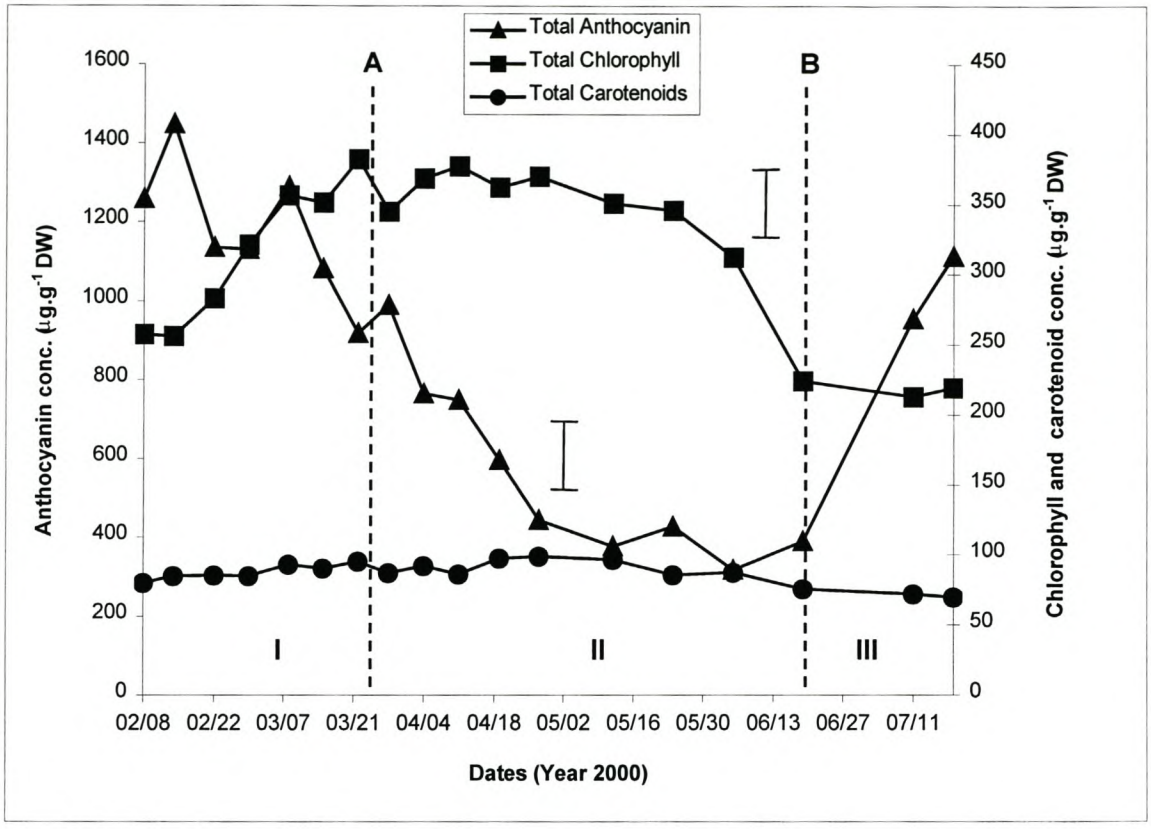

Fig. 1. Anthocyanin and chlorophyll fluctuations in the flower heads of the *Leucadendron* cultivar 'Silvan Red' during the 2000 season. Stages: (**A**) start of floret production; (**B**) protrusion of stigma between floral bracts. Phases: (**I**) production of floral bracts; (**II**) floral development in axils of floral bracts along a central axis; (**III**) pollination phase. Bars represent 5% LSD value.

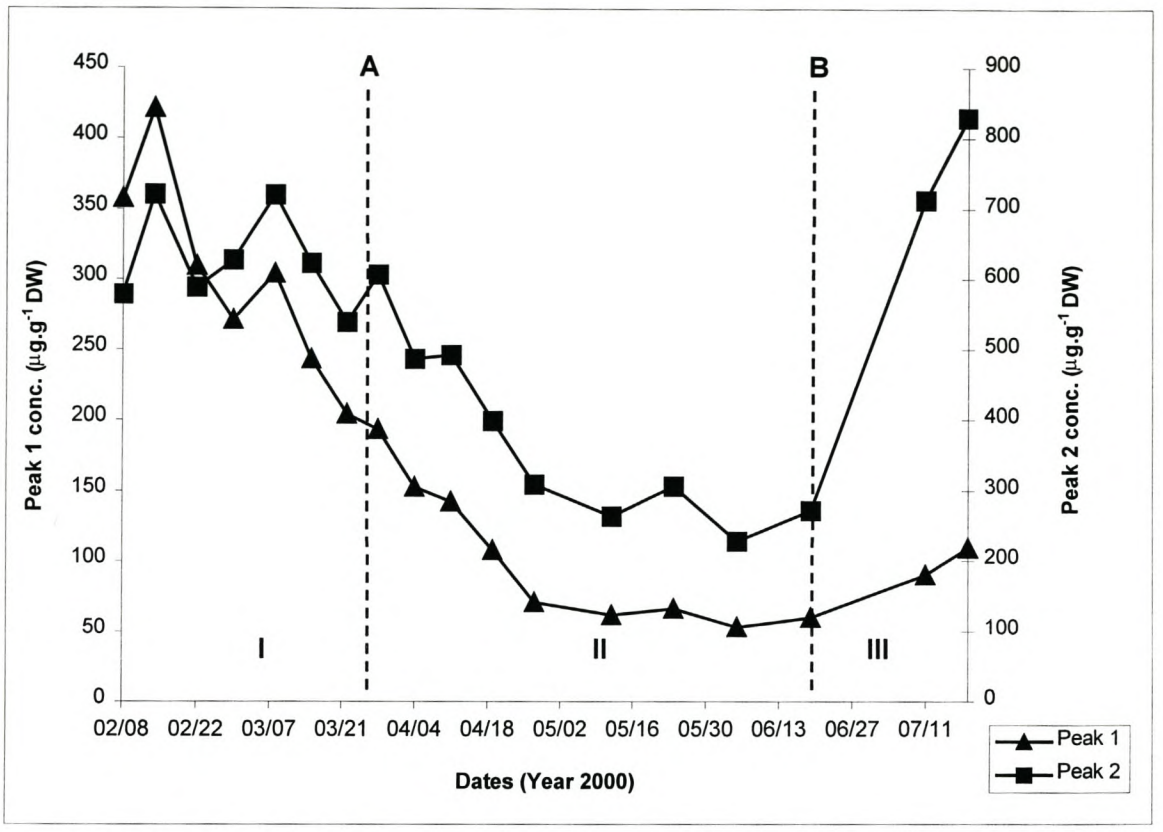

Fig. 2. Fluctuations of the two major anthocyanins (peak **1** and peak **2**) found in flower heads of the *Leucadendron* cultivar 'Silvan Red' during the 2000 growth season. Stages: (**A**) start of floret production; (**B**) protrusion of stigma between floral bracts. Phases: (**I**) production of floral bracts; (**II**) floral development in axils of floral bracts along a central axis; (**III**) pollination phase.

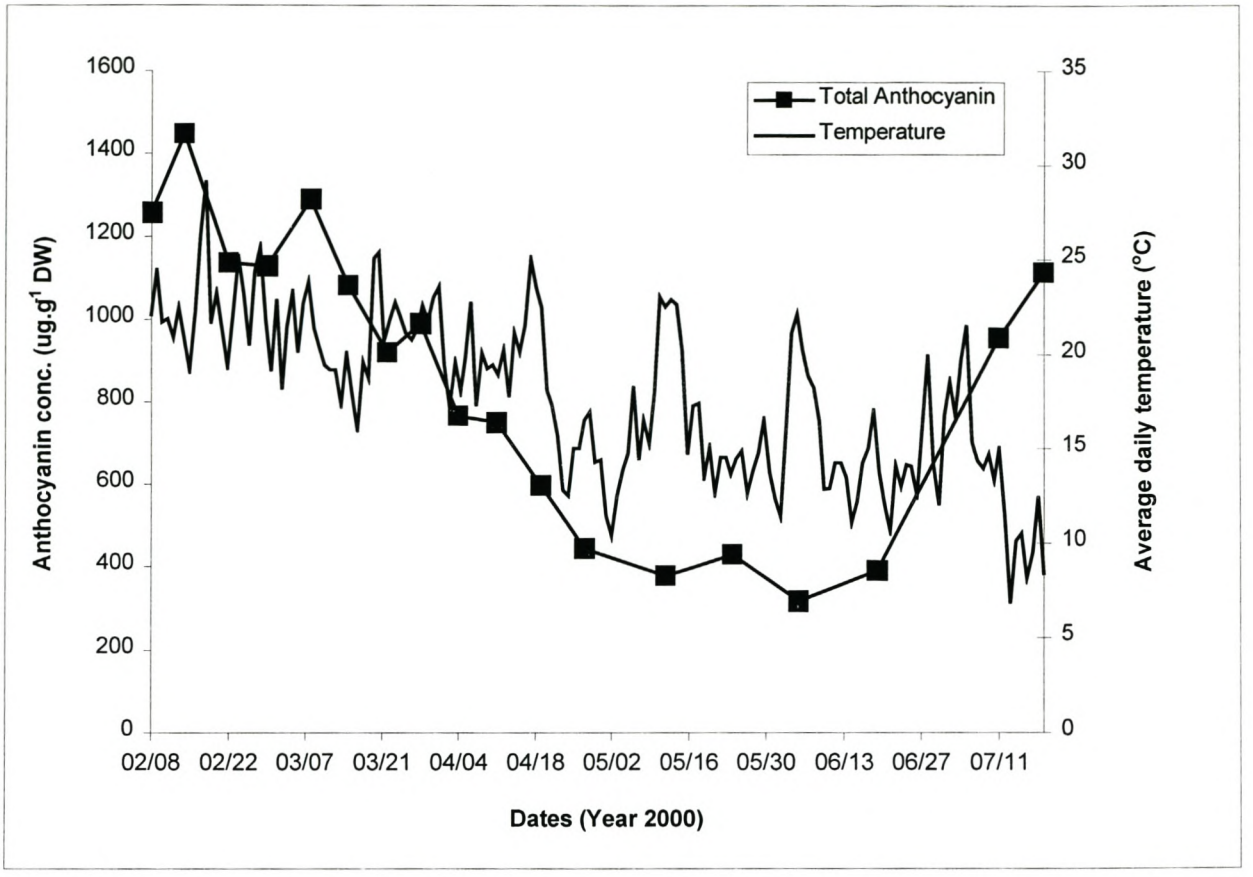

Fig. 3. Fluctuations of average daily temperature and anthocyanin concentration during the 2000 growth season of the *Leucadendron* cultivar 'Silvan Red'.

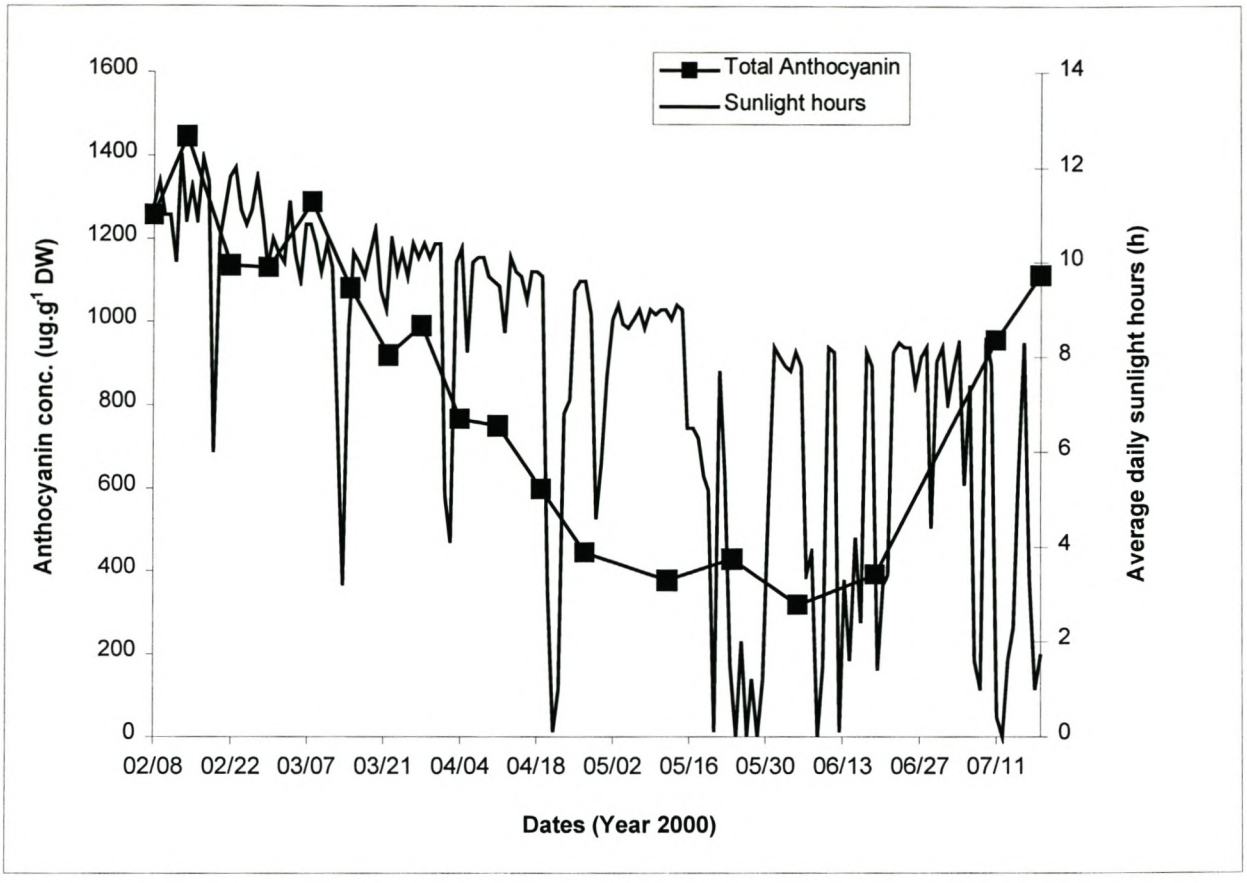

Fig. 4. Fluctuations of average daily sunlight hours and anthocyanin concentration during the 2000 growth season of the *Leucadendron* cultivar 'Silvan Red'.

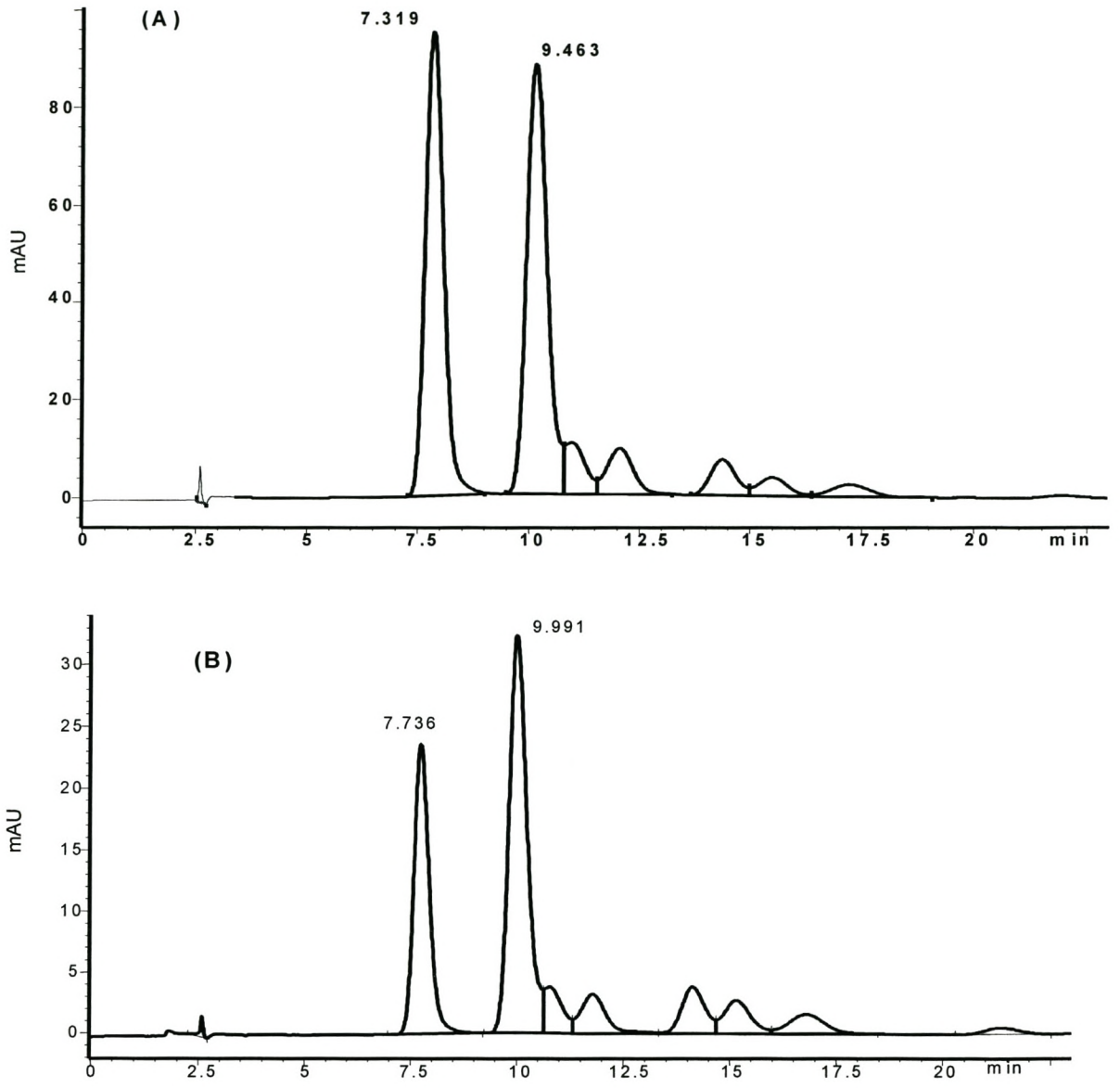

Fig. 5. HPLC chromatograms of anthocyanins in 'Safari Sunset' (**A**) and 'Silvan Red' (**B**) leaves (detected at 520 nm).

PAPER III: RED COLOUR DEVELOPMENT IN THE *PROTEA* CULTIVARS 'IVY', 'CARNIVAL' AND 'SYLVIA'

Abstract

Colour development of the innermost involucral bracts of three commercial *Protea* cultivars, Ivy (probably *Protea aurea* x *P. punctata*), Carnival (*Protea compacta x P. neriifolia)* and Sylvia (*P. eximia x P. susannae*), was followed from an initially selected inflorescence size to commercial harvest (when flowers have opened slightly). 'Ivy', 'Carnival' and 'Sylvia' show a light pink, dark pink and red colouration respectively, which has been ascribed to differences in total anthocyanin concentration. Total concentration of the major anthocyanin peak reported at harvest was 386 $\mu g.g^{-1}$ for 'Sylvia', about 259 $\mu g.g^{-1}$ for 'Carnival' and 68 $\mu g.g^{-1}$ for 'Ivy'. Colour development in 'Carnival' showed a quadratic trend with time, with the highest rate of anthocyanin synthesis occurring a week prior to harvest. Colour development in 'Ivy' and 'Sylvia' was shown to be linear with time. As inflorescences are closed during their development and anthocyanin synthesis in the innermost involucral bracts occurred in darkness, light does not appear to have a major influence on colour development. Temperature did not appear to have a significant effect on flower colour, as flowers developing later in the season, when ambient temperatures were lower, showed no significant differences in anthocyanin concentration to those harvested earlier. Hence, it appears that colour development in all three cultivars is developmentally regulated.

Proteas have shown great potential as cut flowers and in recent years are produced in commercial plantations in South Africa, to ensure products of more uniform quality (Gerber, 2000). High production potentials and especially visual characteristics such as colour are important selection criteria in determining the suitability of a cultivar for commercial production. Flowers of high colour quality are generally desired on international markets and tend to attain higher prices.

Pigments responsible for the pink to red colouration in the three commercial cultivars 'Carnival' (*Protea compacta x P. neriifolia)*, 'Ivy' (probably *P. aurea x P. punctata*) and 'Sylvia' (*P. eximia x P. susannae*) are anthocyanins, situated in the vacuoles of epidermal cells. Pigments *in vivo* may be influenced by external environmental factors, of which temperature and light are probably the most

influential. Increased light intensity and low temperatures have been shown to have a promotive effect on anthocyanin synthesis in numerous fruit, vegetables and cut flowers (Ben-Tal and King, 1997; Biran and Halevy, 1974; Saure, 1990). High temperatures have been shown to prevent anthocyanin accumulation and enhance the degradation thereof (Deal et al., 1990; Marais et al., 2001; Oren-Shamir and Levi-Nissim, 1999).

The colour development in Protea cultivars has, at least to out knowledge, not been investigated thus far. The objective of this paper was to investigate colour development in Protea during the season.

Materials and Methods

Plant material. 'Ivy', 'Carnival' and 'Sylvia' shoots were obtained from the commercial farm Protea Heights in Stellenbosch, Western Cape, South Africa (33°58'S, 18°50'E). Sampling commenced on 14 February 2001 for 'Ivy', 8 February 2001 for 'Carnival' and 25 February 2001 for 'Sylvia' and ended when flowers had reached commercial harvesting maturity, which is generally, when the flowers have opened slightly. Shoots were collected every two days for 'Ivy', weekly for 'Carnival' and every six days for 'Sylvia'. Colour development was followed again on a new set of flowers for 'Ivy' and 'Carnival' from 11 March 2001 onwards until commercial harvest, with the same sampling intervals as mentioned before. The two periods for 'Ivy' and 'Carnival' will be referred to as 'early flowers' and 'late flowers' respectively. As Sylvia shoots became unavailable we were unable to follow colour development for a second run.

On the day that sampling commenced, 15 shoots of similar inflorescence size (diameter and length) were selected in the field for each cultivar. Flowers were selected from different rows and bushes, tagged and left as designated reference shoots, on which all subsequent sample collection was based. To determine initial inflorescence dimensions for each cultivar, several flowers of different sizes (length and diameter) were dissected and the starting size chosen as the one,

where the innermost involucral bracts did not show any red colouration. The following starting sizes were determined (basal diameter x length): 30mm x 50mm for 'Ivy', 30 mm x 65 mm for 'Carnival' and 40 mm x 50 mm for Sylvia. The same initial inflorescence sizes were used at the start of measurement for 'late flowers' in 'Carnival' and 'Ivy'.

On each subsequent sampling date, the reference shoots where again assessed for their flower dimensions. Accordingly, 30 shoots similar to the reference shoots were picked and taken back to the laboratory for further processing. Shoots were divided into 3 repetitions with 10 shoots per replicate. Inflorescence fresh weight, dry weight, length and diameter were determined.

For 'Carnival' and 'Sylvia', 30 and for 'Ivy', 20 of the innermost involucral bracts were removed for pigment analysis. Their fresh mass was determined and then frozen in liquid nitrogen. In their frozen state, they were placed in brown paper bags and stored at –80°C awaiting further analysis. Involucral bracts were freeze-dried, milled to a fine powder and stored in a dark and dry cupboard in lidded plastic vials.

Pigment extraction and anthocyanin quantification by reverse-phase HPLC was done as described in Paper I.

Data analysis. The data was analysed with the General Linear Models (GLM) procedure of the SAS program (SAS release 6.12P, SAS Inst., Cary, NC).

Results and Discussion

HPLC analysis showed that the pigments responsible for red colour in 'Ivy', 'Carnival' and 'Sylvia' are anthocyanins. The same major anthocyanin in terms of highest concentration is present in all three cultivars (peak 2), as indicated by the similar retention time of about 10 min and λ max of 520 nm. Chromatograms showed the presence of four to six minor peaks, whose occurrence was found to

be extremely variable. As peak 2 is the main anthocyanin found in all cultivars and responsible for the majority of red colouration, we report only on this peak.

For both 'Ivy' and 'Carnival', the flowers were significantly smaller than those of the first sampling period (Table 1). It has been reported for various *Protea* cultivars, that the flowers harvested later in the season tend to be smaller and of lower quality than those obtained early in the season (G. Jacobs, personal communication). At harvest, 'Ivy' was the smallest inflorescence, as indicated by the 31 mm x 80 mm (basal diameter x length) compared with the dimensions of 44 mm x 117 mm and 48 mm x 102 mm for 'Carnival' and 'Sylvia' respectively. This is also evident from the inflorescence fresh weight of 209 g for 'Ivy', 526 g for 'Carnival' and 851 g for 'Sylvia' (Table 1). The larger size of 'Carnival' and 'Sylvia', necessitates for a longer development period of 21 and 27 days respectively in comparison to the 14 days till harvest in 'Ivy'.

During inflorescence development of 'Carnival' the fresh weight (whole inflorescence) showed an almost two fold increase from the start of measurement to harvest in both the first and second sampling period (Fig 1). This increase showed a quadratic trend with time in early flowers and a strong linear tendency in the late flowers. Dry weight accumulation of the 30 innermost involucral bracts was linear with time in both early and late flowers, showing an almost three fold increase. Concurrent with floral development as indicated by fresh and dry mass accumulation, was the synthesis of anthocyanins in the innermost involucral bracts. Anthocyanin synthesis showed a strong quadratic trend with time (Fig. 2) in both late and early flowers. During the first week, after the start of measurements, anthocyanin concentration showed a slower increase (an average of 57.5 $\mu g.g^{-1}$), than during the second week, which shows an average increase of 127.7 $\mu g.g^{-1}$. In the last week, just prior to harvest, a slight decrease in anthocyanin levels are observed for both sampling periods (10 to 20 $\mu g.g^{-1}$). Hence, it appears that the majority of colour development in 'Carnival' takes place just about two weeks prior to harvest. The concentration of peak 2 increased five fold from an initial content of 46.2 $\mu g.g^{-1}$ at the start of measurement to 227.5 $\mu g.g^{-1}$ at harvest in the early flowers and a 4.4 fold increase from 53.4 $\mu g.g^{-1}$ to 259.7 $\mu g.g^{-1}$ was observed in the late flowers. Although the difference in total concentration of peak 2 between

the first and second sampling period at harvest was about 32 $\mu g.g^{-1}$, it was not statistically significant and a visual effect was not noted (Table 1). There was no significant difference in chlorophyll or carotenoid levels at harvest between the two sampling periods.

Fresh weight accumulation in 'Ivy' showed a strong linear relationship with time in early flowers, whereas in the late flowers a quadratic trend was noted (Fig. 3). Similarly, the dry weight accumulation was linear in the early flowers and quadratic trend with time in the late flowers. Anthocyanins accumulated linearly for both sampling periods reaching a maximum at harvest of 47.8 $\mu g.g^{-1}$ and 68.1 $\mu g.g^{-1}$ respectively, which was not significantly different and a visual difference was not noted (Table 1). However, a significant difference in peak 2 content was encountered at the start of measurement between the two sampling periods, with an anthocyanin concentration of 19 $\mu g.g^{-1}$ and 46.5 $\mu g.g^{-1}$ respectively (Fig 4). This has been ascribed to the fact that the start of sampling was based on the same initial inflorescence size. As flowers harvested later in the season were shown to be significantly smaller than those harvested earlier, these flowers were developmentally more advanced, until they reached the desired inflorescence size as was evident from the red colour of the involucral bracts at the first harvest date. This is supported by the difference in dry mass of 3.9 g at the start of measurement of the early flowers, compared to 6.1 g dry mass at the start of sampling of late flowers. The early flowers only attained a dry mass of around 6 g, six days later, when the anthocyanin concentration was 39.8 $\mu g.g^{-1}$, similar to that at the start of the late flowers. Similarly to 'Carnival', no significant difference was found between chlorophyll and carotenoid concentration at harvest between late and early flowers (Table 1).

Fresh weight and dry weight accumulation during inflorescence development in 'Sylvia' showed a linear trend over time, with an almost three fold increase from the start of sampling to harvest. Associated with the floral development was a linear increase in anthocyanin concentration, reaching a maximum at harvest of 386.1 $\mu g.g^{-1}$ (Fig. 6). This was considerably higher than the concentrations encountered in both 'Ivy' and 'Carnival'.

The flowers of 'Ivy', 'Carnival' and 'Sylvia' show light pink, dark pink and red colouration respectively. This has been ascribed to the difference in the concentration of peak 2 encountered in the involucral bracts at commercial harvest, when flowers have opened slightly (Table 1). Red colour intensity is generally directly related to the total anthocyanin content (Brouillard, 1983). The presence of minor anthocyanins may further modify the colour perceived, despite their significantly lower concentrations.

Inflorescences are closed during the development and only open shortly before the harvest date. As we followed the colour development of the innermost involucral bracts, light is not expected to be a major influential factor in anthocyanin synthesis, as they accumulate in complete darkness. Furthermore, 'Ivy' shoots that were bagged for the entire duration of the first sampling period, did not show a significant difference in anthocyanin levels to control shoots (data not presented). Temperature did not appear to have a significant effect on colour development as in 'Ivy' and 'Carnival', flowers developing later in the season, when average temperatures were lower, showed no significant differences in anthocyanin concentration than those harvested earlier.

Hence, in conclusion, it appears that colour development in Protea appears to be developmentally regulated. In 'Ivy' and 'Sylvia', anthocyanin accumulates gradually throughout the development period until harvest. In 'Carnival', however, the majority of colour development appears to take place a week prior to harvest.

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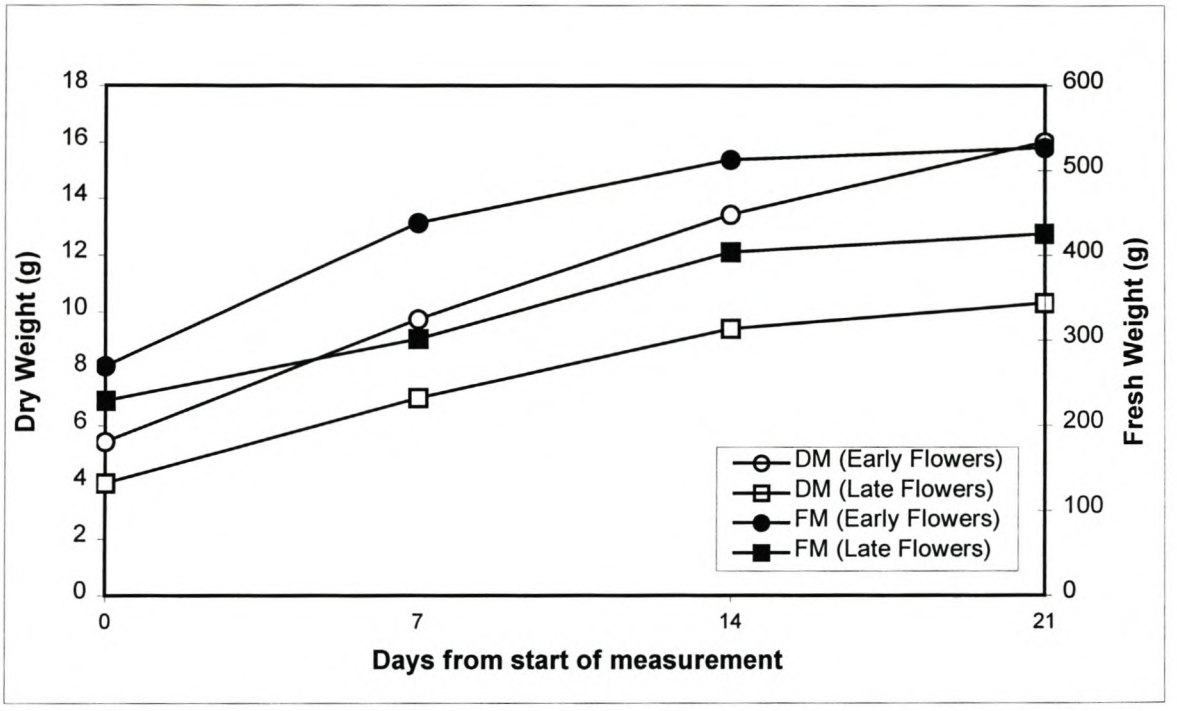
Table 1. Concentration of the major anthocyanin (peak 2), flower size, fresh weight, chlorophyll and carotenoid concentrations of the *Protea* cultivars 'Ivy', 'Carnival' and 'Sylvia' at commercial harvest (when flowers have opened slightly) during the 2001 growth season.

	Harvest Date	Fresh Weight (g)	Length (mm)	Diameter (mm)	Chlorophyll conc. ($g \cdot g^{-1}$)	Carotenoids conc. ($g \cdot g^{-1}$)	Peak 2 conc. ($g \cdot g^{-1}$)
Ivy	28 Feb. [x]	208.86 a [z]	80.04 a	31.03 a	44.07 a	43.96 a	47.82 a
	23 Mar.	177.78 b	74.86 b	29.10 b	67.23 a	51.67 a	68.06 a
Carnival	1 Mar. [y]	526.72 a	117.40 a	43.51 a	62.66 a	22.80 a	227.49 a
	1 Apr.	472.70 b	112.85 b	36.80 b	61.86 a	25.67 a	259.65 a
Sylvia	24 Mar.	851.17	102.39	47.58	56.57	35.45	386.05

x Harvest dates for 'Ivy' of the early flowers (14 February to 28 February) and the late flowers (11 March to 23 March).

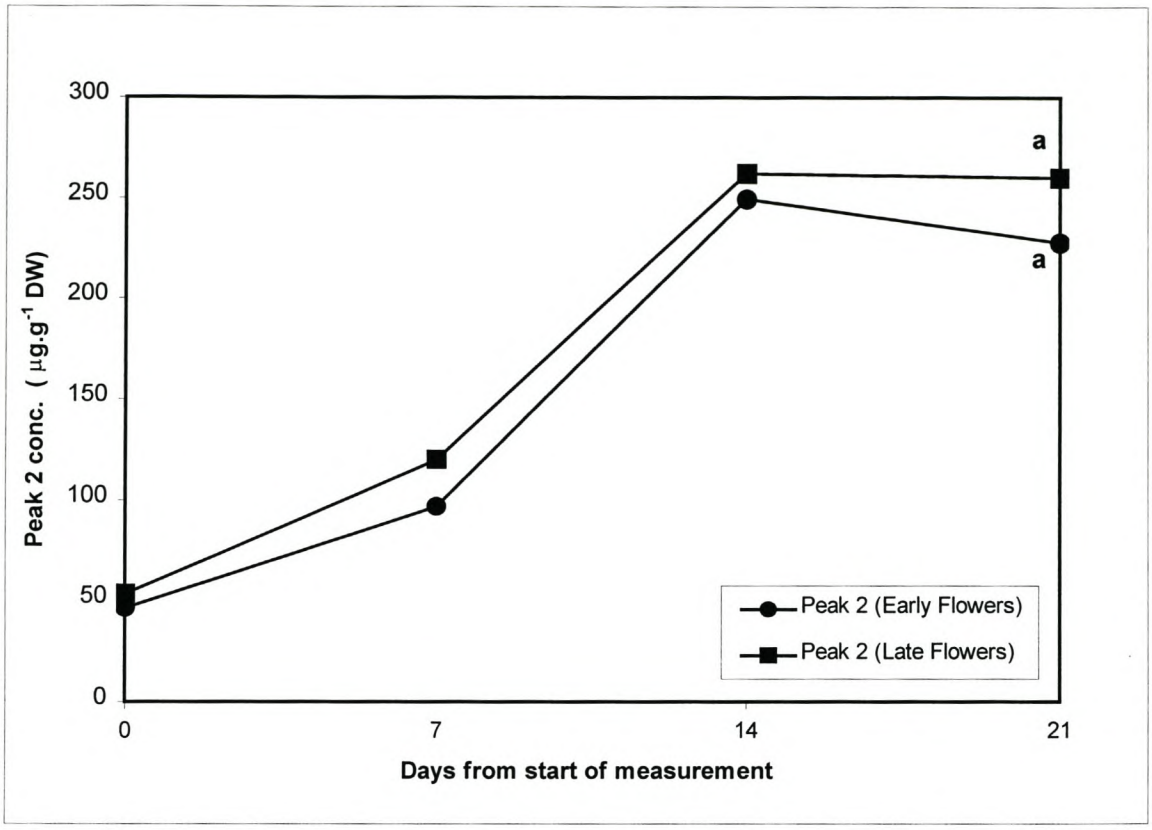
y Harvest dates for 'Carnival' of the early flowers (8 February to 1 March) and the late flowers (11 March to 1 April).

z Means within each column of the respective cultivar followed by the same letter are not significantly different.



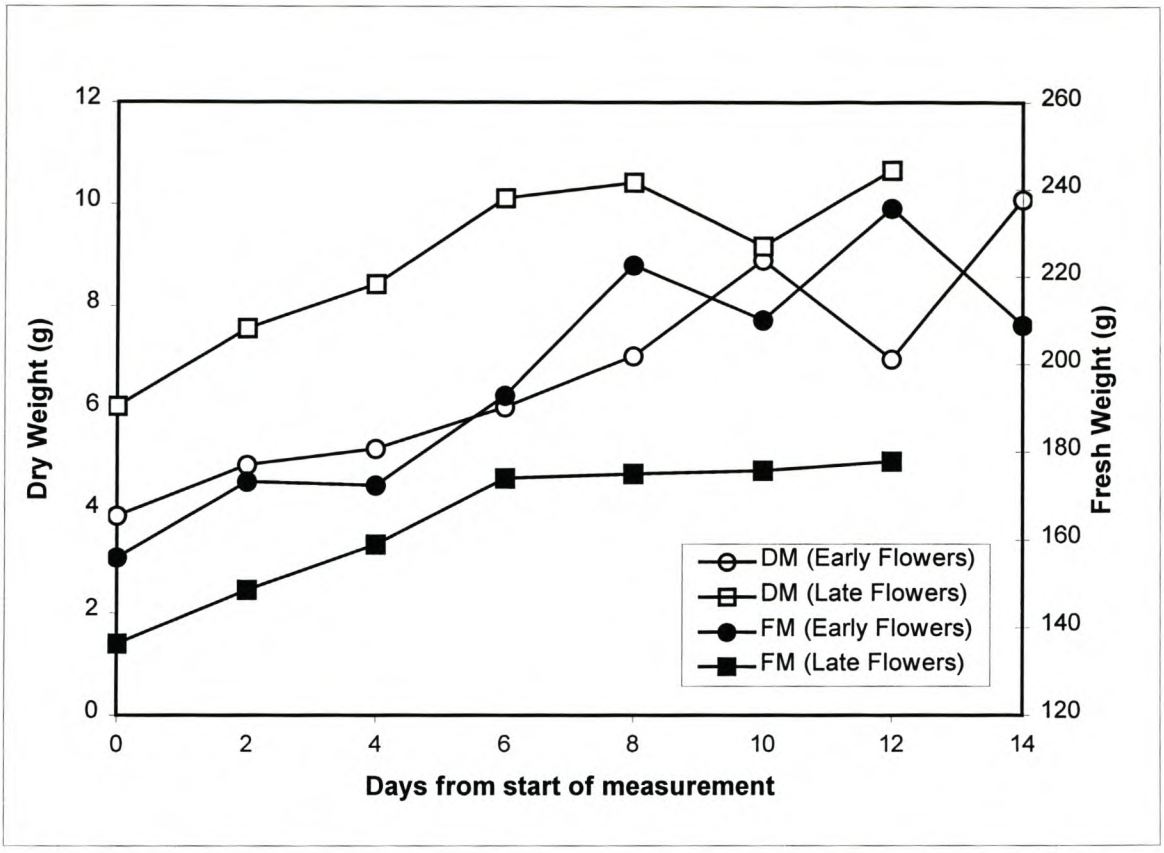
CONTRASTS	Dry Weight (g)	Fresh Weight (g)
(Early Flowers)		
Days Linear	0.0001	0.0001
Days Quadratic	0.4349	0.0038
(Late Flowers)		
Days Linear	0.0001	0.001
Days Quadratic	0.4928	0.8103

Fig 1. Dry weight of the 30 innermost involucral bracts and fresh mass of the whole inflorescence in 'Carnival' during development in the 2001 season (mean of 30 shoots). Early flowers representing measurements taken from 8 February till harvest at 1 March. Late flowers representing measurements starting on 11 March to harvest at 1 April.



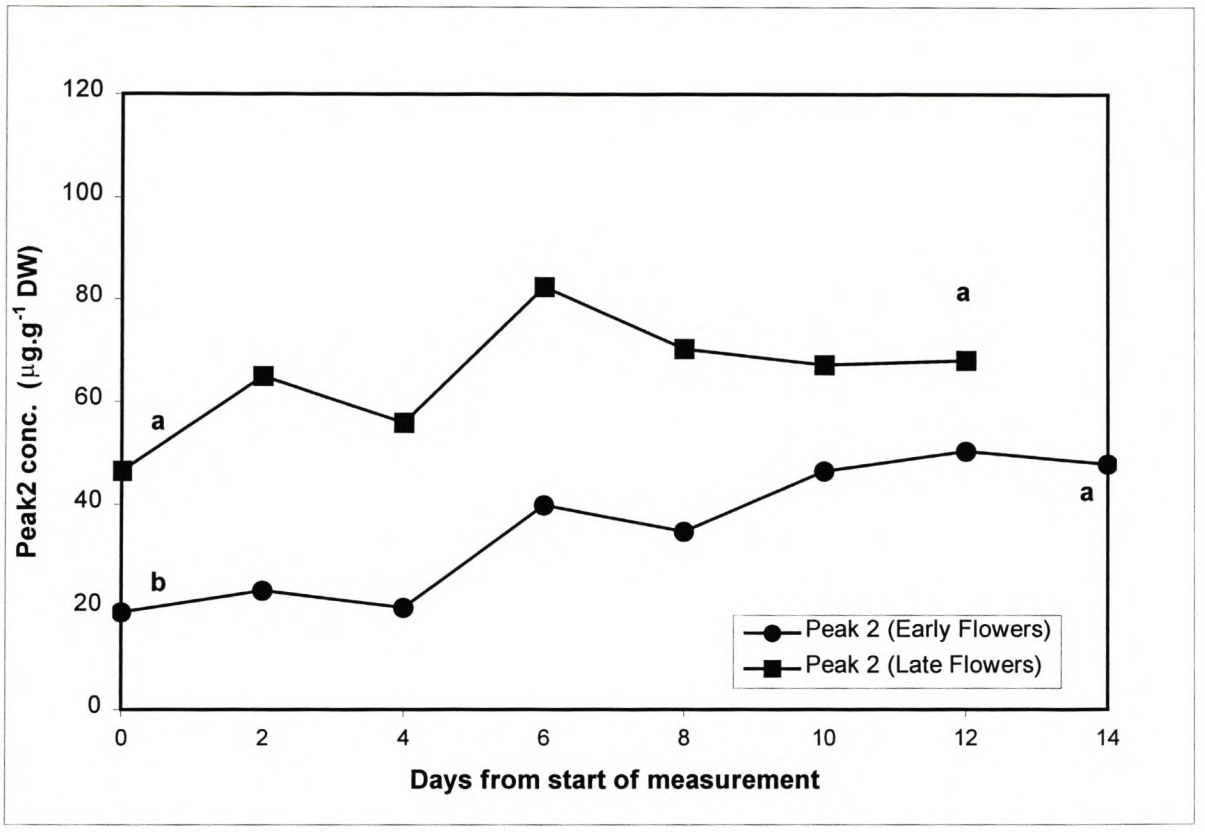
CONTRASTS	Peak 2 conc. ($g \cdot g^{-1}$)
(Early Flowers)	
Days Linear	0.0001
Days Quadratic	0.0038
(Late Flowers)	
Days Linear	0.0001
Days Quadratic	0.0001

Fig. 2. Concentration of the major anthocyanin (peak 2) present in the *Protea* cultivar 'Carnival', as measured in the 30 innermost involucral bracts during the 2001 season. Early flowers representing measurements taken from 8 February till harvest at 1 March. Late flowers representing measurements starting on 11 March to harvest at 1 April.



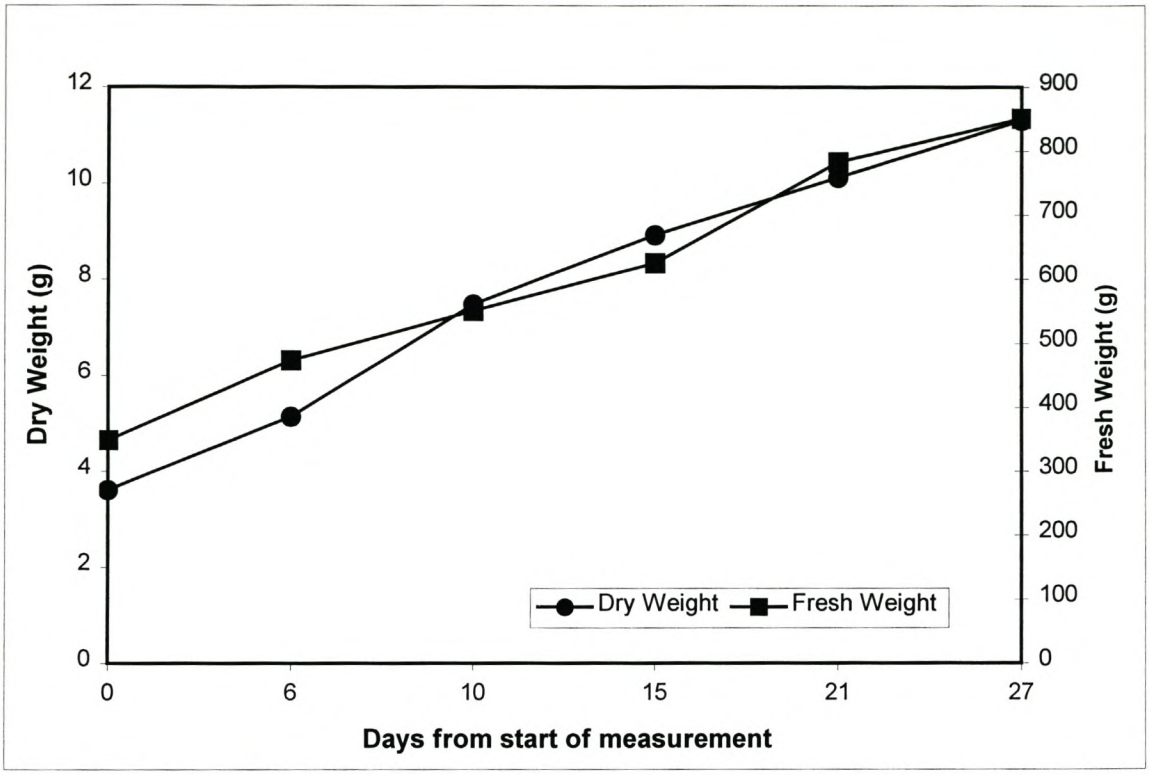
CONTRASTS	Dry Weight (g)	Fresh Weight (g)
(Early Flowers)		
Days Linear	0.0001	0.0001
Days Quadratic	0.2859	0.3301
(Late Flowers)		
Days Linear	0.0001	0.0001
Days Quadratic	0.0002	0.0195

Fig 3. Dry mass of the 30 innermost involucral bracts and fresh mass of the whole inflorescence in 'Ivy' during development in the 2001 season (mean of 30 shoots). Early flowers representing measurements taken from 14 February till harvest at 28 February. Late flowers representing measurements starting on 11 March to harvest at 23 March.



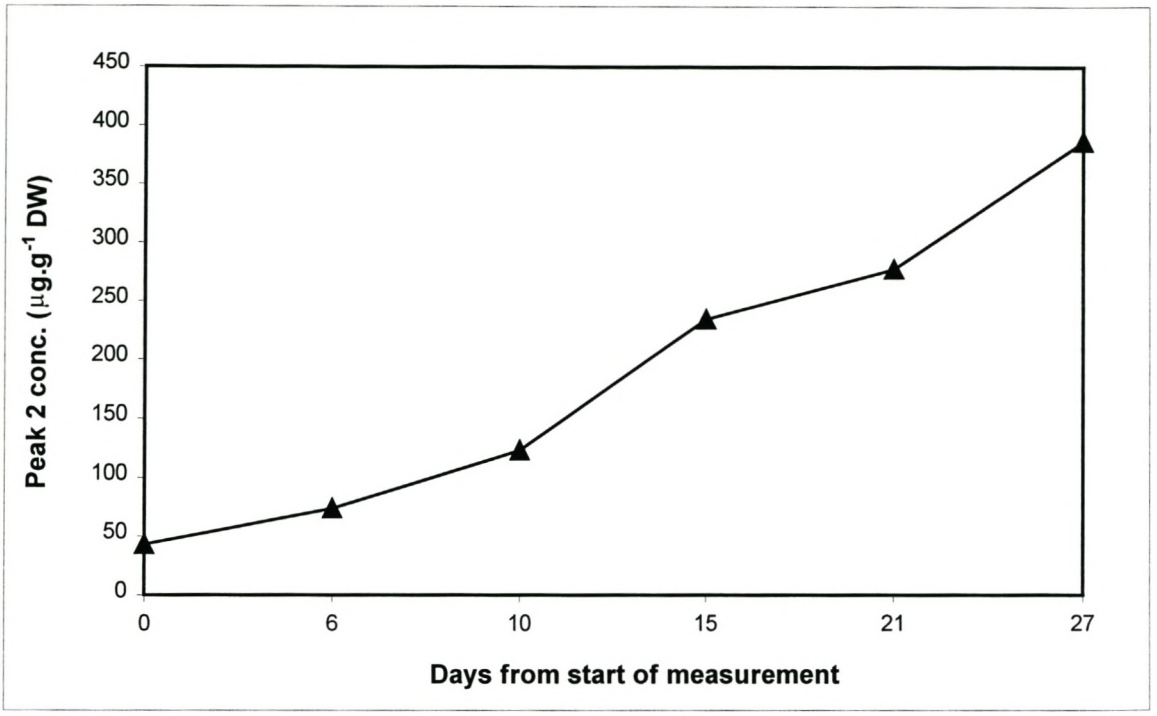
CONTRASTS	Peak 2 conc. ($g \cdot g^{-1}$)
(Early Flowers)	
Days Linear	0.0001
Days Quadratic	0.3706
(Late Flowers)	
Days Linear	0.0150
Days Quadratic	0.0679

Fig 4. Concentration of the major anthocyanin (peak 2) present in the *Protea* cultivar 'Ivy', as measured in the 20 innermost involucral bracts during the 2001 growth season. Early flowers representing measurements taken from 14 February till harvest at 28 February. Late flowers representing measurements starting on 11 March to harvest at 23 March.



CONTRASTS	Dry Weight (g)	Fresh Weight (g)
Days Linear	0.0001	0.0001
Days Quadratic	0.2557	0.0442

Fig 5. Dry mass of the 30 innermost involucral bracts and fresh mass of the whole inflorescence in 'Sylvia' during development in the 2001 season (mean of 30 shoots).



CONTRASTS	Peak 2 conc. ($g \cdot g^{-1}$)
Days Linear	0.0001
Days Quadratic	0.2764

Fig 6. Concentration of the major anthocyanin (peak 2) present in the *Protea* cultivar 'Sylvia', as measured in the 30 innermost involucral bracts during inflorescence development in the 2001 season.

6. GENERAL DISCUSSION

Reverse-phase HPLC analysis showed that the pigments responsible for red colour in the *Leucadendron* cultivars 'Silvan Red' and 'Safari Sunset' and the Protea cultivars 'Ivy', 'Carnival' and 'Sylvia' are anthocyanins. The initially encountered problem of insoluble compound precipitation during anthocyanin extractions with 5% formic acid in methanol from *Leucadendron* leaves, was overcome by using 80% acetone instead. This solvent was shown to be even more efficient in terms of total anthocyanins extracted than the classical 5% formic acid in methanol extraction solvent. A one hour extraction with 80% acetone was shown to be sufficient to study colour development in *Leucadendron* throughout a season, as trends will become evident. However, the 80% acetone and 5% formic acid in methanol showed differences in types of anthocyanins extracted (different number of peaks on chromatograms), and further anthocyanin identification is required to account for this discrepancy. Similarly in *Protea,* the 80% acetone extractions resulted in differences in the number of peaks extracted from involucral bracts of the same cultivar on the same sampling date and again further structure elucidation is suggested to explain these differences and whether further adaptation of the extraction method is required.

Colour development in the *Leucadendron* cultivar 'Silvan Red' and Protea cultivars 'Carnival', 'Ivy' and 'Sylvia' were shown to be developmentally regulated, as they did not appear to respond to changes in light or temperature, which are both factors known to significantly influence anthocyanin synthesis. Exclusion of light, by bagging, did not result in a significant visual difference to non-bagged shoots in 'Silvan Red' nor 'Ivy'. Furthermore, preliminary temperature regime trials conducted with 'Silvan Red', did not appear to influence colour.

Red colour loss in 'Silvan Red' was the result of anthocyanin degradation, which appeared to coincide with cessation of shoot growth and subsequent development of the inflorescence. Therefore, it is recommended that future research, concerned with colour manipulation in 'Silvan Red', should focus on horticultural practices that delay or alternatively shift shoot development. A trial, where 'Silvan Red' bushes

are pruned at different times of the year is suggested. Firstly, to obtain detailed information on the capability of 'Silvan Red' to flower at different times of the year and the effect of pruning on development and yield, which has not been studied thus far. Secondly, if shoot growth cessation can be shifted to occur for example in December (when cut flowers generally attain higher prices on international markets), to re-evaluate colour development and compare colour intensity with those harvested in the current mid February to mid April period.

A complete identification of the types of anthocyanins present in all *Protea* and *Leucadendron* cultivars mentioned, as well as their respective copigments (if present) and potential copigments, is suggested, due to their significant influence on colour expression. This may further elucidate why 'Safari Sunset' shows a more intense red-purple colouration and might indicate why it does not show a loss of red colour during the season.

In vitro studies on colour expression of solutions containing the different anthocyanins identified in combination with the different potential copigments encountered might prove to be useful data for future breeding programs. For example, if a flavone 3-glucoside copigment shows great colour stabilisation (i.e. colour intensity) with a specific type of anthocyanin *in vitro*, it may behave similarly *in vivo*. Therefore, if one were to cross the pink Protea cultivar 'Carnival' with another cultivar known to contain high levels of flavone 3-glucoside, it might lead to an increase in colour intensity. This broadens the scope of potential crosses, in comparison to breeding programs that are just based on the inheritance of a specific anthocyanin.