The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' but 'That's funny...'

Isaac Asimov (1920-1992)

Science fiction writer

Chapter 5

LENTICEL DISCOLOURATION OF MANGO (*Mangifera indica* L.) FRUIT EXPLAINED BY CHANGES IN PHENOLIC PROFILES

5.1 ABSTRACT

The chemical and cytological nature of lenticel discolouration was investigated as part of a physiological study of mango fruit. It is a visible self-defence response by the fruit, manifesting with unacceptable reddened to darkened spots. Differential extracts of phenolics from discoloured areas were analysed by means of reverse phase-high performance liquid chromatography. Discernable shifts in profiles of the phenolics correspond to the progressive colour changes of the lenticels. Fluorescence microscopy of fixed material revealed the localisation and transition of various forms of phenolics, as associated with various degrees of discolouration after staining with vanillin-HCl. The results confirmed that the pigments responsible for the colour of the lenticels are flavonones rather than anthocyanidins. Conservation of cellular structure and metabolic activity were shown using transmission electron microscopy. Reverse phase-high performance liquid chromatography results also correlate with transmission electron microscopy of the lenticels, which indicated that the accumulation of cell wall-bound phenolics do not result from vacuolar collapse or membrane disintegration. Although lenticel discolouration is considered a cosmetic defect, it is an indication of an inherent self-defence mechanism.

5.2 INTRODUCTION

Fresh mango (*Mangifera indica* L.) fruit enjoys popularity worldwide. This has lead to development of cultivars that must satisfy several specifications regarding its horticulture and market access (Bompard, 1993). Meeting these requirements, however, is not enough to assure success. Consumer preference is an important and demanding factor, with appearance, regardless of aroma or taste, the first sensorial criterion of fresh produce

(Pollack, 2001). It is of particular importance for export markets, where competitive agents exploit cosmetic preferences to manipulate market access of suppliers, with resultant financial consequences. Lenticel discolouration, a superficial blemish that affects some cultivars by causing the fruit to exhibit a speckled appearance, illustrates this point. Such fruit is branded as less desirable and downgraded, even rejected, despite the fact that the speckled appearance does not affect the internal quality of the fruit. Blemish development is limited to the lenticel perimeter and immediately adjacent area, not extending deeper than the outermost layers of the fruit rind. The development of a red halo is the first indication of discolouration, with the redness sometimes intensifying and ending in the eventual localised darkened appearance of the lenticel. Lenticels on unaffected fruit display a confluent colouration with the normal rind colour of each cultivar.

Fruit rind, or more correctly, exocarp (*sensu* Esau, 1977) is a structure that is primarily composed of natural wax, cutin, epidermal layer, sub-epidermal tissue and structures such as stomata and trichomes. The stomatal origin of mango fruit lenticels gives the best indication of their morphological characterisation, since they are structurally different from the classic lenticels present on other plant species (Tamjinda *et al*., 1992; Du Plooy *et al*., 2003). Lenticels on mature mango are epidermal structures that form part of the exocarp due to their superficial nature and integration with sub-epidermal layers. By comparing only the cuticle and epicuticular wax layers, mango cultivars cannot be distinguished with certainty at microscopic level. However, combinations of macro morphological lenticel characteristics can be attributed to specific cultivars. Combined with the intricate matrix of resin ducts in the mango peel (Joel, 1980) and the resin chemistry (John *et al*., 1999), it could give some explanation why some cultivars are more prone to lenticel discolouration.

Reasons for the development of the condition have been investigated from various horticultural and post harvest management points of view (O'Hare & Prasad, 1992; Du Plooy *et al*., 2002; Willis & Duvenhage, 2002). Tamjinda *et al*. (1992) described the micro morphology and histochemistry of the discoloured lenticels on the cultivars Namdokmai and Falan. Du Plooy *et al*. (2003) and Bezuidenhout *et al*. (2003) investigated the development of this condition on 'Keitt' and 'Tommy Atkins', two mango cultivars that are commercially important to South African growers, with particular susceptibility to lenticel discolouration. The red colouration is linked to either anthocyanin (Kangatharalingam *et al*., 2002) or flavonoid (Dixon & Paiva, 1995) production and is suspected to be a mechanism of self-protection. Both groups of biochemicals originate from phenylalanine, a product of the Shikimate pathway. Secondary metabolites from the subsequent flavonoid pathway have a role in plant responses associated with biotic and abiotic stress signals

(Dixon & Paiva, 1995; Dixon *et al*., 2002; Robards, 2003) (Fig. 1). Flavonoids, for example, have been implicated in deterrence of herbivory (Berenbaum & Zangerl, 1992; Simmonds, 2003). Flavonoids comprise of a total of 12 subgroups, including the flavanoids. Anthocyanocides can be further subdivided into proanthocyanidins and anthocyanidins (Dixon & Paiva, 1995; Dixon *et al*., 2002; Robards, 2003). Several flavonoids and anthocyanins have been indicated as physiologically functional in screening against UV-A and UV-B by scavenging for reactive oxygen species (Robards *et al*., 1999; Heim *et al*., 2002). This functionality stems from the diversity of molecular structures that originate as hydroxylated derivatives of the benzo-γ-pyrone nucleus. These can undergo further conjugation between the aromatic rings, glycosidic units and methoxy groups. The number of structural elements creating the complexity of a flavonoid group is linked to its physiological functionality. It is proposed that lenticel discolouration is primarily a cosmetic condition (Du Plooy *et al*., 2003), and probably due to a physiological stress response by fruit tissue surrounding the lenticel entrance (Grassmann *et al*., 2002). Insight into the chemical nature of lenticel discolouration will give a better understanding of mango fruit physiology. This study is an investigation into the development and changes of the phenolic profiles of discoloured lenticels (in comparison to unaffected lenticels) and the corresponding cytological changes.

5.3 MATERIALS AND METHODS

5.3.1 Plant material

Physiologically mature mango fruit exhibiting varying intensities of lenticel discolouration (cultivars 'Tommy Atkins' and 'Keitt') was collected fortnightly from the packhouse of Bavaria Fruit Estates (Hoedspruit, Limpopo Province, South Africa) throughout the 2002/2003 and 2003/2004 seasons. For the cytological study and analysis of the phenolics, lenticels were collected in three predetermined colouration groups, namely non-discoloured (no visible discolouration), reddened and darkened lenticels (intense or dark red colouration).

5.3.2 Chemicals

Vanillin and phenolic substances used as authentic standards were purchased from Sigma Aldrich, (Munich, Germany). The electron microscopy reagents were purchased from SPI Supplies (SPI Supplies, West Chester, PA, USA). Solvents used in the extraction and HPLC were of analytical or HPLC grade and obtained from Merck Chemicals (Merck, Halfway House, South Africa).

5.3.3 Experimental

5.3.3.1 Microscopy

Samples for fluorescence microscopy and transmission electron microscopy (TEM) were dissected from mature mango fruit and immediately fixed in a mixture of 2.5 % glutaraldehyde and 2.5 % formaldehyde in a 0.1 M $NaPO_4$ buffer (pH = 7.3 ± 0.05). After standard rinsing and dehydration, the fluorescence microscopy samples were embedded in L.R. White resin, whilst the TEM samples were post fixed in OsO_4 and imbedded in Quetol 651 (Van der Merwe & Coetzee, 1992).

5.3.3.1.1 Fluorescence microscopy

Thin sections (0.5 - 1.0 µm) of the material embedded in L.R. White were cut on a Reichert Ultracut E (Reichert AG. Vienna, Austria) ultra microtome. Sections were heat fixed (60 °C) to glass microscope slides. Fluorescence was achieved by staining the tissue with Vanillin-HCl (1 % in concentrated HCl, w/v) (Guerin *et al*., 1971). Sections were viewed with a Zeiss Axiovert 200 (Zeiss, Göttingen, Germany) microscope fitted with a Nikon digital camera DXM1200 (Nikon Instech Co., Kanagawa, Japan). Digital images of emission at 397 nm, 515 nm and 590 nm were captured with Nikon ACT-1 version 2.

5.3.3.1.2 Transmission electron microscopy

Lenticel material fixed in Quetol 651 was cut on hte Reichert Ultracut E ultra microtome. Sections (ca. 90 nm thickness) were contrasted in 4 % aqueous uranyl acetate and Reynold's lead citrate (Reynolds, 1963) and viewed with a Philips EM301 transmission electron microscope (TEM) (Philips, Eindhoven, Netherlands) set at 20kV.

5.3.3.2 Extraction of phenolics

For the extraction of phenolics, discoloured lenticels were dissected from fresh fruit and immediately plunged into liquid N_2 after dissection, to terminate enzyme activity (polyphenol oxidase) (PPO), and freeze dried under vacuum (Vacutech Dura-Dry II™ MP, FTS Systems, Stone Ridge, NY, USA) for 72 hours. Freeze dried lenticels were ground in a marble mortar and pestle, keeping the three predetermined discolouration groups separate. For each discolouration category 0.05 g of powder was weighed into an Eppendorf tube and 1ml of a cold mixture of methanol: acetone: water (7:7:1, v:v:v) added and maintained at 4 °C to prevent oxidation. No antioxidant (ascorbic acid or $Na_2S_2O_5$) was used, since it would interfere with the Folin-Ciocalteau reagent used for determination of total phenolics (Regnier, 1994). The combined solvent/powder sample was placed in a vortex (Heidolph REAX, Jencons Scientific Ltd, Leighton Buzzard, UK) for 1 min, before

placing in an ice bath on an orbital shaker at 200 oscillations per min. After 30 minutes the supernatant containing the extract was separated by centrifuging at 10 000 rpm for 3 minutes. This extraction procedure was repeated three times and the supernatant fractions pooled. Organic solvents in the solvent mixture were removed by evaporation under N_2 flow and the volume adjusted to 1 ml with cold methanol. Free acid, glycosidically bound phenolics, ester bound phenolics and cell wall bound phenolics were isolated according to De Ascensao and Dubery (2003), and the fractions stored at 4 °C until further use.

5.3.3.3 Quantification of phenolics by the Folin-Ciocalteau method

The reaction mixture used was scaled down to facilitate the use of an ELISA plate technique for the quantification of phenolics (Table 1) (Sivakumar *et al*., 2005). According to this method, gallic acid was used to determine the equivalent phenolic acid content, which is a modification to the Folin-Ciocalteau method as described by Regnier and Macheix (1996). Spectrometric measurements of the phenolic concentrations in the various extracts was calculated from a standard curve ($y=0.0013x + 0.0177$, $r^2 = 0.9982$) determined by ANOVA and expressed as mg gallic acid equivalent per gram of dry weight (Table 2).

5.3.3.4 Reverse phase - high performance liquid chromatography

Thin layer chromatography gave non-distinctive results (data not shown); therefore HPLC was used as the preferred method of determination of phenolic constituents. Extracted phenolic fractions were analysed by means of reverse phase - high performance liquid chromatography (RP-HPLC). Triplicate separations of each extract were run without the addition of an internal standard, because overlapping could not be excluded.

The system for the HPLC analyses consisted of a Varian ProStar 230 solvent delivery system with manual injection and a Varian ProStar 310 UV-Vis detector fitted with a Degassit on-line solvent degasser (Varian, Walnut Creek, California). System control and results analysis was done using the Varian Star version 5.5 software. An excess injection volume of 25 µl of each sample was used in a 20 µl loop. The column was a C-18 reverse phase Inertsil ODS-3 column (250mm length, 4.6mm inner diameter, 5µm particle size) with an integrated guard column (Varian, Walnut Creek, California). The column was kept at 25 °C, with elution performed using a gradient program (Table 3) at a flow rate of 1ml/min.

Samples were run using two solvents and following the gradient program in Table 3. Mobile phase A consisted of distilled water acidified with trifluoroacetic acid (TFA) (pH = 3.58 ± 0.02) and mobile phase B of acetonitrile without any added modifier. After each run, the column was re-equilibrated with the initial conditions for 10 minutes. The detector was programmed for peak detection at 280 nm, which, although not optimum for ferulic acid and its derivatives, allowed simultaneous detection of hydroxybenzoic and hydroxycinnamic acids and their derivatives (Zhou *et al*., 2004). Identities of some peaks were by suggested by comparing their retention times to those of authentic standards run at the same gradient and conditions (Table 4).

5.4 RESULTS AND DISCUSSION

5.4.1 Microscopy

5.4.1.1 Fluorescence microscopy

Higher plants efficiently utilise phenylpropanoids such as hydroxycinnamic acid, cinnamoyl esters, and flavonoids, including flavones and flavonols, and anthocyanins to provide UV-A and UV-B screening (Dixon & Paiva, 1995; Dixon *et al*., 2002). Each species has a specific range of flavonoids responsible for UV screening, with combined capabilities more effective than screening by a single compound. The presence of these compounds in plant tissue was detected using Vanillin-HCl as a fluorescent dye to locate phenolics specifically (Guerin *et al*., 1971; Regnier, 1994).

No pronounced auto fluorescence could be observed in any of the unstained sections, although distinctive blue colouration was visible in cell walls (Fig. 2). The shorter excitation wavelength (365 nm) with emission of 397 nm gave the most useful distinction between different types of phenolics. Phenolics such as ferulic acid, caffeic acid and flavonoids were visible as bright white, blue, and red to brown-red zones (Fig. 3 - 5), with colouration indicative of different phenolic classes encountered (Table 5). No fluorescence in yellow and orange hues to signify anthocyanins, were detected. The limited auto fluorescence observed was attributed to the use of resin-stabilised material. At the time of sampling, it was not possible to provide fresh sections. However, L. R. White resin did not quench the effect of fluorescent stains, and the results were satisfactory.

Tissue surrounding non-discoloured lenticels exhibited minute amounts of red fluorescent phenolics (flavonoids). These were primarily located intracellular in vacuoles, but traces of cell wall bound red fluorescence were also present. Bright white fluorescence of the wax

fraction lining the cavity was indicative of simple phenolics such as gallic acid trapped in lenticular wax fractions (Fig. 3). No other fluorescence was detected in visible light, corresponding to the non-discolouration of the lenticel and the tissue surrounding it.

The visible discolouration of reddened lenticels was due to an accumulation of phenolics in the cell walls and vacuoles of the tissue surrounding the lenticel cavity (Fig. 4). The cell walls were mostly affected in areas of tissue associated with the lenticel and adjacent resin ducts. Cells that constituted the lenticel cavity walls also showed an accumulation of soluble ester bound phenolics. Deep red fluorescence, indicative of the presence of flavonoids, was limited to the cell walls.

In the darkened lenticels, some cells still contained fluorescent cytoplasm, indicating that the vacuoles were still intact. A bright white perimeter of the vacuole in some cells represented the protoplasm. The cell walls still fluoresced red, but less brightly so, with an orange to brown tone, indicating the deposition of polymeric phenolics (Guerin *et al*., 1971; Regnier, 1994), that tended to mask the red of the flavonoids. On the fruit surface, this masking effect was clearly seen as pertinent dark lenticels. In the final stages of discolouration, lenticels appeared darkened, and may become blackened. Growers and traders regard such darkened lenticels as necrotic lesions, giving rise to the use of the misnomer ‘lenticel damage’.

5.4.1.2 Transmission electron microscopy

Transmission electron microscopy gave a visual representation of the chronology of events and chemical changes during discolouration. Cell walls of mesophyll cells in the immediate vicinity of the lenticel lumen were investigated. In non-discoloured lenticels (Fig. 6A & B), cell walls were characteristically striated and granular, corresponding to multidirectional layering of cellulose in the secondary cell wall (Swanson & Webster, 1977). There was no evidence of development of any electron dense material, which would indicate deposition of secondary metabolites in the cell wall. Cellular contents consisted of large vacuoles, plastids, nuclei, endoplasmic reticulum, ribosomes and other micro-organelles, and Golgi apparatus. Vacuolar contents were granular and highly dispersed, often with a somewhat coagulated appearance due to precipitation during fixation (Scalet *et al*., 1989).

Membrane integrity and subsequent continuation of metabolic activity was clear through the presence of intact endoplasmic reticuli and Golgi apparatus visible in both reddened (Fig. 7A & B) and darkened (Fig. 8A & B) lenticels. This indicated that the intensifying

discolouration was not due to a release of PPO when phenolics accumulate (Beckman, 2000), which was contrary to previous conclusions (Bezuidenhout *et al*., 2003). Rather, phenolics were incorporated into the cell wall and storage compartments of the implicated cells. Lenticel discolouration, therefore, was observed as a controlled process whereby the lenticel cavity was encapsulated with cells containing phenolic-laden vacuoles (Fig. 7A & 8A) that were prepared for resistance against pathogen attack (Dixon & Paiva, 1995; Beckman, 2000). A diversity of factors has been observed as the trigger or signal for this self-protection mechanism, which has made lenticel discolouration difficult to manage (Loveys *et al*., 1992; Taminja *et al*., 1992; Willis & Duvenhage, 2002; Bezuidenhout *et al*., 2003). In this study, phenolic deposition was only observed in, and therefore limited to, tissue in the vicinity of the lenticel cavity. Evidence from some sections viewed suggested that resin ducts may act as the transport route for elicitors of the phenolic reactions associated with cell wall deposition (Diaz *et al*., 1997). Furthermore, electron-dense material in the cell wall gradually increased from the outside of the cell wall to the inside (Fig. 7B & 8B), indicating the arrival of cell wall linking reactants through the apoplastic route.

5.4.2 Quantification of phenolics by the Folin-Ciocalteau method

Values obtained through quantification of phenolics by the Folin-Ciocalteau method were indicative of the presence of soluble phenolics. Total phenolics were highest in darkened lenticels, which meant that the soluble phenolics present in vacuoles of cells in dissected lenticel tissue have increased. This concurred with findings reported in the review by Beckman (2000) that specialised phenolic storing cells are located at localities where infection or stress can be contained. As were seen from the TEM micrographs, there was no membrane damage signalling the liberation of PPO. The increasing reddening and eventual darkening of the lenticels is therefore due to the build-up of phenolics in the vacuoles (Regnier & Macheix, 1996; Wink, 1997). A build-up of phenolics could be toxic to adjacent tissue, and cells containing vacuoles filled with phenolics were isolated by cross linking of polymeric ferulates (insoluble) in the cell wall (Beckman, 2000). Leaking phenolics are contained by the denser, strengthened walls (Fig. 8A & B).

5.4.3 Reverse phase - high performance liquid chromatography

Extraction of the phenolics from both non-discoloured and discoloured lenticels yielded highly concentrated samples. The modified Folin-Ciocalteau method (Sivakumar *et al*., 2005) for the determination of phenolic acid content was particularly suited to the small quantities of sample material available for this study. Apart from a crude extract, four targeted extractions were done to obtain free phenolic acids, glycoside bound phenolic

acids, ester bound phenolic acids, and cell wall bound phenolic acids. A gallic acid equivalent calibration curve (Fig. 9) was used determine the abundance of each fraction in the sample material (Fig. 10). These values are representative of the relative abundance of each fraction in the extract. Non-discoloured lenticels had lower concentrations of all the phenolic fractions, followed by red discoloured and lastly, dark discoloured lenticels. This is in agreement with phenolic acid functionality as discussed by several studies (Dixon & Paiva, 1995; Beckman, 2000; Dixon *et al*., 2002; Grassmann *et al*., 2002; Zhou *et al*., 2004). Although high, crude extract values does not reflect the combined values of the four other phenolic acid fractions extracted with more specific hydrolysis reactions.

Fluorescence microscopy gave some indication of phenolic acids that could be expected after extraction. Derivatives of cinnamic and ferulic acid would be polar and hydrophilic, while polymeric phenolics would be less polar and more hydrophobic. Any co-extracted biopolymers such as terpenes would be very hydrophobic (Guerin *et al*., 1971; Regnier & Macheix, 1996). Effective separation by gradient elution was achieved by exploiting the differences in the hydrophilic/hydrophobic nature of the mobile phase constituents, as well as the polarity of eluted molecules. Phenolics were adsorbed onto the stationary phase at low solvent strength through Van der Waals interactions, and were selectively released according to their decreasing ability to participate in hydrogen bonding at distinct solvent strengths (Cunico *et al*., 1998).

Results from the RP-HPLC investigation could not be quantified to satisfaction and were therefore be presented qualitatively only (histograms in Fig. 11 - 15). Representative chromatograms for non-discoloured, reddened and darkened lenticels extracts were included for each group of isolated phenolics (chromatograms in Fig. 11 - 15). Despite the small quantity of tissue that was used for extraction, highly concentrated extracts were obtained. Previous studies into mango phenolics involved the fruit pulp only, and the results from this study could not be related directly to these studies.

Changes in the phenolic composition within each colouration group were referenced against a single major peak present within all three chromatograms of the compared extraction set (histograms in Fig. 11 - 15). Bar heights in the histograms, therefore, did not indicate absolute peak sizes, but rather relative changes in the ratio of compared peaks. That meant that higher bars indicated bigger numerical differences. In this way, trends of changes in concentrations within a set of extractions were indicated by changes in the heights of the histograms for each peak investigated.

From each of the extracts evaluated, the more polar, labile phenolics such as gallic acid, proto catechuic acid and its derivatives, epicatechin and *p*-coumaric acid (Rodríguez-Delgado *et al*., 2001) were eluted from the column within 23 minutes. After this time, the gradient increased from favouring predominantly hydrophilic compounds to favouring more hydrophobic oligo- and polymeric compounds such as quercetin and kaempherol (Regnier & Macheix, 1996; Markham *et al*., 2000; Rodríguez-Delgado *et al*., 2001). All extracts from non-discoloured lenticels had the lowest concentrations of the more hydrophobic oligomeric phenolics, whereas reddened lenticels had the most oligomeric phenolics compounds. This indicated that free acids became increasingly incorporated into complex phenolics through metabolically driven defence strategies (Dixon & Paiva, 1995; Beckman, 2000; Passardi *et al*., 2004). Moreover, increases in polymeric phenolics were correlated with decreases in the concentration of soluble and glycosidically bound phenolics, such as feruoyl glycoside, in the vacuoles. Ferulic acid in particular becomes bound to the cell wall structure (Lozovaya *et al*., 1999), with analogous situations described by Beveridge *et al*. (2000). This shift could be seen in increased numbers and complexity of peaks between 30 - 50 minutes. Increased retention time was indicative of a decrease in analyte polarity and thus solubility. The result correlated with previous findings on the physiological activity of various flavonoids and phenolics (Ketsa & Atantee, 1998; Dixon *et al*., 2002). Some peak broadening that occurred may be alleviated, and the sensitivity increased, by adding TFA to the acetonitrile as well. The TFA acts as an ion pair agent and reduces the pH of the mobile phase, thereby minimising the interaction of the phenolics with the silanols of the column packing (Cunico *et al*., 1998). However, not all peaks were affected and sufficient differences could be identified.

Crude extracts (Fig. 11) of non-discoloured, reddened and darkened lenticels pointed towards the presence of both polar and less polar compounds. Individual separation of the latter peaks was difficult to achieve due to their polymeric nature. Nevertheless, distinct differences between the phenolic profiles of non-discoloured, reddened and darkened lenticels were detected. According to retention times from table 3, peak *d* at t_r = 14.212 min was chlorogenic acid (chromatograms, Fig. 11). The ratio of this peak to the first three polar compounds in all crude extracts was the biggest in that of the *d:a* ratio of reddened lenticels. Due to the increase in concentration of chlorogenic acid for reddened lenticels, and a simultaneous decrease of the compound at t_r= 26.109 min, there was a marked increase of the *d:e* ratio. The *d:h* ratio for both reddened and darkened lenticels, relative to this ratio of non-discoloured lenticels, indicated that there was an increase in the compound at t_r= 32.161 min in both types of discoloured lenticels. Finally, the chlorogenic acid concentration in the crude extract of both reddened and darkened lenticels exceeded

that of non-discoloured lenticels. This result correlates with previous findings in which chlorogenic acid has been associated with induced stress before (Mayr *et al*., 1994; Robards *et al*., 1999).

In the extract of non-conjugated phenolics (free acids, Fig. 12), all the peaks were compared to the chlorogenic acid peak (*c*) at t_r= 14.467 minutes. There were significant changes between the ratios of initial peaks and those associated with oligomeric phenolics. The *c:a* and *c:b* ratios (i.e. ratios of small, polar free acids) for reddened lenticels were markedly different from the corresponding ratios for non-discoloured and darkened lenticels. Furthermore, decreasing ratios between more complex compounds and chlorogenic acid in non-discoloured and reddened lenticels indicated that these compounds were increasing in darkened lenticels (Kahn, 1985; Beveridge *et al*., 2000). Non-discoloured lenticels had very large ratios for *c:h*, *c:i* and *c:j*, indicating a predominance of free acids to other phenolics. These free acids were less prevalent in reddened lenticels (chromatogram, Fig. 12) than in non-discoloured and darkened lenticels.

Hydrolysed extracts of cell wall bound compounds from non-discoloured, reddened and darkened lenticels yielded two distinctive peaks (Fig. 13). Peak *d* (t_r= 24.837 min) and peak *e* (t_r= 26.055 min) were present in a similar ratio for all three groups of extracts. However, when peak *d* was compared to other peaks from each extract, the metabolic translocation of polar components to the cell wall became clear (Passardi *et al*., 2004). Hydrolysis depolymerised the glycans bound to the cell wall matrix, which were present in higher concentrations in discoloured lenticels than in non-discoloured lenticels (histograms in Fig. 13). In discoloured lenticels a small, polar fraction at t_r= 4.602 min was absent from non-discoloured lenticels, indicating the functionality of this molecule in cell wall deposited phenolics (Lozovaya *et al*., 1999). Oligo-and polymeric phenolics were present in stable quantities in all three groups of extracts, which is a further indication that the disassembled polar compounds originate from the primary components that contribute to cell wall reinforcement (Kahn, 1985; Lozovaya *et al*., 1999; Beckman, 2000). Redistribution of the constituent compounds in cell wall bound phenolic extracts was most noticeable in reddened lenticels, as were seen from differences in the *d:a*, *d:b*, *d:c*, and *d:f* ratios.

For the chromatograms of glycoside bound extracts from non-discoloured, reddened and darkened lenticels (Fig. 14), peaks *a-g* within each colouration set were compared to peak *b*, a small, polar phenolic acid at t_r= 6.852 min that corresponded closest to gallic acid.

Removal of the more soluble glycoside moieties by mild hydrolysis liberated the phenolic free acids at these early retention times. Within each of the three lenticel colour groups, the peak sizes showed relatively small changes in the peak ratios (histograms in Fig. 14). This corresponds with enzymatic hydrolysis during cellular utilisation of glycoside bound phenolics (Ferreres *et al*., 1997; Heim *et al*., 2002), with increasingly bound phenolics showing in more dense vacuolar contents (Fig. 6 - 8).

Apart from free acids, ester bound phenolics are the most labile and is reflected as such in the chromatograms in Fig. 15. Peaks *a* - *i* within each set of lenticel extracts were compared to peak *c* (t_r = 14.416 min), with the peak ratios of *c:a*, *c:f* and *c:l* in non-discoloured lenticels markedly higher than the same ratios in the discoloured lenticels (histograms in Fig. 15). This was indicative of the conversion of these compounds into less labile (cell wall bound) compounds (Lozovaya *et al*., 1999; Beckman, 2000; De Ascensao, & Dubery, 2003) that contribute to cell wall reinforcement (Ketsa & Atantee, 1998; Russel *et al*., 1999).

5.5 CONCLUSION

Lenticels are points vulnerable to possible infection in ripening, senescent fruit. Phenolic accumulation presents rapid and efficient blocking and protection against such infection (Matern, 1994; Wink, 1997; Robards, 2003). Discolouration was morphologically characterised by vacuolar phenolics as well as accumulated phenolics in the cell walls of the mesophyll cells surrounding the lenticel cavity and proximal tissue. The resultant externally visible differences between non-discoloured, reddened and darkened lenticels were substantiated by both fluorescence microscopy and TEM. Chronological cell wall discolouration was illustrated by TEM results as increased electron density. The discolouring process happens from the outer boundaries of the cell wall towards the inside of the cell, showing apoplastic transport of the reaction elicitors. There was a progression in bound phenolics in the cell walls and the vacuoles as the intensity of lenticel discolouration increased. The reddening of the encapsulating tissue surrounding the lenticels were related to the build-up of soluble phenolics in the vacuoles of mesophyll cells in the lumen walls (Robards, 2003). Deposition and cross linking of phenolics in cell walls further contributed to darkening of the lenticels. Initial vacuolar inclusions consisted of simple phenolics that appeared granular in TEM micrographs due cross-reaction during fixation. In reddened and later, darkened lenticels, the vacuolar contents become increasingly coagulated, electron-dense and hardened, with corresponding changes in the

fluorescent qualities. Although hardening of phenolics could be described as an artefact of the fixation reactions, their chemical reaction to fixatives is linked to increasing complexity of the phenolics in the vacuoles as they become more conjugated in advanced stages of lenticel discolouration (Scalet *et al*., 1989). The observations were correlated by the results of the total phenolics determination, where an increase in colour is directly related to an increase in total soluble phenolics.

Cell wall bound phenolics form red zones with Vanillin-HCl staining. The red, however, was slightly masked by the brown colouration of quinone-related compounds in discoloured lenticels (Guerin *et al*., 1971; Regnier, 1994). Nevertheless, it is still possible to deduce that the conversion of soluble phenolics into cell wall bound polymeric compounds ultimately produced discoloured lenticels. No evidence could be found that the red discolouration is related to anthocyanins accumulating in the lenticel and surrounding tissue, since the results in this study indicated the presence of hydroxycinnamic acid derivatives and flavonols.

Blackened lenticels that are sometimes observed may be the result of cell death when toxic phenolics are released from ruptured vacuoles. Permeation or infiltration of such phenolics would be restricted by the reinforced cell walls. The lenticel, immediate surrounding tissue and sub-cuticular mesophyll are protected from infection by an efficient barrier of toxic phenolics and a process of structural reinforcement.

Extracts from isolated lenticels rendered fewer flavonoids than anticipated. Comprehensive arrays of peaks representing a wider collection of phenolics were only obtained from the extracts for non-conjugated phenolic acids and ester bound derivatives. RP-HPLC results indicated a high level of reproducibility between lenticel extracts of similar colouration, but extraction validation was inadequate, with consequent unsatisfactory quantification. This was due to the following reasons:

- lenticels were dissected from fruit by hand.
- lenticel tissue was not cut from fruit of similar acidity, ° Brix and firmness.
- slow progress in dissection meant that lenticel tissue was not cut in the same time period. Fruit physiology was therefore not exposed to the same environmental conditions before and during sample collection.
- dissection by hand could lead to poor reproducibility of the quantity of fruit pulp included in each section surrounding the lenticel.

- small quantities of lenticel tissue sampled (0.5 g) will amplify any interference from pulp tissue.
- internal standards were not initially added to extracts, since peak overlapping could not be predicted, and could not be added and accurately quantified after initial removal of any volume of extract for qualitative sampling.

HPLC results confirmed that the discolouration is a self-defence strategy to protect the lenticel and immediate surrounding tissue (Dixon & Paiva, 1995; Robards *et al*., 1999; Beckman, 2000; De Ascensao & Dubery, 2003) and correlated with results from fluorescence microscopy and TEM of the lenticels. No disrupted membranes were observed, and discoloured lenticels were still alive; this supports the idea that lenticel discolouration is the manifestation of an active self-defence mechanism, due to environmental stress being applied to the fruit. The signal for stress related phenolics to develop, and the cultivar sensitivity towards this signal is still unclear. Lenticel discolouration can not be eradicated, but could be controlled through adjusted management of horticultural and packhouse practices.

5.6 REFERENCES

Beckman, C.H. 2000. Phenolic-storing cells: keys to programmed cell death and periderm formation in wilt disease resistance and in general defence responses in plants? *Physiol. Mol. Plant Pathol*. 57: 101 - 110.

Berenbaum, M.R. & Zangerl A.R. 1992. Genetics of secondary metabolism and herbivore resistance in plants, pp. 415 - 438. In: *Herbivores: their interactions with secondary plant metabolites. Vol. II.* (Eds.) Rosenthal G.A. & Berenbaum M.R. Academic Press, San Diego, CA, USA.

Beveridge, T., Loubert, E. & Harrison, J.E. 2000. Simple measurement of phenolic esters in plant cell walls. *Food Res. Internat*. 33: 775 - 783.

Bezuidenhout, J.L.J., Robbertse, P.J., Van der Merwe C.F. & Du Plooy, W. 2003. Lentiselverkleuring op vrugte van Tommy Atkins en Keitt mango's. *S. Afr. Mango Growers' Assoc. Res. J*. 23: 122 - 131.

Bompard, J.M. 1993. The genus Mangifera rediscovered: the potential contribution of wild species to mango cultivation. *Acta Hort*. 341: 69 - 77.

Cunico, R. L., Gooding K. M. & Wehr T. 1998. *Basic HPLC and CE of Biomolecules*, pp. 205 - 233. Bay Bioanalytical Laboratory, Richmond, California.

De Ascensao, A.R.F.D.C. & Dubery, I.A. 2003. Soluble and wall-bound phenolics and phenolic polymers in Musa acuminata roots exposed to elicitors from *Fusarium oxysporum* f.sp. *cubense*. *Phytochem*. 63: 679 - 686.

Diaz, J., Ros Barceló, A. & de Cáceres, F.M. 1997. Changes in the shikimate dehydrogenase and the end products of the shikimate pathway, chlorogenic acid and lignins, during the early development of *Capsicum annum*. *New Phytol*. 136: 183 - 188.

Dixon, R.A. & Paiva, N.L. 1995. Stress-induced phenylpropanoid pathway. *Plant Cell* 7: 1085 - 1097.

Dixon, R.A., Achnine, L., Kota, P., Lui, C.J., Reddy, M.S. & Wang, L. 2002. The phenylpropanoid pathway and plant defence - a genomics perspective. *Mol. Plant Pathol*. 3 (5): 371 - 390.

Du Plooy, W., Van der Merwe, C. & Korsten, L. 2002. Changes to the epicuticular wax layer of mango (cv Keitt) due to treatment along a commercial packline. *S. Afr. Mango Growers' Assoc. Res. J.* 22: 32 - 37.

Du Plooy, W., Van der Merwe, C. & Korsten, L. 2003. Ontwikkeling en morfologie van die epidermale laag van mango vrugte, insluitend 'n ondersoek na lentiselstrukture. *S. Afr. Mango Growers' Assoc. Res. J*. 23: 114 - 121.

Esau, K. 1977. *The Anatomy of Seed Plants*, 2nd ed, pp. 183 - 197. Wiley, New York, USA.

Ferreres, F., Castañer, M. & Tomás-Berbarán, F.A. 1997 Acylated flavonol glycosides from spinach leaves (*Spinacia oleracea*). *Phytochem*. 45: 1701 - 1705.

Grassmann, J., Hippeli, S. & Elstner, E.F. 2002. Plant's defence and its benefits for animals and medicine: role of phenolics and terpenoids in avoiding oxygen stress. *Plant Physiol. Biochem*. 40: 471 - 478.

Guerin, H.P., Delavue, P.G. & Paris, R.R. 1971. Localisations histochimiques. II: Procédés simples de localisation de pigments flavoniques. Application à quelques Phanérogames. *Bull. Soc. Bot. Fr*. 118: 29 - 36.

Heim, K.E., Tagliaferro A.R. & Bobilya, D.J. 2002. Flavonoid antioxidants: chemistry, metabolism and structure activity relationships. *J. Nutr. Biochem*. 13: 572 - 584.

Joel, D.M. 1980. Resin ducts in the mango fruit: a defense system. *J. Exp. Bot*. 31 (125): 1707 - 1718.

John, K.S., Rao, M.J.L., Bhat, S.G. & Rao, U.J.S.P. 1999. Characterization of aroma components of sap from Indian mango varieties. *Phytochem*. 52: 891 - 894.

Kahn, V. 1985. Effect of proteins, protein hydrolyzates and amino acids on o-dihydroxyphenolase activity of polyphenol oxidase of mushroom, avocado and banana. *J. Food Sci*. 50: 111 - 115.

Kangatharalingam, N., Pierce, M.L., Bayles, M.B. & Essenberg, M. 2002. Epidermal anthocyanin production as an indicator of bacterial blight resistance in cotton. *Phys. Mol. Plant Pathol*. 61 (3): 189 - 195.

Ketsa, S. & Atantee, S. 1998. Phenolics, lignin, peroxidase activity and increased firmness of damaged pericarp of mangosteen fruit after impact. *Postharvest Biol. Technol*. 14: 117 - 124.

Loveys, B.R., Robinson, S.P., Brophy, J.J. & Chacko, E.K. 1992. Mango sapburn: Components of fruit sap and their role in causing skin damage. *Aust. J. Plant Physiol*. 19: 449 - 457.

Lozovaya, V.V., Gorshkova, T.A., Yablokova, E.V., Rumyantseva, N.I., Valieva, A, Ulanov, A. & Widholm J.M. 1999. Cold alkali can extract phenolic acids that are ether linked to cell wall components in dicotyledonous plants (buckwheat, soybean and flax). *Phytochem*. 50: 395 - 400.

Markham, K.R., Ryan, K.G., Gould, K.S & Rickards, G.K. 2000. Cell wall cited flavonoids in lisianthus flower petals. *Phytochem*. 54: 681 - 687.

Matern, U. 1994. Natural phenols as stress metabolites. *Acta Hort*. 381: 448 - 462.

Mayr, U., Batzdorfer, R., Treuter, D. & Feucht, W. 1994. Surfactant-induced changes in phenol content of apple leaves and fruit skins. *Acta Hort*. 381: 479 - 487.

O'Hare, T.J. & Prasad, A. 1992. The alleviation of sap-induced mango skin injury by calcium hydroxide. *Acta Hort*. 321: 372 - 381.

Passardi, F., Penel, C. & Dunand, C. 2004. Performing the paradoxical: how plant peroxidases modify the cell wall. *Trends Plant Sci*. 9 (11): 471 - 478.

Pollack, S.L. 2001. Consumer demand for fruit and vegetables: the U.S. example. *In: Changing Structure of Global Food Consumption and Trade*. (Ed.) Regmi. A. Economic Research Service USDA WRS-01-1: 49 - 54.

Regnier, T. 1994. *Les composés phénoliques du blé dur (Triticum turgidum L. var. durum): Variations au cours du developpement et de la maturation du grain, relations avec l'apparition de la moucheture*. PhD Thesis, pp. 43 - 45. Montpellier University. France.

Regnier, T. & Macheix, J. J. 1996. Changes in wall-bound phenolic acids, phenylalanine and tyrosine ammonia-lyases, and peroxidases in developing durum wheat grains (*Triticum turgidum* L var. Durum). *J. Agri. Food Chem.* 44: 1727 - 1730.

Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell. Biol.* 17: 208 - 221.

Robards, K., Prenzler, P.D., Tucker, G., Swatsitang, P. & Glover, W. 1999. Phenolic compounds and their role in oxidative processes in fruits. *Food Chem*. 66: 401 - 436.

Robards, K. 2003. Strategies for the determination of bioactive phenols in plants, fruit and vegetables. *J. Chromatogr. A* 1000: 657 - 691.

Rodríguez-Delgado, M.A., Malovaná, S., Pérez, J.P., Borges, T. & García-Montelongo, F.J. 2001. Separation of phenolic compounds by high-performance liquid chromatography with absorbance and fluorimetric detection. *J. Chromatogr. A* 912: 249 - 257.

Russel, W.J., Burkitt, M.J., Provan, G.J. & Chesson, A. 1999. Structure-specific functionality of plant cell wall hydroxycinnamates. *J. Sci. Food Agric*. 79: 408 - 410.

Scalet, M., Crivellato, E. & Mallardi, F. 1989. Demonstration of phenolic compounds in plant tissues by osmium-iodide postfixation procedure. *Stain Technol*. 64: 273 - 280.

Simmonds, M.S.J. 2003. Flavonoid–insect interactions: recent advances in our knowledge. *Phytochem.* 64: 21 - 30.

Sivakumar, D., Regnier, T., Demoz, B. & Korsten, L. 2005. Effect of different post-harvest treatments on overall quality retention in litchi fruit during low temperature storage. *J. Hort. Sci. Biotechnol*. 80 (1): 32 - 38.

Swanson, C.P. & Webster, P.L. 1977. *The Cell*, 4th ed, pp. 88-96. Prentice/Hall International Editions, London, UK.

Tamjinda, B. Siriphanich, J. & Nobuchi, T. 1992. Anatomy of lenticels and the occurance of their discolouration in mangoes (*Mangifera indica* cv. Namdokmai). *Kasetsart J.* 26: 57 - 64.

Van der Merwe, C.F. & Coetzee, J. 1992. Quetol 651 for general use: a revised formulation. *Comm. E. M. Soc. S. Afr*. 22: 31 - 32.

Willis, A. & Duvenhage, J.A. 2002. Evaluation of dips for reduction of sapburn, lenticel damage and postharvest diseases of mango. *S. Afr. Mango Growers' Assoc. Res. J*. 22: 46 - 48.

Wink M. 1997. Compartmentation of secondary metabolites and xenobiotics in plant vacuoles. *Adv. Bot Res.* 25: 141 - 169.

Zhou, Z., Robards, K., Helliwel, S. & Blanchard, C. 2004. The distribution of phenolics in rice. *Food Chem*. 87: 401 - 406.

5.7 TABLES

Table 1 Table with absorbance values of gallic acid used as a phenolic standard to construct a calibration curve (y=0.0013x + 0.0177, r2 = 0.9982) (De Ascensao & Dubery, 2003)

c (ug/ml)	A
0	0.000
10	0.033
50	0.091
100	0.153
150	0.221
200	0.287
300	0.402
350	0.476
400	0.541

Table 2 Table with gallic acid equivalents of the phenolics acid fractions present in each of the three colour groups for the lenticels. Values for phenolic concentration is expressed as gallic acid equivalent per gram dry weight

Phenolic fraction for which gallic acid equivalent was determined	Colour grouping used for lenticels		
	Non-discoloured lenticels	Red discoloured lenticels	Dark discoloured lenticels
Cell wall bound	0.021	0.167	0.330
Ester bound	0.143	0.554	0.420
Glycocidically bound	0.268	0.830	0.702
Free acids	0.132	0.745	0.485
Crude extract	0.825	2.421	2.116

Table 3 Gradient program used for the HPLC analysis of phenolics extracted from mango lenticels

Time	Flow Rate (ml/min)	%A	%B
Initial	1.0	90	10
20.00	1.0	80	20
33.00	1.0	75	25
38.00	1.0	65	35
43.00	1.0	53	47
55.00	1.0	30	70
57.00	1.0	20	80
59.00	1.0	20	80
60.00	1.0	90	10

Table 4 Table with retention times for selected standards, with suggested occurrence of these phenolics in investigated lenticel extracts. Non-conjugated phenolics are referred to as free acids

Standard	Class of Phenolics (Robards *et al*., 1999)	Retention time (minutes)	Lenticel extract occurrence
Gallic acid	Phenolic acid	7.866	Crude extracts, Ester bound extracts, Glycosidically bound extracts
Chlorogenic acid	Cinnamic acid ester	14.966	Crude extracts, Free acid extracts, Ester bound extracts, Glycosidically bound extracts
Mangiferin	Xanthone	17.690	Glycosidically bound extracts
Caffeic acid	Cinnamic acid	19.283	Crude extracts (red, dark only), Free acid extracts
Quercetin	Flavonol	23.501	Cell wall extracts
Sinapic acid	Cinnamic acid	27.592	Crude extracts (red, dark only), Free acid (non-discoloured only), Ester bound extracts, Glycosidically bound extracts (red, dark only)
Ferulic acid	Cinnamic acid	29.037	Crude extracts, Free acid (non-discoloured, red only), Glycosidically bound extracts (non-discoloured, dark only)
Coumaric acid	Cinnamic acid	40.201	Crude extracts, Free acid (non-discoloured, dark only), Ester bound extracts (non-discoloured, red only), Glycosidically bound extracts (non-discoloured only), Cell wall extracts

Table 4 Table of fluorescence colours and the associated phenolics groups (Guerin *et al*., 1971; Regnier & Macheix, 1996)

Fluorescence colour	**Group of Phenolics**
White	Gallic acid / Caffeic acid
Blue-white	Coumaric acid
Light Blue	Hydroxycinnamic acid & Ferulic acid derivatives
Red	Flavonoids
Brown	Quinone
Deep blue / Purple	Not phenolic - Biopolymers, Terpenoids

5.8 FIGURE CAPTIONS

Figure 1 Overview of the biochemical pathway along which anthocyanocides and flavonoids are formed. Only key intermediates and enzymes, and products of interest are indicated.

Figure 2 Weak auto fluorescence in the unstained sections still exhibited distinctive, albeit faint, blue colouration in cells that were filled with phenolics (a).

Figure 3 Fluorescence of a non-discoloured lenticel sectioned through the lenticel cavity. Insignificant build-up of phenolics in the cell walls of surrounding mesophyll tissue (a), and some vacuolar phenolics were visible inside cells (b). Intra-cavital wax phenolics were visible as bright halos on the outer surfaces of cells lining the lenticel walls (c).

Figure 4 Fluorescence of reddened lenticel sectioned through the area adjacent to the lenticel cavity. Random build-up of phenolics in the cell walls of surrounding mesophyll tissue was visible (a). The contents of cells forming part of the cavity wall was differentiated from that of cells further away by the blue fluorescence (gallic and ferulic acid derivatives) (b), with cytoplasmic cinnamic acids evident as white fluorescence on their inner surfaces (c) (Guerin *et al*., 1971).

Figure 5 Fluorescence of darkened lenticel sectioned through an area adjacent to the lenticel cavity. Build-up of phenolics in the cell walls of surrounding tissue became more confluent (a), with encapsulation of the lenticel evident. Fluorescence of the vacuolar content of cells lining the cavity wall changed from blue to blue-white, indicative of caffeic acid (b). The white fluorescence due to cinnamic acids in the cytoplasm surrounding the vacuoles was visible (c). Deposition of polymeric phenolics in the cell walls caused the red fluorescence to have a brownish tinge (Guerin *et al*., 1971).

Figure 6 Transmission electron micrograph of cells from the wall of the cavity of a non-discoloured lenticel. Organelles and membranes are intact, with the endoplasmic reticulum indicated by the black arrows (A). There is no indication of electron dense areas developing in the cell walls (B). Vacuolar contents appeared granular (white arrows in A & B) due to some precipitation of phenolics during the fixation process.

Figure 7 Transmission electron micrograph of cells from the wall of the cavity of a reddened lenticel. All intracellular structures were intact, with abundant endoplasmic reticulum visible (black arrows, A). Deposition of electron dense cell wall material was visible from the outer boundary of the cell wall (B), indicating transport by the apoplastic route rather than secreted from inside the cell.

Figure 8 Transmission electron micrograph of cells from the wall of the cavity of a darkened lenticel. Dense and phenolic rich vacuolar content is signified by uneven cutting marks typical of the phenolic contents hardening during fixation of the material (1 in A, arrow in B). Metabolic functionality was apparent from the intact cellular structures (2 in A). The cell walls were impregnated with electron dense material (3 in A).

Figure 9 Calibration curve of gallic acid as a phenolic acid standard for determination of equivalent phenolic concentrations in the sample material.

Figure 10 Graph of gallic acid equivalents of each isolated phenolic fraction in the dried sample material.

Figure 11 Chromatograms of crude extracts from non-discoloured, reddened and darkened lenticels, with the combined histogram of the changes in peak ratios between the different lenticels. Peaks a - h within each colouration set were compared to peak d.

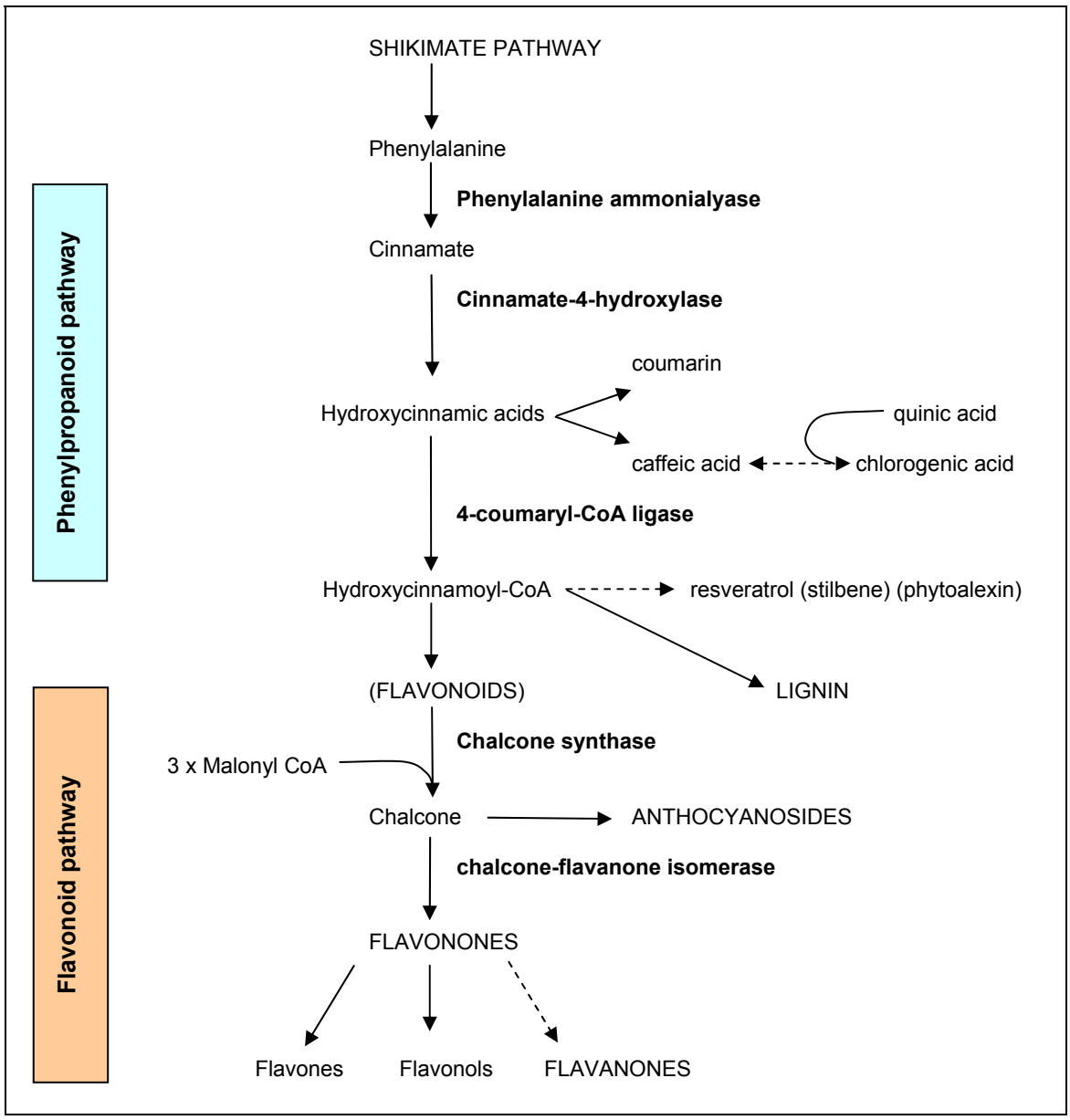
Figure 12 Chromatograms of non-conjugated phenolics (free acids) extracted from non-discoloured, reddened and darkened lenticels, with the combined histogram of the changes in peak ratios between the different lenticels. Peaks a - j within each colouration set were compared to peak c.

Figure 13 Chromatograms of extracts of cell wall bound components from non-discoloured, reddened and darkened lenticels, with the combined histogram of the changes in peak ratios between the different lenticels. Peaks a - j within each colouration set were compared to peak d.

Figure 14 Chromatograms of glycosidically bound extracts from non-discoloured, reddened and darkened lenticels, with the combined histogram of the changes in peak ratios between the different lenticels. Peaks a - g within each colouration set were compared to peak b.

Figure 15 Chromatograms of ester bound extracts from non-discoloured, reddened and darkened lenticels, with the combined histogram of the changes in peak ratios between the different lenticels. Peaks a - i within each colouration set were compared to peak c.

5.9 FIGURES



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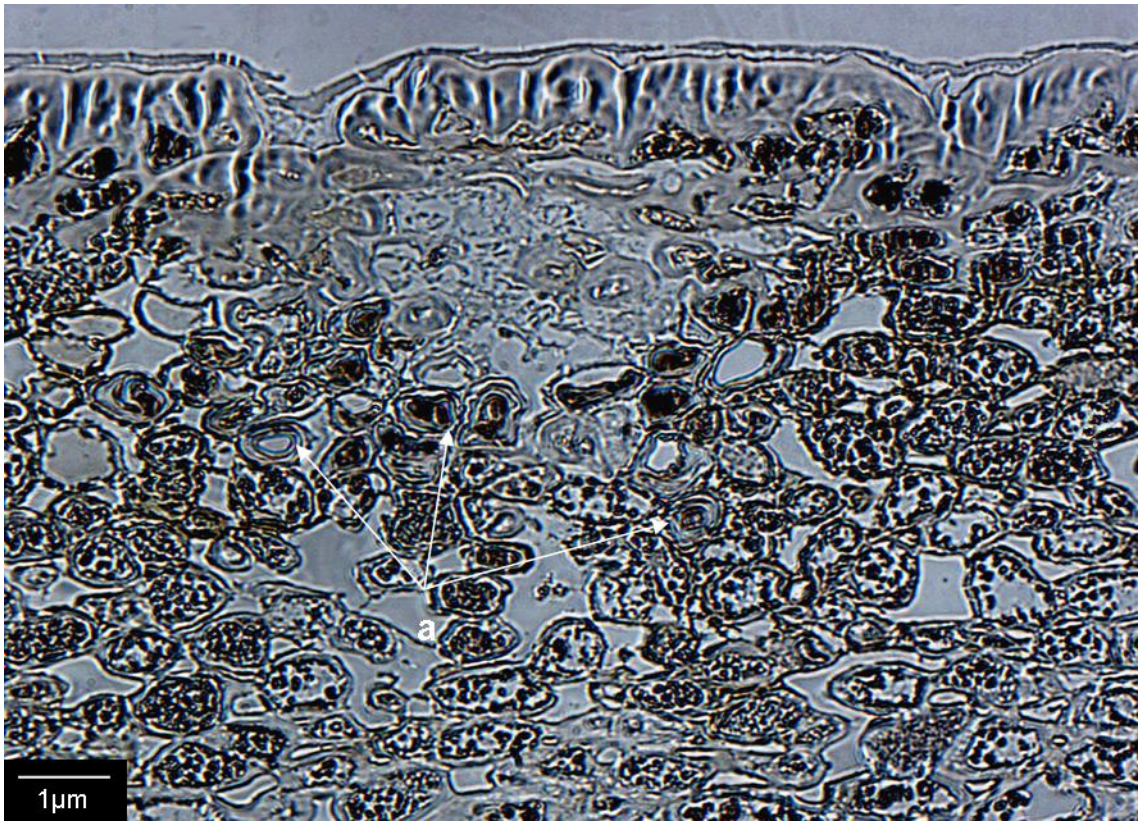
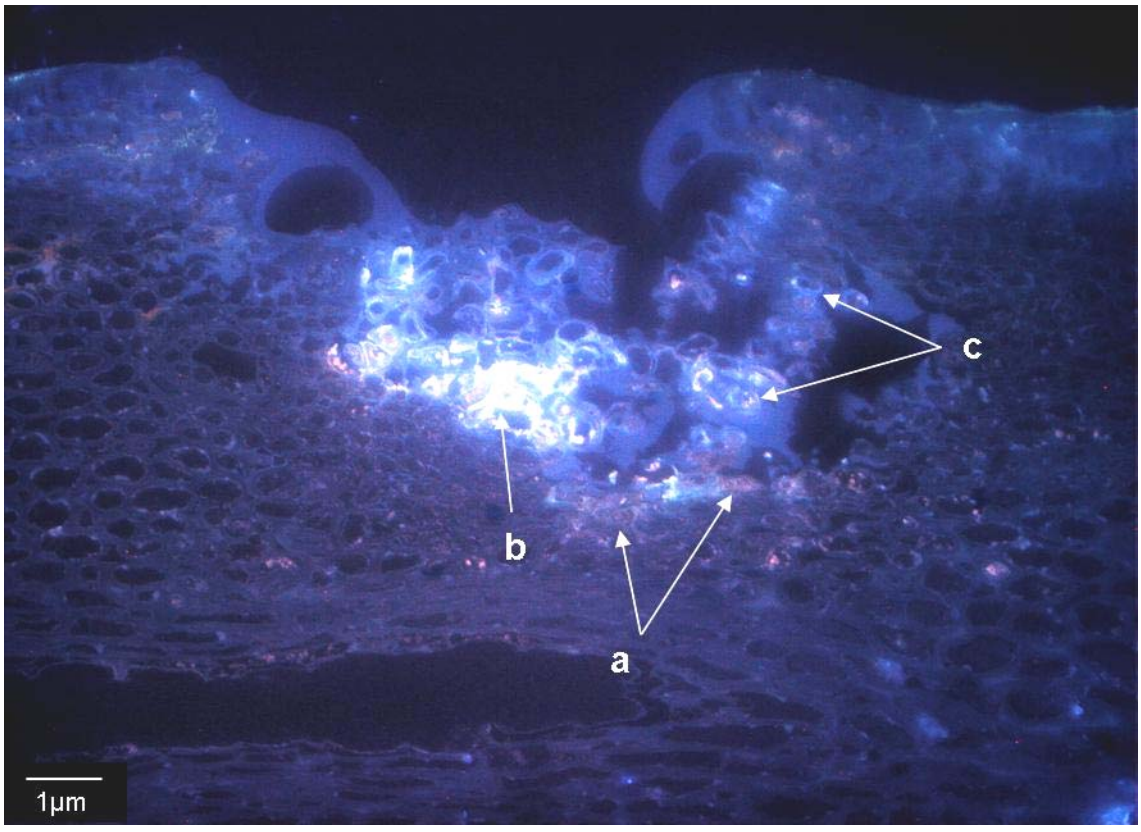


Figure 2



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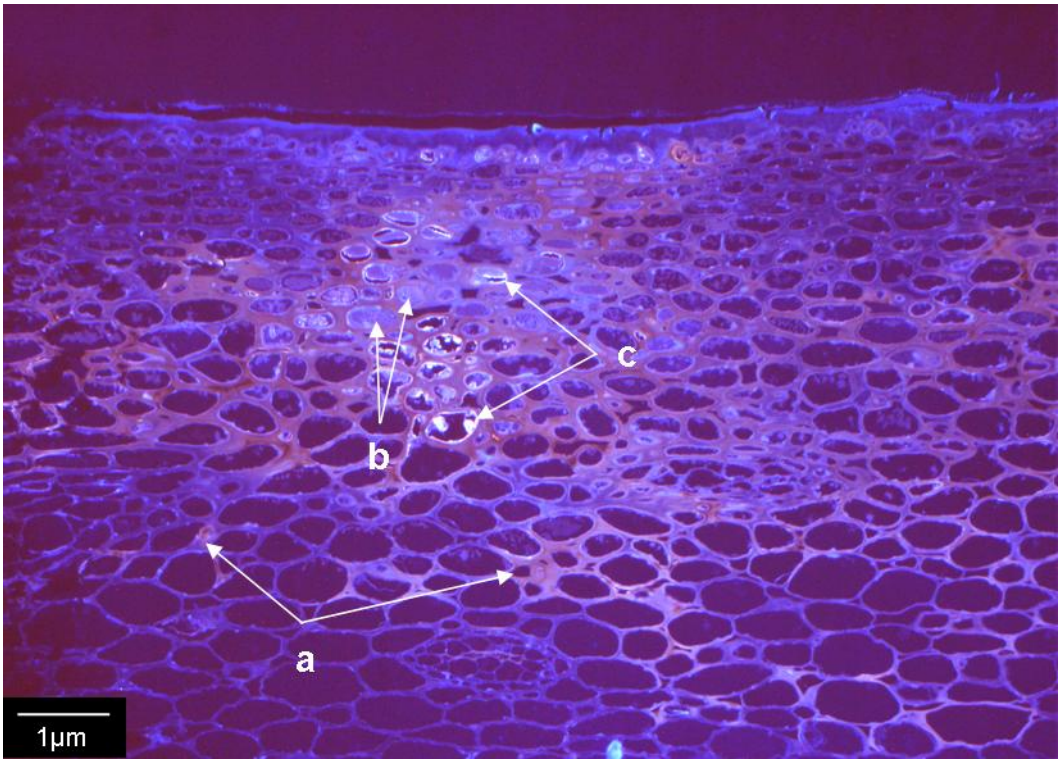

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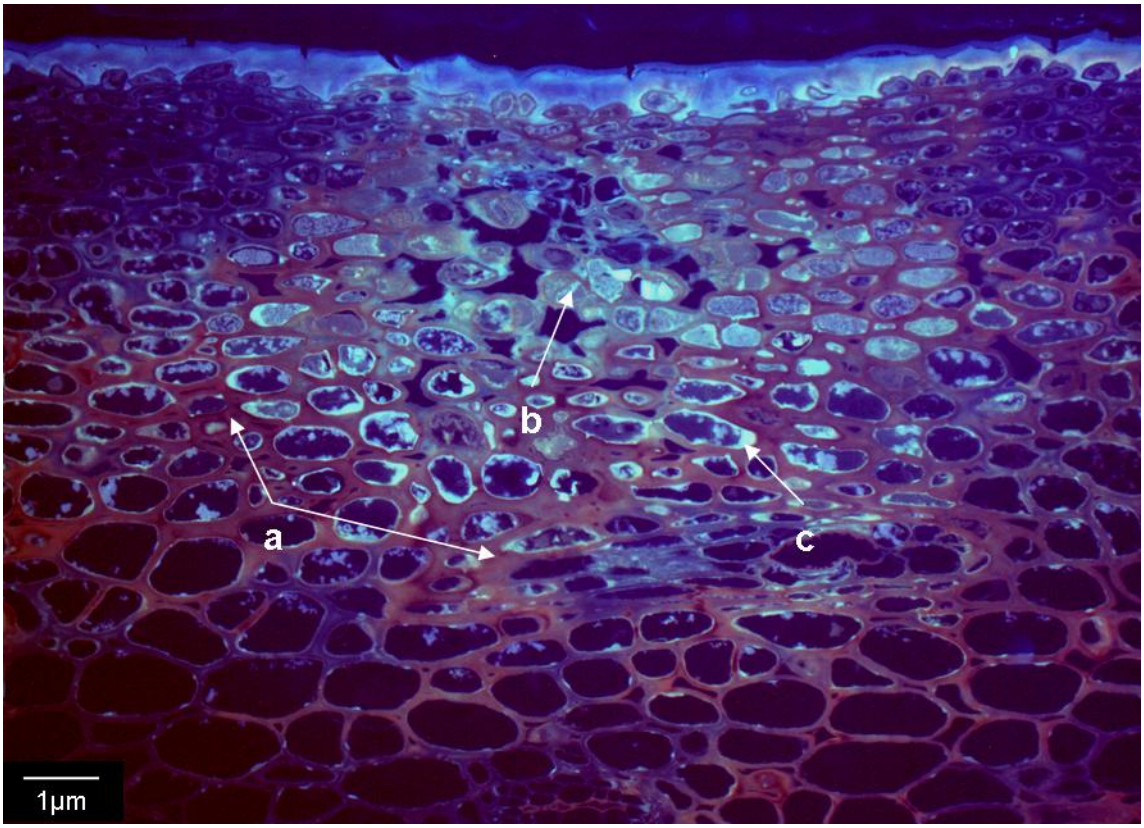

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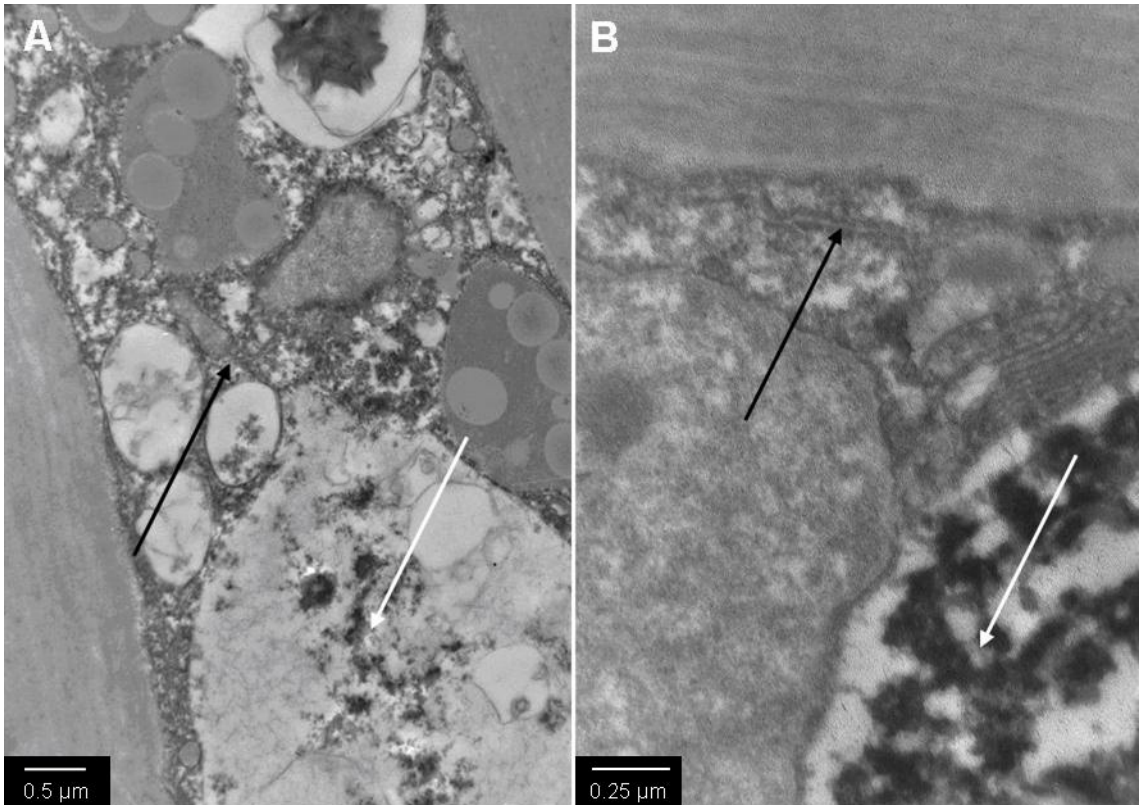

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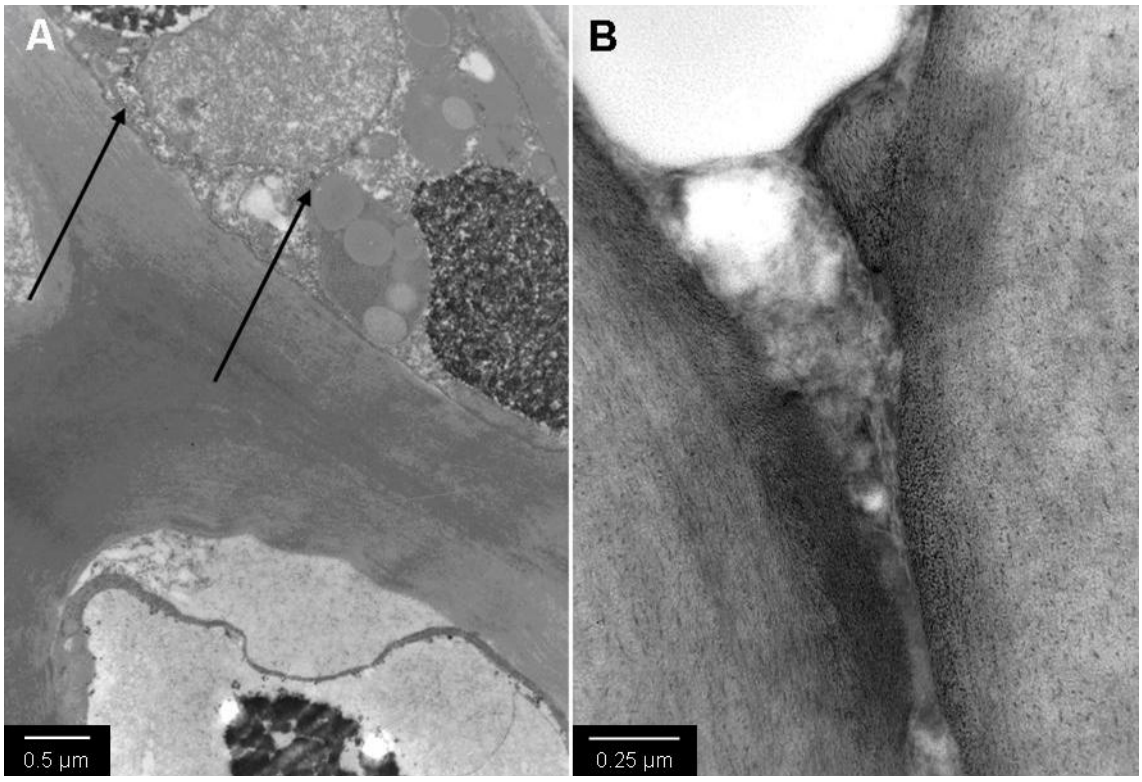

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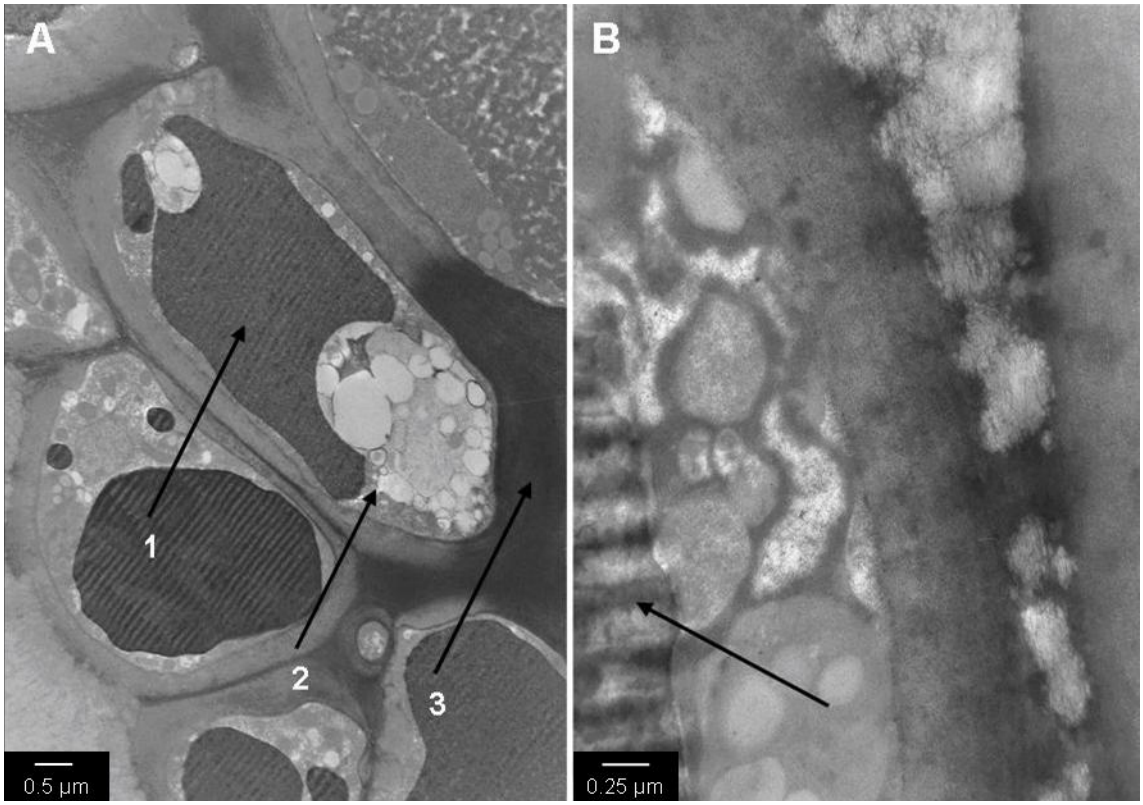

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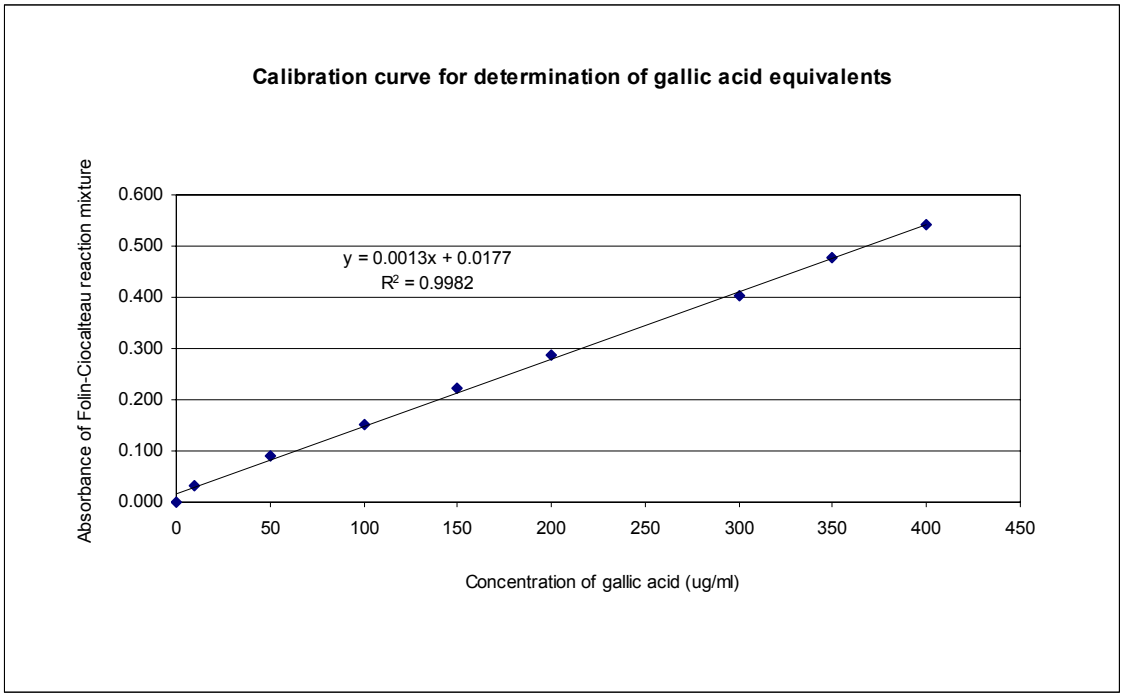


Figure 9

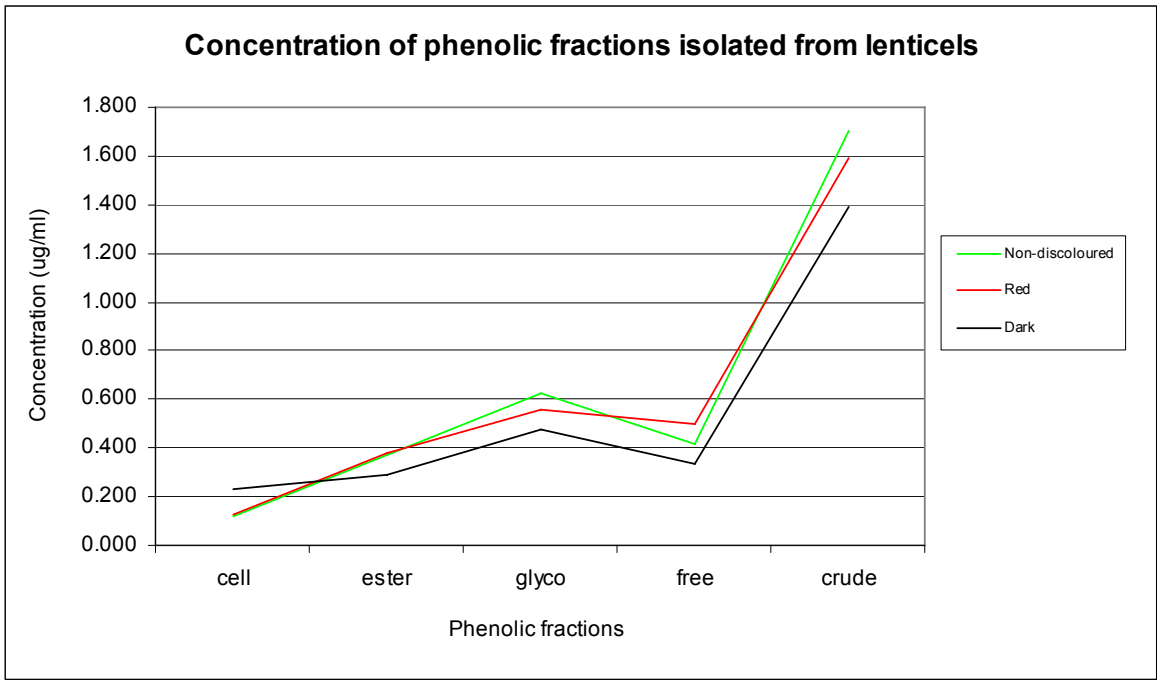


Figure 10

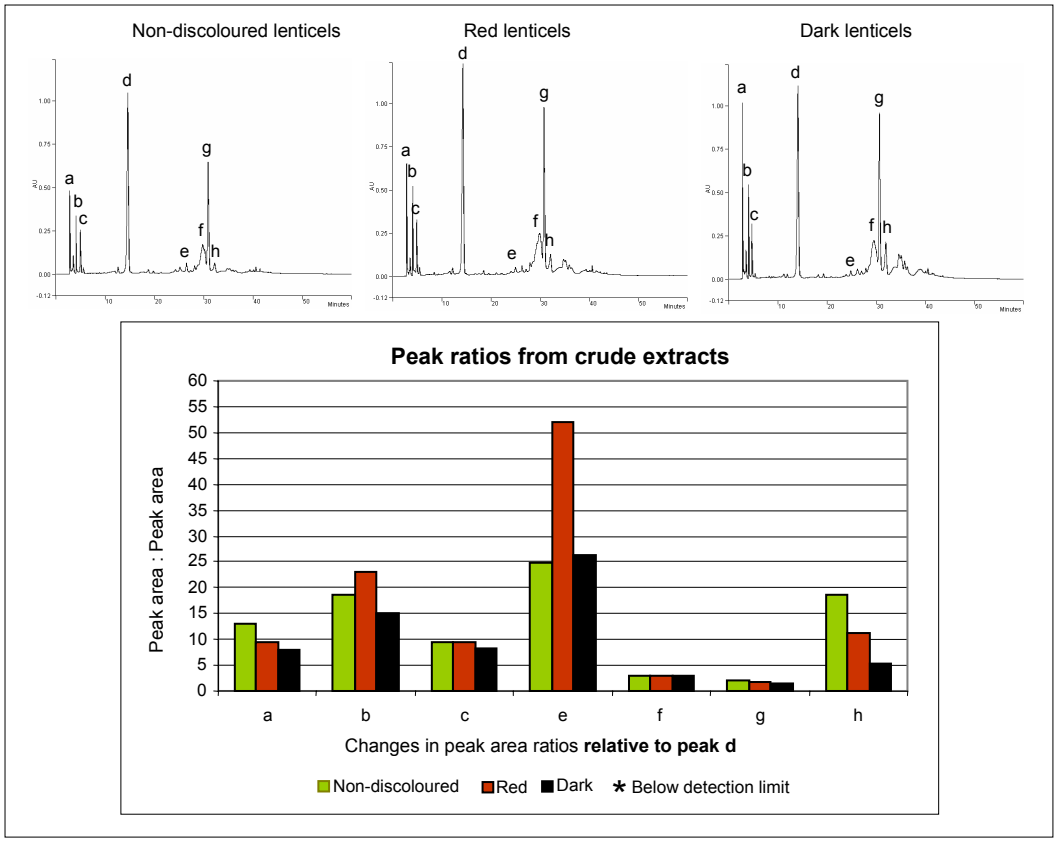
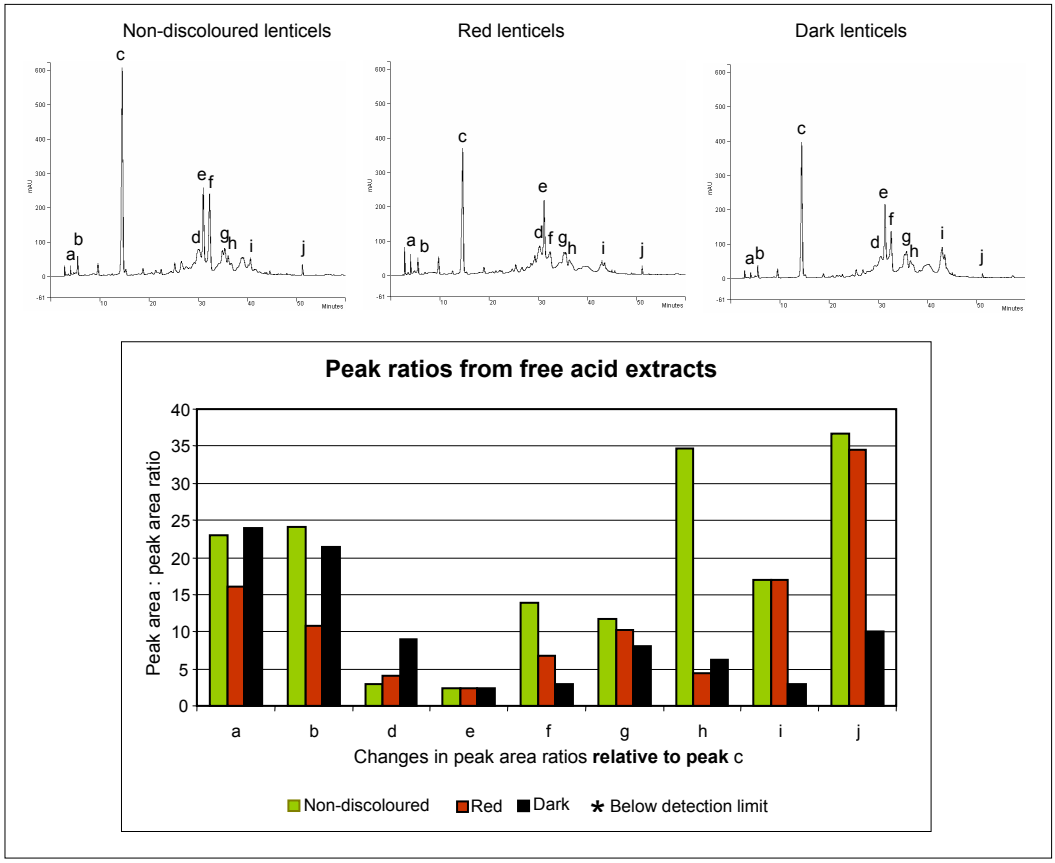


Figure 11



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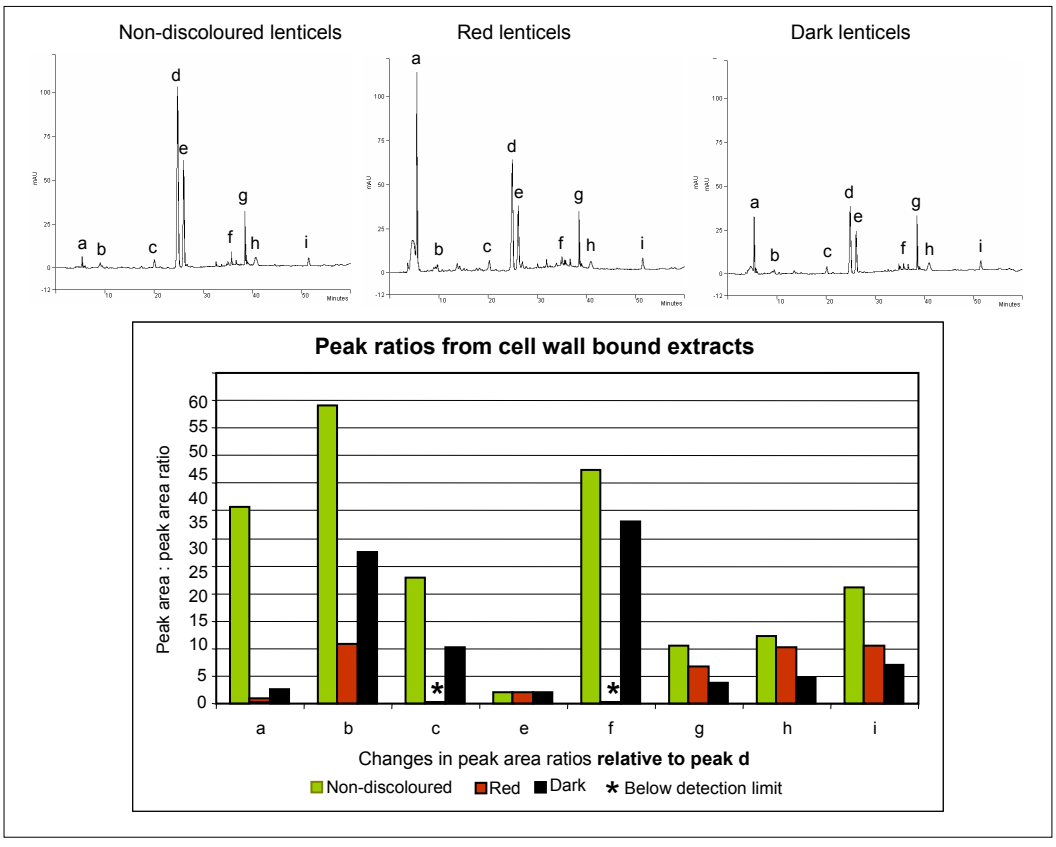

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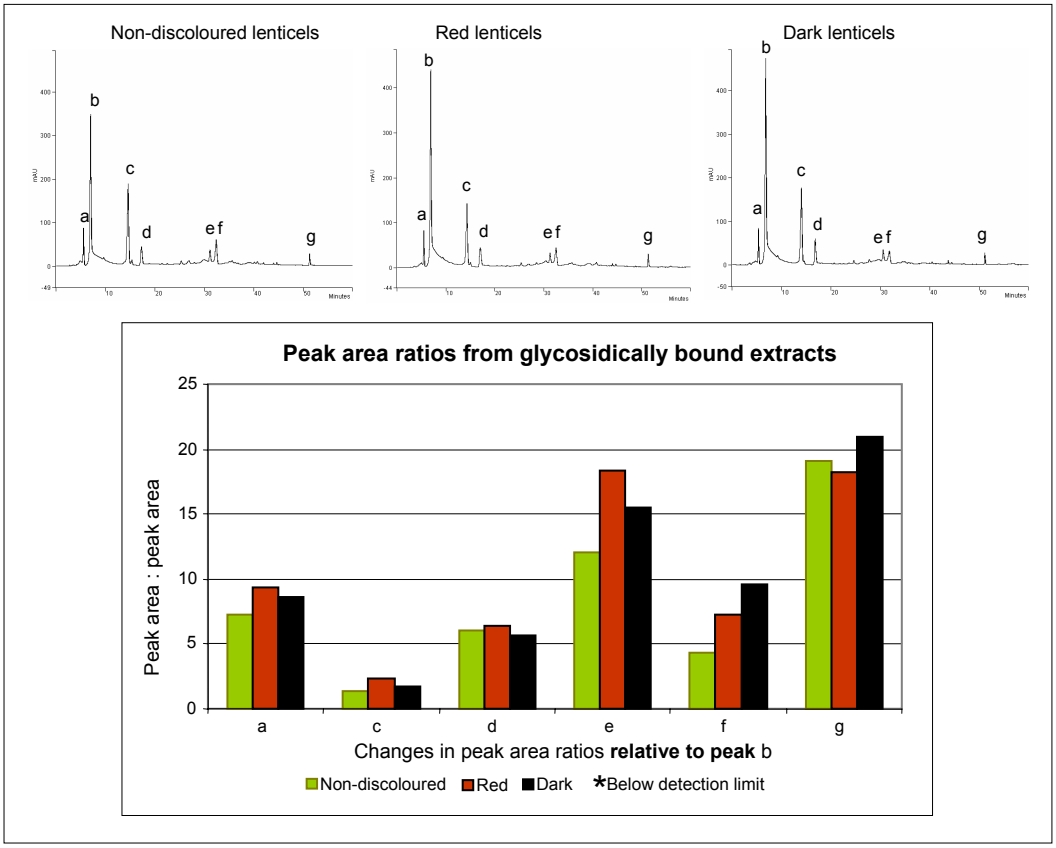

Figure 14

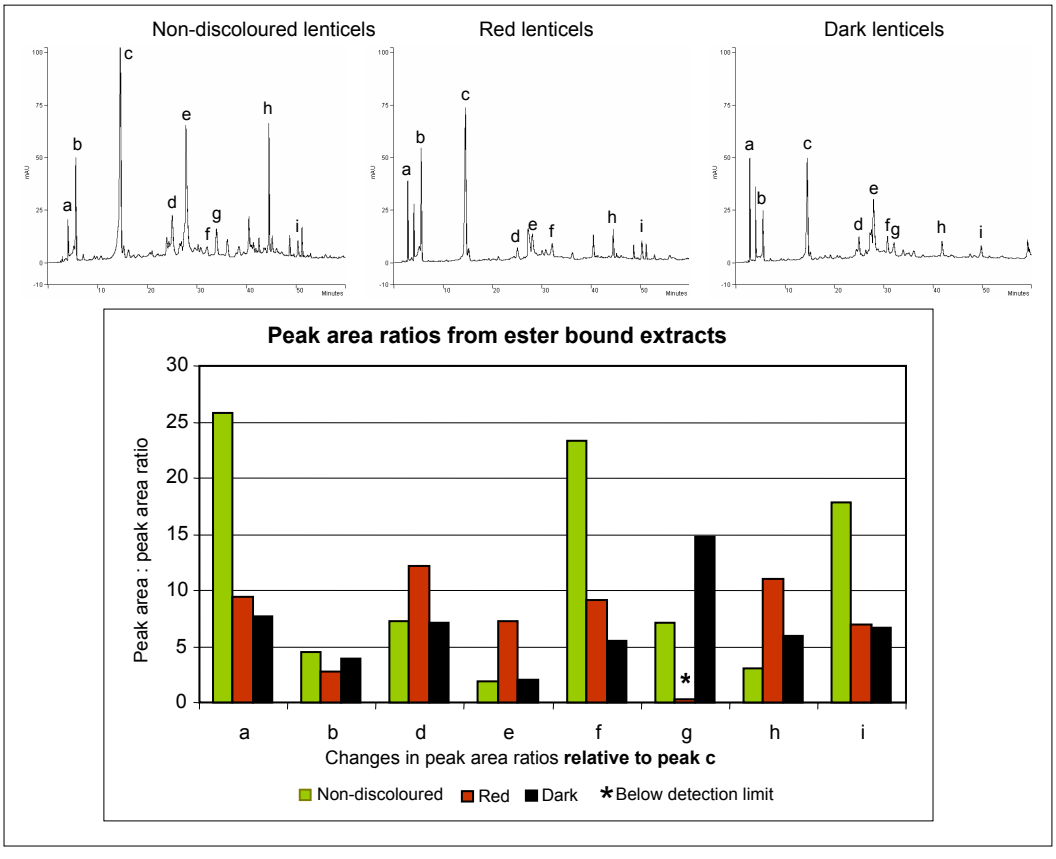

Figure 15