IDENTIFICATION AND QUANTIFICATION OF CAROTENOID AND FLAVONOID PIGMENTS IN AUSTRALIAN WHEAT

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The objective of this research was to identify and design accurate and objective measures for the assessment of pigment type and concentrations in Australian Standard White wheats. This would facilitate the characterisation of individual flavonoid, phenolic and carotenoid compounds all of which can influence wheat flour colouration and therefore colour of products made from the flour. Flour colouration is influenced by the amount and type of pigments and phenolic compounds within the flour. Other factors that influence flour colour include; the protein content, the milling process, storage conditions and time, bleaching treatments, seasonal growth conditions, moisture content and foreign material. Flour colouration influences end product use. Flour high in flavonoid content may not be suitable for white bread making but would be ideal for alkaline noodle production where yellow colouration influence the perception of the noodle quality.

In terms of colour the major distinguishing differences that separates flavonoids from carotenoids is that they absorb light in both the ultra violet and visible regions where as carotenoids absorb light in the visible region only. This characteristic may therefore make a flour with a lower carotenoid content more desirable as this flour will be lighter and whiter. Flavonoids may be more desirable in wheats as their colour impact may not be observed unless under alkaline conditions such as when used for Chinese noodle production. The addition of an alkaline salt (Kansui) during the preparation stage enhances flavonoid yellow colouration.

At the time of this research there were no RP-HPLC methodologies for the characterisation and quantification of carotenoid and flavonoid compounds in wheat. The methodologies for detection of these pigments from other plant materials and foods was researched and applied to identify and develop a successful extraction, detection and quantification process for wheat flavonoid and carotenoid compounds. This research focused on developing methods of compound extraction, purification and then RP-HPLC compound separation and detection using UV-visible and
Photodiode Array Detection. Later experiments were conducted to try to isolate individual pigment compounds from wheat for conclusive structural determination.

Firstly a number of different RP-HPLC separation methodologies where trialled involving the testing of solvent types and combinations using different gradient elution and some isocratic elution systems. The method that provided the best baseline to baseline separation of compounds, the detection of the greatest number of different compounds and good duplication of results with less than 10 % RSD between duplicates, was chosen for further optimisation. Completely different extraction, separation and detection mythologies were needed as flavonoids and carotenoids differ markedly in their polarities. Carotenoids may be either hydrocarbon compounds or xanthophyll compounds that are more polar containing hydroxyl end groups. Flavonoids are essentially polar compounds.

The flavonoid and phenolic traces of wheat flour were found to be very complex with more than fifty different compounds detected and with many co eluting compounds with little baseline to baseline separation. Different solvent elution systems using polar and non polar solvent combinations resulted in different flavonoid and phenolic compound profiles. For separation and detection of flavonoid and phenolic compound the most effective method was found to be a formic acid-water and methanol gradient elution system with compound detection at 280 nm by UV-visible spectroscopy. This method facilitated the best separation of the many compound detected. This method was further optimized to encourage maximum separation of compounds.

Research then focused on trialling and developing different extraction and purification methods to enable rapid characterisation and quantification of flavonoids and phenolic compounds and to perhaps increase the opportunity to separate individual compounds. Different chromatographic profiles were observed for wheat bran extracted with solvents of different polarities. An alkaline-water extraction method and a rapid extraction method using methanol were found to produce the greatest number of compounds with the best compound resolution. These methods
were further compared and found to provide chromatographic traces of similarity for major compounds with eight being detected using both methods, but differences in the number of minor compounds detected. For the first time, it has been established that wheat flour contains some 57 different flavonoid and phenolic compounds. A formic acid-water and methanol gradient elution system using C18 RP-HPLC chromatography enabled the separation of methanol extracted flours. Seven major pigments were consistently observed in fifteen different wheat varieties including two different samples of Rosella wheat flour, Tammin, Hartog, Trident, Cadoux, Machete, Goldmark, Yanac, Vectis, Merring, Batavia, Suneca, Janz and Katunga. RP-HPLC analysis coupled with UV-Visible detection enabled the tentative identification of ferulic acid, chlorogenic acid and apigenin. In the wheat varieties Cadoux, Machete, Vectis and Katunga, two different forms of apigenin were observed. Euradu flour contained three forms. Unfortunately as purification of individual compounds was not achieved conclusive structural differences of these three apiginen type compounds was not achieved. Future analysis using an LC-MS system may facilitate definitive identification of these apigenin forms.

Wheat flour total flavonoid and phenolic concentrations ranged from as low as 3.94 µg.g\(^{-1}\) to as high as 17.35 µg.g\(^{-1}\) exposing the variability in the concentration of these compounds between different Australian Standard White wheat varieties. More work to understand the impact of wheat pigment concentration variation may be of benefit to further identify wheat variety suitability for specific end products further refining the existing segregation system of different wheats.

Consistently observed in low concentrations was ferulic acid, with concentrations ranging from 0.01 µg.g\(^{-1}\) to 0.13 µg.g\(^{-1}\). Chlorogenic acid was not observed in all wheats and was detected in concentrations of 0.11 µg.g\(^{-1}\) to 0.51 µg.g\(^{-1}\) in Trident, Cadoux, Machete, Vectis, Suneca, Janz and Katunga. The presents of phenolic compounds such as ferulic acid and chlorogenic acid may further influence the yellow colouration of flours in a similar manner to flavonoids. The most predominant form of apigenin was observed in all wheat varieties at concentrations ranging from 0.2 µg.g\(^{-1}\) for Rosella wheat to 1.57 µg.g\(^{-1}\) for Katunga.
Methods for the separation of individual pigments for later conclusive structural elucidation using mass spectral techniques were examined in detail. This involved; fraction collection after RP-HPLC separation of compound, examination of the effectiveness of open column purification and fraction collection of solutions at different times during the process, thin layer and paper chromatography. Unfortunately none of these methods or combinations of these methods were able to separate the individual flavonoid compounds from the complex mixture. Semi purified extracts and standard flavonoid compounds were examined using both traditional and more advanced methods for compound structural determination and included using EMS (La Trobe University and Victoria University), FAB (CSIRO), GC-MS (Victoria University) and NMR (Victoria University). Full identification of flavonoids and carotenoids in wheat was not achieved due to the lack of success in isolating individual compounds. These methods were all successful in the structural characterisation of standards. Advancements in analytical instrumentation with the use of LC-MS may now open the way forward for the successful identification of the many wheat flour pigments detected.

With regard to examination of the carotenoid pigments a similar approach was taken for the identification of a suitable RP-HPLC elution system. A number of different solvent elution systems were trialled which resulted in the detection of an excellent chromatographic elution system using acetonitrile-water and ethyl acetate over a gradient elution program with compound detection at 450 nm. This method enables excellent baseline to baseline separation of the carotenoid standards lutein and β-carotene and the detection and separation (with minimal co-elution) of five major pigments groups within the carotenoid wheat flour extract. This solvent elution system was optimised to reduce the overall run time to make analysis of the carotenoids in wheat flours more efficient. It was established that at a 60% acetonitrile-water and 40% ethyl acetate combination the separation of carotenoids occurred. This enabled the gradient elution profile to be modified which reduced the overall runtime of the program from fifty minutes to 35 minutes, saving time and solvent. The carotenoid extract of wheat flour was much simpler than that of the
flavonoids and an available standard β-apo-8’carotenal was able to be used as an internal standard.

Research to identify the most appropriate extraction method for the removal of wheat flour carotenoids involved comparing several extraction methods that varied in the use of solvent type and combination. Extraction efficiencies were able to be calculated with the use of the β-apo-8’carotenal IS. The extraction process utilising diethyl ether, achieved a carotenoid extraction rate of 100%. This was far superior to other methods such as the one utilising acetone and petroleum ether that resulted in an extraction efficiency of 39%. Similar chromatographic profiles using each of the four solvent extraction methods were obtained.

Lutein and β-carotene were amongst the carotenoid compounds detected by comparison with authentic standards in the wheat flours trialled. For the first time, the presence of five other major carotenoid compounds was observed in Meering, Machete, Vectis, Janz, Eradu, Hartog, Yanac, Suneca, Goldmark, Rosella, Batavia, Trident and Cadoux. Quantification of these compounds found that lutein is not always the predominant compound within wheat flour with five other carotenoids observed in concentrations greater than lutein dependant on the variety. Lutein concentrations ranged from 0.4 µg.g\(^{-1}\) to 0.59 µg.g\(^{-1}\). Cadoux contained the highest concentration of lutein observed at 0.59 µg.g\(^{-1}\) which displayed higher concentrations of compound 2 and 3 at 0.64 µg.g\(^{-1}\) and 0.67 µg.g\(^{-1}\). Total pigment concentration of carotenoid ranged from as low as 1.97 µg.g\(^{-1}\) to as high as 4.21 µg.g\(^{-1}\).

Using colour space analysis, the influence of these pigment groups was readily observed when the flour of Batavia wheat was extracted for flavonoids, extracted for carotenoids and extracted for both pigment groups. Flavonoid pigment appears to have more influence on the hue of wheat flour and noodles, carotenoids on the lightness and yellowness of flour, noodle sheets and noodles. The removal of both pigment groups caused a further reduction in yellow coloration suggesting that flavonoids and phenolic compounds also contribute to yellow colouration.
The traditional approach to carotenoid pigment concentration determination using the AACC methodology was compared to the developed RP-HPLC methodology for total carotenoid pigment content and showed little correlation between the two methods. Higher total carotenoid concentrations were observed using the RP-HPLC methodology as expected with a higher degree of precision and accuracy from the improved detection sensitivity of the analytical instrumentation. It is therefore recommended that for wheat characterisation and quantification that the RP-HPLC method be used over the traditional AACC method.

This research has been successful in developing RP-HPLC chromatographic methodology for the detection and quantification of wheat flavonoid, phenolic and carotenoid compounds.
DECLARATION

“I, Samantha Ward, declare that the PhD thesis entitled ‘The Identification And Quantification Of Flavonoid And Carotenoid Pigments In Wheat,’ is no more than 100,000 words in length, exclusive of tables, figures, appendices, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work”.

Signature Date 1/11/2007
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At many a stage in this work there have been times when the end was nowhere in sight. I have finished this work for myself and most importantly to show my growing family that what can be dreamed of can be achieved with will and determination. Thank you mother for your unfailing support and belief that I can achieve just about anything. To Darren and Kari for never doubting my ability and not even considering that anything but success would be obtained.
SCIENTIFIC PUBLICATIONS AND PRESENTATIONS

Publications in Non-Refereed Journals


Presentations

Abstract and Poster Presentations


LIST OF ABBREVIATIONS

RSD relative standard deviation
Av average
RP-HPLC reverse phase-high performance liquid chromatography
HPLC high performance liquid chromatography
LC liquid chromatography
HPLC-MS high performance liquid chromatography-mass spectroscopy
IS internal standard
ES external standard
FAB fast atom bombardment mass spectroscopy
EMS electrospray mass spectroscopy
DMF dimethylformamide
PDM perdeuteriomethylation
PM permethylated
TMS trimethylsilylation
TFA trifluoroacetic acid
DCM dichloromethane
DMSO dimethyl sulfoxide
BSA (N, O-bis[Trimethylsilyl]acetamide).([CH$_3$Si]$_2$OCH$_3$)C-N.
GC-MS gas chromatography mass spectroscopy
FD field desorption
DCI desorption chemical ionisation
GC gas chromatography
PAD photodiode array detection
RDA retro-Diels-Alder
NMR nuclear magnetic resonance spectroscopy
$^1$H NMR proton NMR
$^{13}$C NMR carbon 13 NMR
MS mass spectrum of spectroscopy
RI refractive index
FID flame ionization detector
TLC  thin layer chromatography
k  transmissive index
v/v  volume for volume
MW  molecular weight
N  normality
UV  ultraviolet spectra
%  percentage
KOH  potassium hydroxide
i.d.  internal diameter
\( t_R \)  retention time
CI  chemical ionization
MALDI  matrix-assisted laser desorption ionization
RDA  retro-Diels-Alder
DCCC  droplet counter current chromatography
EIMS  electron impact mass spectrometry
CV  coefficient of variation
TEA  triethylamine
\( \mu g.mL^{-1} \)  micrograms per millilitre
\( mg.mL^{-1} \)  milligrams per millilitre
\( g \)  gram
\( \mu L \)  microliter
\( ^\circ C \)  degrees Celsius
C1  first concentration
V1  first volume
C2  second concentration
V2  second volume
\( mm \)  millimetre
\( cm \)  centimetre
\( \mu m \)  micrometre
Da  Dalton

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<table>
<thead>
<tr>
<th>Symbol</th>
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<tr>
<td>L*</td>
<td>A colour space parameter used to measure lightness with a Chroma Meter</td>
</tr>
<tr>
<td>a*</td>
<td>a colour space parameter that indicated colour direction towards red</td>
</tr>
<tr>
<td>b*</td>
<td>a colour space parameter that indicates colour direction towards blue and the degree of yellowness.</td>
</tr>
<tr>
<td>atm</td>
<td>atmospheres</td>
</tr>
<tr>
<td>rpm</td>
<td>revs per minute</td>
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<tr>
<td>cm.sec(^{-1})</td>
<td>centimetres per second</td>
</tr>
<tr>
<td>µM</td>
<td>micro mole</td>
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<td>UV-Vis</td>
<td>Ultraviolet-visible spectroscopy</td>
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CHAPTER ONE. GENERAL INTRODUCTION

1.0 Introduction to the Australian wheat market

Wheat is the largest grain crop produced in Australia making it the most important seasonal agricultural crop for the country with a gross value of $17.8 Billion for the season of 2004/05 (ABS, 2007). Australia is the fourth largest exporter of wheat in the world, following the US, Canada and the EU with an average export level of 17 million tonnes a year. Domestic consumption is around 5.5 million tonnes per year (AWB, 2007).

Australia’s success in the international market is due to two major factors. First, all Australian wheats are white-grained, characterised as clean, sound and dry (Martin and Stewart, 1994) and depending on the variety, growth site and season, have a range of protein contents (Simmonds, 1989). Wheats grown in the Northern Hemisphere are either red with a reddish-brown seed coat or white. White wheats are often preferable to red wheats because they contain less pigmentation and can therefore be used in a wider variety of wheat-based products. Secondly, Australian wheat is specifically marketed and customized to meet the wheat usage requirements of targeted countries. The AWB was formed in 1939 (Martin and Stewart, 1994) to support and locate market areas for Australian wheat and developed a new wheat classification system that segregated wheats largely on the basis of protein and grain hardness. Research on consumer requirements and the delivery of grains that specifically met consumer specifications resulted in an increase in the export of Australian wheat from 7 million tonnes to 13.5 million tonnes within 20 years (PIE newspaper, 1994).

The segregation of wheat on the basis of physical and chemical characteristics has lead to the classification of Australian wheats into seven major categories. These categories include; Australian Prime Hard, Hard, Premium White, Standard White, Soft, Noodle and Durum. From this
classification the AWB is able to further segregate wheat into fifty different categories to cater for specific market requirements (AWB, 2007). Today, Australian wheat’s are exported to many different regions and are used in a range of different types of products. For example, Australian Prime Hard wheat is principally used for Chinese-style and Ramen-style alkaline noodles as well as the production of high quality, high volume breads (AWB, 2007). Australian Standard White Wheat is used for the production of European-style breads; Asian-style breads and noodles; Indian and Middle Eastern style flat bread (Martin and Stewart, 1994). The inherent properties of these wheat enable their use in specific applications for particular market situations.

1.1 Australian noodle wheat and noodles

Over 80 % of the wheat grown annually in Australia is exported (AWB, 1998). Approximately 40 % of this is exported to the Asia Pacific regions where some 22 % is used in the production of alkaline noodles (AWB, 1993). In Australia, the majority of wheat sold for noodle production is used to make white salted and ramen or alkaline noodles (AWB, 1998). In 1997, five percent of the National wheat crop was used for the production of white salted noodles. Australian wheat is well suited to the production of both Chinese salted and alkaline noodles due to its inherent protein, grain hardness and colour attributes. Generally noodles are made from Australian Standard White (ASW) wheats (AWB, 1996). Noodle wheats are characterised by a protein content ranging between 8 and 13.2 %, as clean and dry, with a carotenoid concentration of approximately 2.5 to 4.6 ppm and Minolta b* reading of ranging between 9.0 to 9.5 (AWB 1996).

There are several different methods for making noodles and these are dependent on the product being made. The basic ingredients are flour, water and salt and in the case of Ramen style noodles alkaline solution, known as Kan-sui, which consists of sodium and potassium carbonate and sodium hydroxide. Initially dry or crumby dough is prepared by the addition of water and salt, or alkaline solution, to the flour. The dough is then passed through rollers repeatedly to form dough sheets of approximately 5 mm thickness. These sheets are then cut into noodles. It is at this stage that different methods of
processing may occur. Further processing such as drying, steaming, frying or par boiling may occur prior to packaging, sale or consumption.

Noodle colour and noodle whiteness may vary according to the pigment concentration and with the flour extraction rate (Moss, 1971). Flour with a high extraction rate (>70%) may contain a greater amount of bran, which may cause an oxidative discouloration observed as brown specks. Protein levels were also found to influence noodle colour (Moss, 1971). The higher the protein content the less bright or white the noodle. The addition of Kan-sui influences the degree of noodle yellowness. This is a result of cationic and anionic exchange on the flavonoid nucleus that results in the development of a stronger yellow colour under the higher pH conditions (Hoseney, 1994). According to Moss (1971) the degree of yellowness can be attributed to the different concentration of flavones and related compounds.

1.2 The importance of flour colour in wheat marketing

To select and develop wheat for specific markets, a good understanding of the quality characteristics of the wheat is essential. There are many different wheat quality factors that contribute to final product characteristics. These include inherent factors; protein, grain hardness and colour, as well as seasonal quality attributes; soundness, moisture content and contamination by foreign material. Wheat colour is recognised as an important quality parameter influencing both the types of products that can be produced from the wheat and manufacturing process, to achieve end product specifications. Yasungaga, and Vemura (1962) stated that “colour is the most important factor in accessing the commercial value” of Japanese noodles. This is because Japanese noodles are made from very basic ingredients, flour, salt and water. The resultant colour of the noodles is directly related to the colour attributes of the flour.

A colour influences consumer preference for wheat based products (Shuey, 1975) and indicates product quality, taste, shelf life, packaging and storage conditions (Little, 1963). In baking, the colour grade is considered as important
as the protein content, (Moss, 1971) influencing the resulting loaf colour. Pasta colour indicates to consumers the taste and texture of the product: consumers preferring a bright yellow transparent pasta (Johnson, et al., 1980). To achieve specific flour colour, wheat with inherent colour attributes may be selected or the colour may be manipulated during milling and processing. Other factors such as storage and moisture may also influence flour colour.

1.3 The inherent properties of wheat

The inherent colour properties are principally due to two major pigment groups, the flavonoids and the carotenoids. The carotenoid pigments determine the yellowness and hue of flour (Ferrari and Bailey, 1929). The degree of flour yellowness is dependent on wheat type and pigment concentration, which is influenced by the wheat variety and the conditions under which the wheat is grown (Yasunga and Vermura, 1962; Hook, 1987). Bratt and McMaster (1976) concluded, after preliminary investigations into the genetic and inherent flour colour of bread wheat, that pigment content showed high hereditability and monogenic or digenic control. Miskelly (1984) examined the effects of flour components on noodle flour and concluded that variation in colour was principally due to genetic factors. To establish if growth conditions were contributing to pigment concentration, Shuey (1976) studied two wheat varieties over two years that were grown in four different locations. He concluded that the different varieties responded differently in the same locations proving that genetics plays a role in pigment content.

The principal carotenoid in wheat is said to be lutein (xanthophyll) and its esters (Palmer, 1922; Markly and Baily, 1935). Minor carotenoids that have been identified include; \( \beta \)-carotene, \( \beta \)-apo-carotenal, cryptoxanthin, zeaxanthin and antheraxanthin (Belitz and Grosch, 1987). Less is known about the types of flavonoid pigments in wheats. Kent-Jones and Herd first detected these pigments in 1927. It was not until 1988, however, that Feng and his colleagues (1988) conclusively identified C-glycosyl flavones in the bran of American Hard Red Spring wheat. Renewed interest in flavonoid pigments
has arisen with the increase in sales of wheat to Asian markets, accounting for over 40% of the total Australian wheat export (AWB, 1993). Much of this wheat is used for the production of alkaline or Chinese noodles. In the preparation of these noodles, an alkaline solution is added to enhance the yellow colouration (Simmonds, 1989), to improve their taste and texture (Miskelly, 1984).

1.4 Other factors that influence wheat colour

It is now understood that wheat colour is influenced by many different factors which include: the milling effect, storage, bleaching treatments, the year of growth, flour granulation, moisture content, bran content, foreign material, carotenoid pigments (Ferrai and Baily, 1929; Geddes et al., 1934) and under alkaline conditions, the flavonoid pigments (Miskelly, 1984).

The different factors that influence flour colour often interact with each other, for example Flour Colour Grade (FCG) is influenced by the extraction rate of the flour. Ziegler and Greer (1971) tested the influence of an increased flour extraction rate on the FCG and observed an increase in the bran content with a higher extraction rate of 70% or more. An increase in the amount of bran results in a lower colour grade. Moss discovered (1971) that both the milling process and the variety of wheat influenced FCG, along with crumb colour, loaf volume and score.

Other factors that may influence flour colour include microbial spoilage and the presence of foreign materials. Microbial spoilage may cause grain discolouration hence it is important to store grain correctly. The presence of foreign materials such as dirt and smut spores (Ferrari and Baily, 1929) depends mainly on the milling process and can be controlled with adequate grinding.

To assess the factors that influence wheat colour, essentially three aspects are measured: A) Flour brightness, B) Carotenoid or yellow pigment content and C) flour colour in terms of its lightness and yellowness.
A)  **Flour brightness**

The brightness of flour may be assessed by determining ash content (Bailey 1925), or by establishing FCG (Symons and Dexter, 1991). Flour colour grade analysis is achieved using either the Agtron (AACC method 14-30, 1983) or Kent-Jones and Martin flour colour grader (AWB, 1998). Both methods involve assigning a score that characterizes the colour. The Agtron colour test involves assigning a score to the paste using light reflectance through a green filter. This test is a measurement of flour colour produced by factors other than carotenoids. The Kent-Jones and Martin method also involves assignment of a score to a flour-water paste that is indicative of the amount of bran contamination (Croes, 1961). A high score is given for flour that is darker, a lower score for flour that is brighter (Moss, 1971). Generally flour with a high colour score contains higher levels of bran, which is undesirable. Bran contains higher concentration of oxidative enzymes (Barnes, 1986; Miskelly, 1984) that may cause flour discolouration. Lower bran content is more desirable as increased bran content may also reduce flour brightness caused by a shadowing effect of the larger bran particles (Miskelly, 1984; Croes, 1961). Ash content is indicative of milling refinement; it is lowest in the endosperm and highest in the aleurone layer of the wheat (Symons and Dexter, 1991). The amount of ash in the flour can only be used as an indicator of colour as it is influenced by both seasonal and growth conditions (Shuey, 1976).

B)  **Carotenoid or yellow pigment content**

Total pigment content involves measurement of the degree of yellowness by establishing the total carotenoid content of solvent extracted flour following standard methods (AACC method 14-50, 1983). Total yellow colouration is expressed in parts per million (ppm). Wheat varieties with high or low pigmentation may be identified and selected for sale for specific wheat markets, or blended to obtain optimum colour grade. Wheat carotenoid
content for the national crop was routinely assessed up until 1999. In the 2000 crop report, however, carotenoid concentrations were not reported.

C) Flour lightness and yellowness

The lightness and yellowness of flour may be assessed using tristimulus colorimetry that measures flour colour following Judd-Hunter values (Oliver et al., 1992). This method involves the use of tristimulus red, green and blue filters together with selected photocells and metering circuits that provide an approximate of Commission Internationale de l’Eclairage, 1931 (CIE) system. This system involves colour matching using all of the three colours red, green and blue.

A common instrument for measuring lightness and yellow colour is with a Chroma meter that against a calibration plate enables the lightness, hue and yellowness of flour (L*a* and b* values respectively) to be measured. As the Minolta colour test requires no sample preparation, this method is preferable to the total carotenoid content method for yellow pigment assessment. Tristimulus colorimetry is also used to assess the colour stability of noodle sheets and to assess the colour of cooked noodles by the AWB. A high correlation between Minolta b* values and total yellow pigment content has been established (Oliver et al., 1992).

1.5 Inadequacies of the current colour analysis systems

With the current methods of flour colour analysis there is some question regarding the accuracy of the methodology as contributing characteristics of the flour and flavonoid pigments may influence the results. There is some doubt about what is actually being measured. Kent-Jones and Herd (1929) observed that the dilute alkaline methanol solution used in the FCG method extracted flavonoids. Hook (1987) investigated the factors that contributed to FCG by adding bran to increase FCG of samples. Using tristimulus colorimetry, he established that the hue of the flour (a* value) for each test sample was different because of differences in sample xanthophyll
concentrations not because of the bran content. The correlation between bran content and colour was low having an \( r = 0.29 \) correlation value. A stronger relationship between FCG and protein content, with a high correlation value of \( r^2 = 0.77 \) was observed. When xanthophylls were removed, protein influenced flour yellowness.

The analysis of total pigment content enables assessment of carotenoids on the basis of \( \beta \)-carotene equivalents. The accuracy of this method is questionable, however, as the major carotenoid pigment in wheat is xanthophyll not \( \beta \)-carotene. This technique of quantification is a crude method for the estimation of a concentration of a complex carotenoid mixture (Simpson et al., 1985).

Assessment of colour using tristimulus colorimetry is fast and simple but does not enable clear distinction between flours with similar colour attributes. Wheat colouration is very subtle and differences between varieties may only be slight (Table 1). Often there is as much colour variation within samples of the same cultivar as there is between different varieties. Because of this variation between cultivar significant differences between varieties have not been established.

Table 1.5 . Minolta colour values of wheat flour derived from different classes of wheat grown in Victoria during the 1995-1996 and 1999-2000.

<table>
<thead>
<tr>
<th>Class</th>
<th>L value</th>
<th></th>
<th>a* value</th>
<th></th>
<th>b* value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hard Wheat</td>
<td>93.1</td>
<td>92.3</td>
<td>-2.0</td>
<td>-1.7</td>
<td>9.1</td>
</tr>
<tr>
<td>Premium White</td>
<td>93.2</td>
<td>92.1</td>
<td>-2.2</td>
<td>-2.2</td>
<td>9.1</td>
</tr>
<tr>
<td>Standard White</td>
<td>93.3</td>
<td>92.6</td>
<td>-2.4</td>
<td>-2.3</td>
<td>9.5</td>
</tr>
<tr>
<td>Noodle Wheat</td>
<td>94.1</td>
<td>93.8</td>
<td>-2.7</td>
<td>-2.5</td>
<td>9.5</td>
</tr>
</tbody>
</table>


The above data suggests that there is very little colour difference between the different grades of wheat, and very little difference between the years.
The current methods of flour colour analysis provide some insight into the type and quantity of pigments but do not enable the assessment of individual compounds that can contribute to colour. These methods do not facilitate the full characterisation and identification of either pigment group responsible for wheat colour. The accuracy of the methods and the integrity of the information they provide is questionable given that colour scores may be influenced by many other factors and not just the two major pigment types.

To ensure that international wheat sales are maintained and increased, an increased understanding of the pigment content in wheat would be beneficial. A comprehensive method of analysis for the characterisation and quantification of wheat pigments will enhance an understanding of how these pigments contribute to wheat colour. The more we understand about the primary factors that influence colour, the pigments, the better placed producers will be to provide specific wheats to meet the needs of buyers.

A wheat quality classification system that ranks wheat according to colour or pigment concentration will provide valuable information to wheat growers and flour manufacturers for selection of the most appropriate wheat variety for growth and for specific end product use. The following research was performed to develop accurate methods of wheat colour analysis that may ultimately be useful for the classification of wheats on the basis of colour.

1.6 Research objectives: Developing accurate methods of pigment detection and quantification.

A challenge to the food industry is to develop more accurate methods for the analysis and identification of nutrients and compounds that influence wheat flour and flour based product quality.

This research was designed to establish accurate and objective measurement of pigment type and concentration. With advancement in computer technology and development of analytical equipment, new methods for the
analysis of wheat colour can be developed. The introduction of High Performance Liquid Chromatography (HPLC) has enabled the detailed characterisation and quantification of many different non-volatile organic compounds such as sugars, amino acids, vitamins and proteins. Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) coupled with identification detector systems such as the Photodiode Array Detector (PAD) or Mass spectrometer (MS) have facilitated the identification of carotenoids in a variety of matrices. Carotenoids have been analyzed in cereal and bakery products (Heinonen, et al., 1989), in fruit and vegetables (Minguez_Mosquera and Hornero-Mendez, 1993, Khachik, et al., 1986). Similarly flavonoids have been separated from plant extracts and quantified using RP-HPLC (Hostettmann, et al., 1984), and from honey (Ferreres, et al., 1991). Similar techniques may be employed to facilitate the identification and comprehensive analysis of pigments in wheat.

1.7 Research Aims

This research was developed to enable the characterisation and quantification of wheat flour pigments. The primary focus of this research was on the development of appropriate techniques. Methodology was developed to enable the separation, quantification and characterisation of carotenoid and flavonoid pigments using High Performance Liquid Chromatography.

- Develop a method for the total extraction of carotenoids and flavonoids from wheat flour.
- Develop RP-HPLC methodology for the characterisation of flavonoid and carotenoid pigments.
- Develop RP-HPLC methodology for the quantification of flavonoid and carotenoid pigments.
- Investigate methodologies for the comprehensive identification of flavonoid pigments.
- Characterise and quantify the flavonoids and carotenoid pigments in different varieties of wheat.
- Examine the relationship between the AACC method for carotenoid
pigment content and the RP-HPLC method of quantification.

- Investigate the carotenoid and flavonoid impact on salted and alkaline noodle colour.

A thorough understanding of the nature and levels of pigments may assist in the classification of wheat on the basis of colour, and help in the selection of wheat for specific end products. It is no longer enough to rely upon the inherent characteristics of the wheat to ensure market competitiveness. Variations in wheat colour, between wheat varieties, growth sites and season must be understood to ensure consumers obtain a consistent product and to enable the development of new markets. This is a first step to understanding some of those interactions by developing methods to obtain comprehensive characterisation and quantification information on specific pigment compounds.

This knowledge gained from this research may benefit Australia’s wheat industry by:

- Facilitating the classification of wheat based on colour attributes to enable selection of the most appropriate varieties for specific end product applications.
- Ensuring importers of Australian wheat receive consistently the type of wheat they specify.
- Enabling wheat colour quality to be maintained.
- Ensuring that wheat colour specifications for specific products are met. Allowing the early detection of wheat with undesirable colour characteristics through wheat breeding programs.
2.0 A history of wheat colour analysis method development.

Flour colour was considered to be a significant attribute of wheat as early in the nineteenth century when advancements in the milling process resulted in the production of whiter flour (Oliver et al., 1992). To obtain a greater understanding of the colour attributes of wheat, methods for the identification and assessment of the factors that influence flour colouration were developed. One of the first methods of flour colour assessment developed in 1911 by Winton, involved extraction of flour with gasoline. Sample concentration was determined by comparison of extract spectral absorption with calibration data obtained with potassium dichromate standards. Instrumental limitations and problems with result reproducibility were encountered. For this reason the method was not considered to be a reliable means of wheat concentration determination (Schertz, 1923).

In 1911 and 1912, it was established that the spectra of wheat extracts resembled that of carotene (Wesner and Teller, 1911; Monier-Williams, 1912). It was assumed that the principal component of wheat was β-carotene. Later research indicated that xanthophyll was the principal carotenoid component (Ferrari and Bayle, 1929). Xanthophyll (lutein) and its esters were conclusively identified as the major carotenoid compound in wheat (Markly and Baily, 1935). In addition to lutein, α and β-carotene were also identified.

With advancements in spectroscopic instrumentation Schertz (1923) determined the amount of carotene in wheat by establishing the transmissive index (k) of β-carotene. The transmissive index was determined by measuring the relative radiant energy at a wavelength of 435 nm in a solution of petroleum ether. Ferrari and Bailey (1929) supported the findings of this method. They confirmed that the use of the carotene transmissive index and consideration of the amount of flour and solvent used for extraction, enabled
calculation of total β-carotene when absorbance of the extract was measured at a specific wavelength. They compared the new method with the gasoline colour test and found that a high correlation between the two methods existed. Problems with reproducibility were encountered with variation between duplicates as great as 40%. This was attributed to quality variation of the gasoline solvent used and by due to differences in instrumentation settings.

Ferrari (1933) improved the method of carotenoid concentration determination by altering the extraction solvent and changing to light cleaners naphtha and absolute alcohol (93:7, v/v).

Binnington et al., (1938) overcame the problem of solvent quality, by identifying a superior solvent after comparing over 60 different solvents. The solvent n-butyl alcohol was selected for its high extractability, low hazard level and high and consistent purity. The specific transmissive index was determined for carotene and xanthophyll in water saturated n-butyl alcohol using a 10 mm cell at 435.8 nm. The $k$ value found for xanthophyll was 1.7225 and 1.6632 for carotene. This method was further refined by Binnington and Geddes (1939) who reduced the extraction time to 45 minutes and improved accuracy by using illuminating radiation. This technique of total carotenoid assessment continues to be used today and became a standard method of concentration analysis adopted by the AACC in 1939.

Kent-Jones and Herd (1927) developed a method for wheat colour analysis based on the amount of bran in a sample and the assessment of flour brightness. The brightness of a flour and water paste was scored and given a FCG. The score was indicative of the level of bran in the flour. Similarly, the Agtron or Pekar slick test involved assigning a colour score to a flour-water paste after assessment of light reflectance through a green filter (Kruger and Reed, 1988). This method measures the colour contribution of pigments other than carotenoids also measuring the mineral content in the flour. As
mentioned previously, there are discrepancies in the accuracy and precision of these methods.

Alternate methods for measuring flour brightness and the milling effect were sought. Measurement using photoelectric tristimulus colorimetry techniques enabled the measurement of flour lightness, hue and yellowness (Croes, 1961). Yasunga and Vemura (1962) dissatisfied with the Pekar slick test examined the colour of different wheats using a photoelectric spectrophotometer and colour notation in CIE chromaticity co-ordinate. This method enabled quick and easy determination of flour lightness and yellowness. To test the accuracy of this method it was compared with the traditional carotene content test using \( n \)-butanol. A high correlation between the carotenoid content and yellowness determined with CIE chromaticity values was established. Hook (1988) was able to develop a more accurate perception of dry flour colour by relating human perception of colour to tristimulus colour values. The relationship between FCG and tristimulus values for both paste and dry flours were examined. \( L \) measures white to black (perfect white, 100), \( a^* \) measures hue, \( a^* \) positive the degree of redness (\( a^* \) negative colour towards green), \( b^* \) the yellowness of flour, grain or wheat based consumables. Flour colour attributes were readily determined. The method was simple to perform and results immediately obtained and the colour values established were closely related to human perception of colour.

Colorimetry was developed for the fast accurate analysis of flour colour. A National standard reference procedure was developed for colorimetric assessment of noodle colour (Allen and Pleming, 1997). A white tile template has been developed for use as a standard with the Minolta chroma meter, for measurement of flour and noodle products by tristimulus colorimetry.

To this day a comprehensive method for total pigment content and pigment identification has not been developed. Other methods of analysis have been sought and the old methods adapted and improved with increasing technological advancement.
2.1 Current wheat pigment identification and quantification methods.

Very little information on the extraction, purification and identification of individual carotenoids and flavonoids from wheat is available. The most common method for carotenoid analysis as mentioned previously involves calculation of total carotenoid content using solution absorbency following the AACC 14-50 method. The use of a Chronometer enables colour differences between wheats and noodle sheet to be objectively determined. Flavonoid pigment isolation and separation has traditionally involved liquid/solid extraction, purification by open column chromatography, followed by separation and collection using paper or thin layer chromatographic separation, then identification by UV-Visible spectroscopy or with authentic standards.

2.2 Carotenoid assessment

Following the AACC official method developed by Binnington and Geddes (1939) for total yellow pigment concentration determination, six samples of each variety were examined and the mean and standard deviation determined in each case. Nineteen different wheats were examined. Concentrations ranged between 1.45 – 5.33 ppm. The semolina wheat Dural and Festival, displayed the highest pigment content of 4.64 and 5.33 ppm respectively. Most wheat had a range of concentrations lower than 2.50 ppm.

Lepage and Sims (1968) were able to isolate and identify lutein as the major wheat carotenoid. They extracted the flour with water-saturated n-butanol, reduced the extract to dryness and redissolved the sample in hexane for later chromatography. The extract was separated into fractions (A, B and C) on a 100-transfer counter-current distributor and portions were further separated by open column chromatography on silica acid. To establish the amount of free xanthophyll in the samples the fractions were subjected to partition between hexane and 90 % methanol. These fractions were subjected to hydrolysis with methanolic potassium hydroxide (0.2 N) and were found after co-chromatography to have similar properties, suggesting that they were esters of
free lutein. To confirm the presence of esters, the fractions were separated by TLC on silica gel with hexane: -isopropyl ether -diethyl ether - acetone: -acetic acid (85:12:1:4:1, v/v) and subjected to transmethylation for GC. The fatty acids (or esters) identified were palmitic (22 %), stearic (4 %), oleic (34 %), linoleic (32 %) and linolenic acid (2 %). The total carotenoid fraction was determined by spectroscopic analysis of the extract prior to separation and was found to be between 3.7 and 2.9 µg.g⁻¹ for Mindum and Thatcher flours. Trans lutein (Figure 2. 1) and lutein diester were identified by comparison of their spectra with previously identified samples as the major components, which was consistent with the findings of Markley and Baily (1935). The percentages of carotenoids were calculated by molar absorption coefficients. Free lutein accounted for 85 % of carotenoids in Mindum wheat and lutein esters accounted for 78 % of total carotenoids in Thatcher wheat. The samples did not contain β-carotene when examined by TLC. The total carotenoid content was found to range between 2.8 to 5.3 µg.g⁻¹ for these two wheats.

![Figure 2.1 Lutein with a C40 carbon chain with hydroxyl groups on the benzene rings.](image)

Belitz and Grosch (1987) reported that the carotenoid content in wheat averaged 5.7 mg/kg and was part of the minor components (1.5-2.5 %) of the lipid fraction of the wheat. These lipids are principally found in the germ and were also present in the aleurone layer of the grain. Durum wheat, renowned for its stronger yellow colouration, was reported to contain 7.3 mg.kg⁻¹ of carotenoids. Lutein was present as the major carotenoid in wheat along with the lesser carotenoids β-carotene, β-apo-carotenal, cryptoxanthin, zeaxanthin and antheraxanthin. The methods for their isolation and identification were not discussed and further references were not given.
More recently Humphries and Khachik (2003) reported the presence of carotenoids in two North American wheat varieties determined using HPLC semi-quantification. Adom et al., (2003) identified and quantified lutein, zeaxanthin and β-cryptoxanthin in eleven varieties of wheat and reported a great range in values between varieties. In a study to understand the potential health benefits of soft wheat varieties from Maryland USA, lutein was identified as the predominant carotenoid with zeaxanthin and β-carotene also reported (Moore et al., 2005).

On an annual basis the AWB conducts colorimetric analysis of wheat flour using tristimulus colorimetry with a Minolta Chronometer CR300 series which measure L, a* and b* attributes. These attributes correspond to the lightness, hue and yellowness of the flour respectively. Lighter flour has the highest L value measured on a scale of 0-100. The b* value or yellowness is measured on a scale of 0-60 with yellow flour having a higher b* value. Hue or redness a* is measured on a scale of 1 to 5 which indicates the degree of green or red colour in the sample, a negative value tending towards red.

Digital image analysis of wheat kernel surface has been used to assess the carotenoid content of wheat (Konopka et al., 2004). They found that the amount of free and non-polar lipids could be predicted on the basis of the colour measurement of the wheat kernel surface.

2.3 Flavonoid analysis

Little advancement in determining the nature and quantities of flavonoids in wheat occurred until 1983, though Kent-Jones and Herd (1927) had detected flavonoid compounds in an alkaline methanol solution. Other researchers have tentatively identified flavonoids in wheat. Anderson (1931 and 1932) published his findings of the flavonoid, tricin in the leaves of wheat. Markly and Bailey (1935), in an attempt to further understand the characteristics of the pigments, studied the dilute alcohol-soluble pigments of whole wheat. They found that 50-67 % alcohol was suitable for the extraction of flavone
pigments. These extracts were acidified and the colour compared with carotene and xanthophyll solutions. Compound colouration was found to be different. The acidified flavone extract was a yellow-brown colour, the carotene an orange-yellow colour and the xanthophyll fraction a lemon-yellow colour. They subjected the alcohol solution to alkaline conditions and found that the solution turned a greenish-yellow, a characteristic of flavonoids (Kent-Jones and Herd, 1927). This extract was found to display a high absorptivity of 435.8 nm with a minor band at 525 nm. Further UV-visible spectral analysis showed that the aqueous alcohol extract of durum wheat displayed a distinct band at 269 nm and a lesser band at 325 nm. The addition of alkali to the solution resulted in an increase in intensity of these bands at 269 and 325 nm and a shift in the spectra from 379 nm to 424 nm. They concluded that this shift was the reason for an increase in colour when solutions were made alkaline.

Feng and his colleges (1988) isolated and identified two flavonoids from Hard Red Spring wheat bran. Isolation involved extraction with dilute sodium hydroxide and separation of the flavonoids from non phenolic compounds using open column chromatography with Amberlite XAD-2 resin followed by size exclusion chromatography on Sephadex G-15 resin. The flavonoid fraction was purified using three dimensional paper chromatography. The paper was developed with ethyl acetate-formic acid-water (66:14:20, v/v), then 15 % acetic acid and finally tert-butanol: acetic acid -water (3:1:1, v/v). Further separation was achieved using TLC on silica gel with the developing solvents, chloroform-hexane (1:1, v/v), for final purification of the flavonoids. Two pigments were isolated and identified using shift reagents by UV-visible spectroscopy, by mass spectroscopy and with 13C-NMR using deuterium oxide to dissolve the samples and acetonitrile as a reference compound (Feng et al., 1988). Acid hydrolysis was performed to establish the sugars attached by oxygen or carbon linkages to the flavonoid aglycone (Markham, 1982). Apigenin was identified as the flavonoid aglycone. Sugar group attachment was found to be by C-glycosidic linkage as acid hydrolysis did not occur. The 13C-NMR chemical shifts of spectra established that the compounds were similar to 6-C-glycosyl-8-C-arabinasylapigenin. Mass spectra of the permethylated compounds enable
determination of the location of sugar groups attached to the flavonoid nucleus. UV-visible spectra (Bouillant et al., 1975) and interpretation of the results of the different identification process enabled them to conclude that the major flavonoid was apigenin-6-C-arabinoside-8-hexoside and the minor apigenin-6-C-hexoside-8-C-pentoside (Figure 2.3).

![Figure 2.2 Apigenin-6-C-arabinoside-8-hexoside (A) and apigenin-6-C-hexoside-8-C-pentoside (B)](image)

Using these flavonoids as standards, Feng and McDonald (1989) identified and quantified flavonoid compounds in several different classes of wheat (Table 2.1). Isolation and purification was achieved by small-scale extraction (2 g) of bran with alkaline water. Isolated samples were identified by TLC, UV-visible and fluorescent colour comparison with the isolated standards. The extracts were quantitatively analysed by preparation of a standard curve using the isolated standard di-C-glycosylapigenin following a procedure described by Mabry et al., (1970). The same flavonoids were identified in four classes of wheat bran. A range of concentrations was observed with a noticeable increase in content for the durum wheats.
Table 2.1. Analysis of flavonoid concentrations in different classes and varieties of wheats.

<table>
<thead>
<tr>
<th>Class and Variety</th>
<th>Mean Concentration (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hard red spring</td>
<td></td>
</tr>
<tr>
<td>Len</td>
<td>149.1</td>
</tr>
<tr>
<td>Alex</td>
<td>245.4</td>
</tr>
<tr>
<td>Stoa</td>
<td>289.0</td>
</tr>
<tr>
<td>Hard red winter</td>
<td></td>
</tr>
<tr>
<td>Roughrider</td>
<td>288.2</td>
</tr>
<tr>
<td>Agassiz</td>
<td>271.1</td>
</tr>
<tr>
<td>Durum wheat</td>
<td></td>
</tr>
<tr>
<td>Rugby</td>
<td>405.7</td>
</tr>
<tr>
<td>Lloyd</td>
<td>358.4</td>
</tr>
<tr>
<td>Vic</td>
<td>291.2</td>
</tr>
<tr>
<td>White wheat</td>
<td>320.3</td>
</tr>
<tr>
<td>Overall mean</td>
<td>290.9</td>
</tr>
</tbody>
</table>

Reproduced from Feng and McDonald (1989).

Flavonoids in buckwheat have also been investigated and quantified (Qian et al., 1999) with the detection of protocatechuic acid or catechin and rutin in some of the cultivars studied.

The roles of these pigments with respect to their influence on wheat colour, before and after processing of specific wheat products (ie. noodles) have not been given serious consideration.

2.4 General properties and the physical and chemical characteristics of carotenoid and flavonoid pigments.

2.4.1 The carotenoid pigments

Carotenoid compounds are orange, red and yellow pigments found in plant material and in a variety of organisms such as algae, animals and microorganisms (Scita, 1992). In humans carotenoids can only be obtained by consumption of plants containing these pigments. Some carotenoids are essential for the metabolism of vitamin A. The most active and common
provitamin A carotenoid is β-carotene (Ball, 1988) others include β-cryptoxanthin and β-apo-8'-carotenal. These carotenoids exhibit provitamin A activity and are converted in the intestine or liver to retinol via retinaldehyde and retinyl palmitate.

Carotenoids are subdivided into two main groups, carotenes and xanthophylls. Carotenes include α and β-carotene and are essentially hydrocarbon compounds (Ball, 1988). Xanthophyll compounds are more polar containing hydroxyl end groups. The term xanthophylls also includes alcohols (lutein and zeaxanthin), esters (spheroidene) ketones (astaxathin), epoxides (violaxanthin) and acids (torularhodin) (Nelis and De Leenheer, 1989). The major difference between the carotene and xanthophyll pigments is that xanthophylls do not possess vitamin A activity (Kruger and Reed, 1988). All carotenoids have a basic C40 structure derived by the addition of two terpene subunits (Lea, 1988) (Figure 2.4).

![Figure 2.3 Basic C40 Structure of carotenoids](image_url)

The conjugated double bonding in the structure of the carotenoid causes the yellow, red and orange colouration and is due to the movement of electrons along the unsaturated chain (Klein, 1992). There are 300 naturally occurring carotenoids. Carotenoids are principally responsible for the yellow and orange colouration of foods. This is due to chromophoric activity across the double bonds of the molecule. The typical carotenoids found in plants are displayed in Figure 2.4. Carotenoids accumulate in the chloroplast of all green leaves and most plants contain the same major carotenoids.
Figure 2.4. Commonly occurring carotenoid compounds in plants.

Carotenoids that are found in wheat are mainly xanthophylls (which contain polar end groups (Lea, 1988) such as lutein and its esters (Heinonen, et al., 1989). Of all the different wheat fractions (the endosperm, bran and embryo), the embryo contains the highest concentration of carotenoids (Chen and Geddes, 1945).

Carotenoids are fat-soluble and therefore principally soluble in organic solvents. According to Lea (1988), however, the carotenoids in plants are mainly water soluble and are associated with the lipids in the chloroplast cells. Carotenoids are sensitive to light and readily degrade by oxidation. Lea (1988) stated that pigment loss can be caused by thermally induced isomerisation from trans to cis forms, epoxidation in the presence of oxygen or peroxide, acidic degradation of epoxides to furinoid oxides and photosensitive degradation catalysed by metals. Scita (1992) examined the stability of β-carotene. Under different laboratory
conditions samples of β-carotene were exposed to light and air over a period of time and measured spectroscopically. Rapid degradation occurred with exposure to light including Ultra-violet light and with exposure to atmospheric air. For this reason he and others (Lea, 1988) recommend that carotenoids be extracted away from direct light and were possible under inert conditions (i.e. under nitrogen).

2.4.1.1 Health influences and applications

Carotenoid compounds are often added to food as a natural colourant. In some cereal products they are added as a dietary supplement. In addition to provitamin A activity some carotenoids have antioxidant activity and may play a role in the immune function of animals (Macrea et al., 1993) and have been linked with cancer prevention in humans (Hendry and Houghten, 1992).

2.4.2 The flavonoid pigments

Flavonoids are considered to be responsible for the majority of flower colours (Harborne and Grayer, 1993). Flavonoid compounds contribute to the red and purple colour of flowers, and grapes and depending on the class of flavonoids, to yellow pigmentation. Flavonoid compounds can be found in higher plants, they are not synthesised by animals but have been found in insects where it is assumed they are obtained by ingestion of plants (Harborne, 1967). They have been found in the roots, stems, leaves, flowers, seeds and the bark and wood of plants (Geissman, 1962) as well as in tea and wine (Middleton and Kandaswami, 1993).

The anthocyanin flavonoids are generally found in the fruit, flower and leaves of plants, the catechins and flavan-3,4-diols in wood and bark and the aurones and chalcones in flower petals. Flavones, flavonols and flavanones are found in all parts of the plant (Geisman, 1962). In angiosperms, the chemical basis of yellow flower colour is from carotenoids, flavonols, anthochlors and carotenoid and yellow flavonoids together. As the different parts of a flower have different
pigment groups, so do the different parts of the wheat grain. It has been reported that the majority of flavonoid pigments have been found in the bran layer (Kent-Jones and Herd, 1927).

Flavonoid function in plants is thought to include resistance of plants to insects (Strack and Wray, 1993). Rutherford et al., (1995) found that three flavonoids of sugarcane were associated with resistance to the sugarcane borer *Eldana saccharina*. Some types of flavonoids are thought to protect the plant by producing a toxin. The anthocyanins flavonoids responsible for the colours red, purple and orange are thought to function by attracting pollinating insects and birds (Mabry and Ulubeless, 1980; Strack and Wray, 1993). The isoflavonoid, vestitol was found to posses antifeeding activity and to deter the root feeding larvae of *Costelytra zealandica* from eating the root of *lotus pendunculatus*, a legume (Russell et al., 1978). In terms of colour the major distinguishing factor that separates the flavonoid compounds from carotenoids and quinones, is that they absorb light in both the UV and visible region. Quinones and carotenoids absorb light in the visible range only.

All flavonoids contain 15 carbon atoms in a parent molecule and share a common structure of two phenyl rings linked by a three carbon oxygenated moiety (Agrawal and Markham, 1989, Geissman, 1962). The 15 Carbon atoms are arranged in a C6-C3-C6 configuration (Figure 2.5) and these rings are labelled A, B and C. The individual carbon atoms are referred to by a numerical system to enable characterisation of the many flavonoid variants.

![Figure 2.5. The basic flavonoid structure.](image)
Flavonoids occur in a variety of structural forms of which there are over 4000 known flavonoids were they are distinguished from each other by the oxidation level on the central ring (Figure 2.6) (Strack and Wray, 1993). The flavones and flavanols are collectively the most abundant group of flavonoids with between 200-300 known substitution (Harbourne, 1965). Flavanols are distinguished from flavones, by an additional hydroxyl group on the C3 central molecule joining the A and B rings of the compound. Both these forms significantly contribute to colour as yellow pigments.

![Chemical structures](image)

**Figure 2.6.** The seven major classes of flavonoid pigments.
Flavonoids and phenolic compounds isolated from wheat have been reported as light yellow. In a review of the literature on flavonoid characteristics (Harbourne and Grayer, 1993) therefore suggest that the flavones and flavanols are most likely to be present in wheat, as they contribute to the yellow colouration of many different plants.

The majority of flavonoids occur as glycosides (Seshadri, 1962, Hasler et al., 1989) monosaccharides and alcohols, (Geissman, 1962) and their solubility is dependent on the position of the sugar group (Seshadri, 1962). Flavonoid glycosides are generally soluble in water and polar organic solvents. Two forms of glycoside linkage exist, flavonoid-O-glycosides were the sugar is attached to the flavonoid nucleus via an oxygen linkage and flavonoid - C-glycosides were a carbon-carbon bond to the flavone nucleus attaches the sugar. C-glycosyl compounds are commonly found amongst the flavone compounds (Harborne, 1967).

Another class of flavonoids that contribute to the yellow colouration of flower pigments are the Aurones. To date, however, their presence has only been identified in Coreopsis grandiflora (Harborne, 1967).

A less common group of flavonoids are the acylated glycosides. The acyl group may be either ferulic, p-coumaric or caffeic acid. These acyl groups are attached to the flavonol by the sugar alcohol molecules. To date these forms have only been isolated from lime flowers (Tiliroside) and pea seedlings (Pentunoside) (Harborne, 1967).

2.4.2.1 Health influences and applications

Flavonoids pigments have been reported to provide antioxidant activity, act as enzyme inhibitors and as precursors to toxic substances (Middleton and Kandaswami, 1993). In addition they have been recognised as being antiviral, anti-inflammatory and as having anticarcinogenic activities.
Highly coloured flavonoids such as the anthocyanins have potential in the food industry as natural food colourants (Strack and Wray, 1993).

2.5 Other compounds which may contribute to wheat colour.

In wheat, phenolic compounds such as polyphenol oxidase (PPO) contribute to the discolouration of wheat products due to enzymatic breakdown of the product. Crosbie and his colleagues (1996) observed that good noodle colour stability was associated with low levels of PPO. Ferulic acid (Figure 2.7) is another phenolic compound present in wheat and was monitored in milled fractions of wheat using HPLC and by microscopy (Pussayanawin, 1988). Ferulic acid was identified in Australian wheat varieties and used for quantification of flavonoids by Wang and Mares (1995). Its concentration in different wheat varieties ranged from 42-70 mg.100g$^{-1}$ of whole meal flours in 13 cultivars. Evidence that ferulic acid contributes to alkali noodle colour was found when a pale yellow colour developed when the ferulic acid was subjected to an alkaline solution.

It is interesting to note that ferulic acid, cinnamic acid, $p$-coumaric acid, caffeic acid and Chlorogenic acid contribute to the formation of various flavonoids such as anthocyanins (Salibury and Ross, 1985) derived from the Shikimic acid pathway. These acids are widely distributed in plants (Klein, 1992).

![Figure 2.7 Ferulic acid](image-url)

Some overlap of colour properties exists between flavonoids and phenolic compound such as xanthones, (also yellow pigments), and quinones. It is, therefore, possible that these polyphenolic compounds may contribute to wheat colour (Harbome, 1967). A common xanthone is mangiferin. The easiest way to distinguish xanthones from flavonoids is by comparison of their UV-visible...
spectra. Xanthones exhibit wavelength maxima in four regions at 230-245, 250-265, 305-330 and 340-400 nm whereas flavonols exhibit wavelength maxima in two band regions, 250-280 and 330-350 nm only (Markham, 1982). Xanthone compounds were isolated along with flavonoids from Gentiana vema by Hostettmann and his colleagues in 1984 (Hostettmann et al., 1980). They successfully isolated and identified mangiferin, isooreintin, swertianin-1primeveroside, gentiacaulein-1-0-primeveroside and decussiatin-1-0-primeveroside using HPLC post column dramatisation to obtain spectral shift information and retention time comparison with authentic standards.

Unlike xanthones, the quinones generally do not contribute to plant colour. Anthraquinones, however, display colours similar to that of anthocyanins, reds and purples. These compounds, however, have only been found in microorganisms. Plumbagin is a typical quinone found in plant roots and leaves. Like the xanthones, it has quite a different spectral pattern to flavonoids. Wavelength maxima are expressed at 220–235, 235–280 and a less intense band at 350-410 nm.

2.6 General methods for the extraction and analysis of flavonoid and carotenoid pigments from plants

Because of a lack of information available on wheat flour and bran pigment analysis, it became necessary to examine the processes of extraction and analysis using methods derived from other plant and food sources. Information on the carotenoid and flavonoid pigments in plants and food products may be derived from analysis of extracts. In general, pigment extraction involves solid/liquid methods by stirring or refluxing. For individual pigment identification a separation technique that enables the collection of individual compounds for later identification, is essential.

There are many methods for carotenoid and flavonoid identification both chemical and physical. The most common and reliable method of analysis involves determination of the physical structure of isolated compounds. Definitive identification of a compound requires supporting structural information.
gained from more than one physical method of structural analysis (Khackik and Beecher, 1987; Minques-Misquera and Homero Mendez, 1993).

Traditional methods for flavonoid and carotenoid identification have involved gas liquid chromatography (GC) (Ball, 1988), paper chromatography and TLC. Chromatographic separation procedures are appropriate for pigment separation, purification, identification and quantification because each individual carotenoid and flavonoid has a different solubility and colour (Agrawal and Markam, 1989; Seikel, 1962). The increased availability of spectroscopic techniques, IR (Agrawal and Markham, 1989), UV-visible (Feng et al., 1988; Feng and McDonald, 1989; Ball, 1988; Agrawal and Markham, 1989; Marby, et al., 1970; Hostettmann and Marston, 1986), Mass Spectroscopy (Agrawal and Markham, 1989, Feng et al., 1988) and NMR spectroscopy (Agrawal and Markham, 1989; Feng et al., 1988; Mabry et al., 1970; Hostettman and Marston, 1986) provide structural information for total assessment of pigments.

Many attempts have been made to simplify the separation and purification process and less commonly used methods for the separation of flavonoids have included Chromotron, centrifugal TLC, flash chromatography, semi-preparative HPLC and support free liquid-liquid partition chromatography (Hostettmann and Marston, 1986). These methods were not routinely used due to the expense of equipment and the requirement for experienced chromatographers to operate the equipment. Successful elution of concentric bands of phenolic compounds was achieved by Hostettmann et al., (1980) from crude plant extracts, spun off with the solvent by centrifugal TLC. Flash chromatography used in conjunction with centrifugal TLC, low pressure Liquid Chromatography (LC) and open column chromatography, permits samples between 100 mg to 10 g to be separated within 1/2 hour. Semi-preparative HPLC was reported by Hostettmann and Marston (1986) to give high resolution. Hostettman et al., (1984) reported that problems of adsorption desorption that occur with solid stationary phases can be overcome by liquid-liquid partition techniques. This technique includes droplet counter current chromatography (DCCC) and rotation locular counter current chromatography. Both techniques have been found to be satisfactory for flavonoid separation.
More recently HPLC has become the dominant tool for the analysis of non-volatile organic compounds from a variety of food sources. Retention time data and UV-visible spectra are most commonly obtained using HPLC and if standards are available, characterisation of pigments can be readily achieved. With on line MS and Photodiode Array Detector (PAD) systems, separated compound identification may be achieved.

In addition to HPLC analysis, the following techniques are still applied for the comprehensive examination of both pigment types. TLC for the purification of pigments, pigment profiles comparison and identification by comparison with authentic standards. UV-visible spectrophotometry for the analysis of pure compounds. Purified compounds may also be conclusively identified using EMS, FAB-MS, and proton and C13 NMR.

2.6.1 General methods for the extraction and analysis of carotenoid pigments from plants

2.6.1.1 Carotenoid extraction and analysis

There are a number of excellent texts on the subject of carotenoids whereby general information on the nature of carotenoids and specific information on their extraction and analysis is available (Ball, 1988; Goodwin and Britton, 1988). There is also much literature on the isolation, purification and identification of carotenoids (Simpson et al., 1985; Hoseney and Faubion, 1981; Goodwin and Britton, 1988; Weedon, 1969; Schwieter et al., 1969). Goodwin (1976) has examined and studied the carotenoid pigments in plants over decades. The distribution, biosynthesis and analysis of carotenoids in plants are discussed in depth in a later publication on plant pigments (Goodwin, 1988). The use of spectroscopy for analysis of carotenoids was examined by Weedon (1969). His account provides information on the chemical and physical attributes of carotenoids; why a UV-visible spectra is produced; the interpretation of absorption bands in Infra-red spectroscopy and tabulates characteristic NMR bands of many different carotenoids.
2.6.1.2 Carotenoid extraction and purification

General methods for carotenoid extraction involve either mixing or refluxing the sample with a lipophilic organic solvent such as petroleum ether. Carotenoids and retinoids may be extracted with hexane: diethyl ether (70:30, v/v), with a recovery of 94% (Kruger and Reed, 1988). Often the type of analysis to be performed will determine the type of extraction and purification process, the sample must undergo (Khachik et al., 1986). If extracts are to be analysed by HPLC, saponification is often employed to simplify the HPLC profiles. Saponification helps in the regeneration of parent hydroxycarotenoids (Haslam, 1975; Lea 1988, Heinoin et al., 1989). Fallon et al. (1987) reported that the best method for extraction from plant material is by saponification, to remove fat and lipophilic compounds, followed by extraction in solvents compatible with HPLC, and separation and detection with HPLC coupled with UV-visible spectroscopy. Various methods for the extraction, saponification and purification of carotenoid are available (Lea 1988, Heinoin et al., 1989: Fallon et al., 1987). Pironen et al., (1984), saponified flour using potassium hydroxide (KOH) and water, in an ethanoic solution. For total carotenoid content following AACC methods (1984) carotenoids are simply filtered prior to spectroscopic analysis. For definitive structural elucidation of unknown compounds samples must be individually purified prior to analysis to ensure other compounds do not interfere with the interpretation of unknowns.

Carotenoids are reported to be prone to degradation (Ball, 1988; Lea, 1988). For this reason, great care must be taken to ensure that pigment loss or degradation does not occur during extraction, purification or analysis. It is recommended that pure solvents be used and that extracts are purged with nitrogen to reduce oxidation. Acetone should be avoided to prevent aldol condensation (Stewart and Wheaton, 1973). Glassware should be covered. Extraction and analysis should be conducted away from direct light where possible, to reduce the incidence of photo-oxidation (Ball, 1988). To reduce the occurrence of degradation, oil may be added to the extract (Thompson, 1986) or an antioxidant (Lepage and Sims, 1968; Geismann, 1962). If open column
purification is employed, silica gel may be added to the separating resin to improve recoveries.

Literature on vitamin A and β-carotene analysis was sought to establish protocol for carotenoid extraction from wheat. Vitamin A and its derivatives have been detected in animal feed (Kruger and Reed, 1988; Analytical Methods Committee, 1985) using saponification and HPLC with UV-visible detection. A successful method for the determination of all-trans and 13-cis vitamin A compounds in foods, was developed by Egberg et al., (1977). Briefly the procedure involved refluxing the breakfast cereal, after the addition of an antioxidant, with ethanol, saponification and filtration before injection on a 25 cm x 3.2 mm i.d column, slurry packed with Vydac 10 µm ODS packing and RP-HPLC with UV detection at 328 nm. This process enables 95 % recovery of standard.

2.6.2 Flavonoid extraction and analysis

Marby et al., (1970) in a work entitled “The Systematic Identification of Flavonoids”, discusses the many methods for flavonoid isolation and purification. Great detail on the UV-visible analysis of flavonoids using shift reagents with diagrammatic representation of 175 different flavonoids is given. Methods for the structural interpretations of flavonoids using 1H NMR are explored making this review incredibly informative and essential reading for those interested in flavonoid analysis. Another detailed review by Markham (1982) compliments the work of Mabry et al., (1970), and gives a detailed account of alternative extraction, purification and identification techniques and focuses principally on methods for the examination of the chemical properties of flavonoids. Other methods for identification include the use of paper chromatography, acid hydrolysis and derivatisation techniques for flavonoids identification.

A great deal of research on flavonoids has been conducted and collected in the edited works by Harborne (1967; 1993). In a book entitled “The Flavonoids”
Harbome collated and edited the work of several researchers. The specific chemical and physical characteristics of the different classes of flavonoids, their distribution, and identification, structural elucidation and in some instances the synthesis of specific compounds is discussed. His most recent edition, “The flavonoids: Advances since 1986.” presents the work of many other researchers on recent developments and discoveries made in flavonoid research (Harbome, 1993).

2.6.2.1 Flavonoid extraction and purification

General procedures for flavonoid extraction, purification, identification and quantification are similar to those for carotenoid extraction though naturally involves the use of solvents and adsorbents for polar compounds. More polar solvents such as methanol and solutions of aqueous alcohol are employed (Court, 1977). Various methods for the extraction, purification and analysis of flavonoids are available (Feng et al., 1988; Markham and Mabry, 1975; Harborne, 1967; Wagner et al., 1983).

Purification may be achieved using a number of techniques. Fractionation and separation is achieved by chromatographic procedures. Column chromatography is often used for large-scale isolation and purification of flavonoid compounds (Seikel, 1962). The eluate from column chromatographic systems can be monitored using UV-visible spectroscopy to check the progress of elution (Ball, 1988) and for fractionation of individual compounds. Traditional methods have involved paper and TLC, as well as preliminary purification for separation of compound with open column chromatography through various resins.

Thin-Layer Chromatography is a common method for flavonoid purification. Many different solvent combinations and development techniques are available (Markam, 1982; Wagner et al., 1983). Good TLC enables the separation and collection of individual compounds. It also provides a useful technique for checking the progress and efficiency of extractions (Feng et al.,
It also permits evaluation of the most appropriate solvents for extraction (Casteel and Wender, 1953).

2.7. HPLC Analysis and identification

Reverse Phase-High performance liquid chromatography (RP-HPLC) is an advanced chromatographic procedure employing high pressure solvent elution across an adsorbent stationary phase. The reverse phase refers to the column packing material, reverse phase for non-polar material, normal phase for polar material. As with all chromatographic procedures compounds are separated on the basis of their polarity characteristics. Many variables influence the chromatographic conditions that include the column stationary phase, column temperature, column packing and particle size, the elution conditions and solvents used, the pH of the solvents and the detection wavelength selected.

For a sample to be separated, it must elute from the column at a different rate to other samples (Macrea, 1988). A detailed description of the theory of the chromatographic process has been reported by Macrea et al., (1993). The theory of HPLC, the different modes of detection available, methods and principle behind different elution systems for quantitative and qualitative analysis are discussed.

RP-HPLC offers the means to separate and quantify non-volatile carotenoid and flavonoid compounds. It is especially good for carotenoid analysis as the incident of compound degradation is lessened, being a closed system; with reduced exposure to air, light and heat (Khachik and Beecher, 1986).

The basic HPLC system comprises a pump, column and detector. Advancements in computer technology now enable automatic sample injection and data collection. As the sample is eluted from the column, the compounds are detected. The type of detector used depends on the type of compounds being examined. There are several different detection systems available, UV-visible, PAD, fluorescence, refractive index (RI). Other less
common forms of detection involve flame ionisation detectors (FID), conductivity detectors and the like (Macrea, 1988). Carotenoids and flavonoids absorb in the UV-visible region of the spectra and are therefore usually monitored with either a variable wavelength UV-visible detector or PAD. In fact absorbance detection is the most commonly used form of detection (Ball, 1988).

When PAD is used information on both the compound spectra and compound retention time is obtained. Compounds can be identified by their wavelength maxima; extinction coefficient and the compound purity can also be determined. UV-visible detectors enable detection of compounds at a specific wavelength providing information on compound absorbance and peak retention time. Samples can be identified by direct comparison with authentic standards. Using this method of detection samples can be quantified by either the Internal standard (IS) or External standard method (ES). The IS method involves addition of a standard to a sample prior to its extraction. Concentration is calculated by plotting peak height or area against an internal standard at different concentrations. The ES method involves preparation of standard solutions of the analyte at various concentrations to establish a calibration curve. The amount of sample may be determined using the coefficient of the curve and the peak area or height of the analyte.

2.7.1. HPLC of carotenoids

Much work on the RP-HPLC analysis of carotenoids in flowers, fruits and vegetables has been reported. A great deal of research in this area has been performed by Khachik and his co-workers, who have achieved considerable advances in the HPLC analysis of carotenoids in fruits and vegetable (Khachik and Beecher, 1987; Khachik et al., 1986). Khachik, et al., (1986) developed a method for the identification and quantification of the major carotenoids and chlorophyll compounds present in green vegetables using a Reverse phase C 18 column. Xanthophylls, chlorophylls and their derivatives together with hydrocarbon carotenoids were detected and quantified.
Khachik and Beecher, (1987) continued development of a quantification system for the analysis of hydrocarbon carotenoids prevalent in yellow and orange coloured vegetables. They stressed that for accurate concentration determination, it was important to select an appropriate internal standard. An ideal IS must resemble as closely as possible, the analyte chromatographically. It must be stable and easy to prepare. Use of an appropriate IS is essential for accurate determination of concentrations and to observe compound loss, if any, during extraction and purification of carotenoids. Khackik and colleagues developed the IS nanopreno-β-carotene, which was considered superior to decapreno-β-carotene which degraded faster, required more preparation during synthesis and showed less chromatographic likeness to β- and α-carotene.

Methods were developed to enable the separation and identification of the fatty acid esters from squash by HPLC (Khackik and Beecher, 1987). Separation of four classes of carotenoids including oxygenated, fatty acid esters, hydrocarbon and carotenol bis (mono fatty acid ester) was achieved with a Reverse phase C18 column using an isocratic and gradient elution system. Structural information on the different groups was obtained by UV-visible and mass spectrophotometry. With advancement in detection methods, analysis and identification of carotenoids became easier. Biacs and Daood (1994) were able to isolate and identify the four classes of carotenoids by RP-HPLC with PAD. For conclusive identification, the analytes were compared with standards extracted by TLC.

Separation and quantification of carotenoid pigments in red capsicum, paprika and oleoresin using LC was achieved by Minquez-Misquera and Homero-Mendez, 1993. After separation and purification with TLC, the extracts were fully characterised using TLC, absorption spectra, IR spectroscopy and chemical tests. These compounds were then analysed by HPLC to obtain retention time information before the identification and quantification by HPLC of two varieties of pepper was achieved.
A comprehensive review by Lea, (1988) reported a selection of HPLC procedures for the analysis of carotenoids in fruit, vegetable, flowers and salmon. Over 34 different methods were described. A range of columns including C18, Zorbax and C 8 were used. Normal phase HPLC will elute carotenes before xanthophylls and that in RP-HPLC the situation is reversed. Ball (1988) discusses the aspects of HPLC analysis of fat-soluble vitamins. Detailed information on sample preparation, quantification modes and chromatography of vitamins is given. Kingston (1979) published a review on current literature on HPLC of natural products such as carboxylic acids, alkaloids, lipids, flavones and phenols.

The column and solvent system employed depended on the class of carotenoid being examined. Gradient elution is reported to be the most effective method for complete separation of carotenes and xanthophylls from natural extracts (Khachik et al.,1986; Annesley et al., 1984). Solvent systems for separation of the different classes of compounds in an extract that relied upon a gradient elution system, starting with a more polar solvent mix, to a less polar system facilitating the separation of oxygenated carotenoids to the hydrocarbon carotenoids. The disadvantages of gradient elution include increased baseline fluctuation that can induce greater variability of compound retention times and reduce sample reproducibility. Isocratic systems are often preferred and as they may be more appropriate for less complex mixtures. Isocratic solvent systems using solvents such as acetonitrile, chloroform and methanol appeared to be more popular for the separation of the hydrocarbon carotenoids.

Only one report on the HPLC analysis of carotenoids from wheat has been published to date (Heinonen et al., 1989). Heinonen and his colleges used HPLC to quantify the carotenoid and retinoid compounds of 22 cereal and bakery products. The samples were extracted from flour with hexane and diethyl ether as described by Ollilainen et al., (1988). This method was highly successful and resulted in 94 % recovery rates. Saponification was achieved with 100 % potassium hydroxide in ethanolic solution (Pironen et al., 1984). Saponification was used for the removal of fats and lipids and was achieved.
with the use of 100 % potassium hydroxide in ethanoic solution (Pironen et al., 1984). It may also be employed for the regeneration of parent hydroxy carotenoids and the evaluation of carotene esters (Fallon et al., 1987) and is reported to simplify HPLC profiles (Haslam, 1975). Separation was achieved using a non-aqueous reversed-phase (NARP) method described by Neils and DeLeenheer, (1989). A Zorbax ODS5-6 um column and ODS2 guard column with isocratic elution of acetonitrile, dichloromethane and methanol (70:20:10) at a flow rate of 2 mL.min⁻¹ was employed. Detection was achieved at 450 nm. Lutein and zeaxanthin were poorly resolved, as was β-carotene which co-eluted with α-carotene and cryptoxanthin. Quantification was based on the external standard method using authentic β-carotene and lutein standards. β-carotene was found in wheat based cereal products; the range in concentration was between <1-62 µg.100g⁻¹ of fresh product. The highest concentration of 62 µg.100g⁻¹ product was obtained from wheat germ, the lowest of <1 µg.100g⁻¹ from wheat flour. Lutein and another carotenoid zeaxanthin were identified and quantified as lutein, values ranged from 190-790 µg.100g⁻¹ of food in wheat based products tested. In durum wheats, the total carotenoid content was found to be 170 µg.100g⁻¹, in wheat germ 790 µg.100g⁻¹ and wheat flour 190 µg.100g⁻¹.

2.7.2. HPLC of flavonoids

HPLC has been applied to the separation and identification of polymethoxylated flavones in orange juice (Heimhuber et al., 1988); from citrus fruits (Kawai et al., 1999); of phenolic and flavonoid compounds in tobacco (Court, 1977) flavonoids in tomatoes (Heimhuber et al., 1988); from berries (Hakkinen et al., 1998) and honey (Ferreres et al., 1991). Hasler et al., (1989) used RP-HPLC with PAD for the isolation of aglycone pigments from medicinal plants.

Strategies for separating flavonoid compounds involve, amongst other things, selection of an appropriate solvent system, adjusting the pH and selection of the flow rate. A common elution system of methanol water and acetic acid has
been used for the separation of flavonoids, chalcones and anthocyanidins (Kingston, 1979; Tittel and Wagner, 1982). The weaker the mobile phase, the longer the analyte is in contact with the stationary phase. The separation of flavonoids using HPLC is based on several factors; the principle consideration is the overall stereochemistry of the flavonoids and their polarity influences (Strack and Wray, 1993). Three major aspects of flavonoid HPLC analysis must be considered: (a) the number and position of OH and CH$_3$ groups; (b) The presence of sugar groups on the aglycone and the position and type of glycosidic linkage; (c) The acylation of sugars with phenolic acids (Strack and Wray, 1993).

A large percentage of water-soluble compounds such as the flavonoids have acidic or basic functional groups (Cross, 1996). This is significant because the state in which the compound exists, in an electrically neutral or ionised form, can influence the separation of that compound. According to Cross, the charge of a compound increases its solubility in a RP-HPLC system and subsequently decreases its retention on the stationary phase. Adjustment of the pH to alter the charge can increase the retention time of a compound that may therefore improve separation. Tittel and Wagner (1982) reported that the use of acidified solutions enhanced separation by reducing band broadening and peak tailing.

Vande Casteele et al., (1982) examined the separation chemistry of 141 standard flavonoid compounds using RP-HPLC. Employing a Lichrosorb RP-C-18 column and a gradient elution system of methanol/formic acid-water (5:95 v/v) they examined the elution sequence of the different classes of flavonoids. They found that flavonoids that differ in the number of OH groups, located on positions other than C3, could be readily separated. Flavonoids differing only in the methoxyl group cannot be separated. Compounds containing sugar groups were found to have the same retention times as their corresponding glucoside. Despite the increase in the molecular weight of glycosylated flavonoids, a general increase in retention time was not observed, smaller compounds with OCH$_3$ functional groups eluting after more polar sugar containing flavonoids.
The use of PAD allows the simultaneous analysis of different type of phenolic and poly phenolic compounds (Andrade et al., 1998). Hostettmann et al., (1984) identified flavonoid and phenolic compounds in plant extracts using on line HPLC with PAD of phenolic compounds in plant extracts using post-column derivatisation. They reported that flavones and xanthones were readily identified, however, UV analysis was not sufficient for the characterisation of closely related compounds. Sample derivatisation after separation provides more information on compound structures. Shift reagent use provided information on the type of compound, oxidation pattern and location of free hydroxyl groups. This method enabled separation and identification of both flavonoid and carotenoid xanthones. Identification achieved by comparing the retention time and UV spectra of authentic standards. Gradients of methanol water were employed. Sulphuric acid was added to prevent peak tailing of phenolic compounds at pH 3.5. Only compounds with the same retention time and spectra of authentic standards were identified.

Barberan et al., 1985 also examined the effect of hydroxyl and methoxy group location on the flavonoid nucleus for chromatographic retention times. They found that the HPLC elution sequence of 5-hydroxyflavones is the result of the hydrophobic interactions and the formation of hydrogen bonds with the mobile phase. Compounds with a greater number of hydroxyl groups had a shorter retention time. The internal interaction within the molecule will also influence retention time ($t_R$). For example if a hydroxyl group is present on the flavonoid nucleus at the C-5 position, strong hydrogen bonding between this group and the carbonyl group occurs. Fewer interactions with the stationary phase occur so the sample elutes with a shorter $t_R$. Interestingly, it was also found that the size of the molecule could influence bonding interactions with the column stationary phase and the mobile phase. In the instance of methoxylated flavonoids, smaller molecules are able to interact readily with C-18 functional groups on the stationary phase. This is due to hydrophobic interactions enabling penetration into the stationary phase. The presence of hydroxylated groups on the molecule reduces the hydrophobic effects.

It is usual for the presence of phenolic and flavonoid compounds to occur
together in the same matrix in plants (Ho et al., 1992). In the review of the literature on flavonoid HPLC, a large proportion of the articles analysed phenolic compounds in conjunction with flavonoid compounds (Hostettmann and Marston, 1986). In an attempt to gain greater understanding of the chromatographic behavior of both phenolic compounds and flavonoids, Wulf and Nagel (1976), examined the two different classes of phenolics together using HPLC. Great detail is used to describe the influence of compound structure on retention time.

In the instance where standards are available HPLC can be a very rapid technique for identification of compounds, especially when coupled with PAD to enable structural characterisation. When standards are not available, it is necessary to isolate the compounds and examine them using several different techniques. Kouçai-Abyazani and his colleagues (1992) developed a rapid method for the identification of flavan-3-ols and phloroglucinol adducts from sainfoin proanthocyanidins. Compounds were readily identified by comparison with authentic standard. Phloroglucinol compounds were found to elute before the flavan-3-ol compounds, due to an increased affinity with the mobile phase caused by H-bonding interactions with substitution on the phloroglucinol group, at the C-4 position.

The combination of chromatographic systems, with absorbency detectors, together with mass spectral systems, provides a powerful analytical tool that enables definitive identification of compounds. Extracts can be isolated as well as identified using coupled systems. Saffron components in a crude plant extract were isolated using HPLC with PAD and MS. Hostettmann et al., (1984) was able to identify the crocetin glycosides, safranal and picrocrocin, in a crude extract. In addition to this method on-line, addition of shift reagents coupled with UV-Visible detection can assist in the identification of flavonoids. Using post column derivatisation they were able to further characterise compounds unidentified due to their similarity in structure. The use of the shift reagents provided further information on the type of compound, the oxidation pattern and the location of hydroxyl and methoxy groups.
The use of HPLC/MS was successful in the separation and identification of procyanidins in cocoa (Hammerston et al., 1999). Using a silica stationary phase and a solvent system gradient, increasing in polarity during the run phase they confirmed the presence of procyanidins in raw cocoa, separating nonomers from dimers and trimers.

Wheat bran and flour have been reported to contain, rutin, quercetin, kaempferol and apigenin flavonoids (Blouin et al., 1981; Feng et al., 1988). Feng et al isolated flavonoids from Hard Red spring wheat using an alkaline water extraction process, purification using open column chromatography, paper and three dimensional TLC to isolate individual compounds. These compounds were derivatised then identified using GC-MS analysis.

As there was not any literature available on the analysis of wheat flour or bran flavonoid compounds using HPLC analysis, literature on HPLC analysis of flavonoids extracted from other plant materials was used to establish appropriate solvent systems and chromatographic conditions.

2.8 UV-Visible spectrophotometry of flavonoids and carotenoids.

UV-visible spectroscopy is one of the principal tools used for identification of carotenoids and flavonoids, each pigment type having a unique absorption spectra. UV-visible spectroscopy is based on the principal of light transmittance from an absorbing sample that is detected by placement of the sample between a spectroscope and a light source (Rao, 1975). Following the principals of Planck's constant for a compound to absorb, its energy must increase. Rao (1975) covers the theory and factors that contribute to UV-visible spectroscopy. Different spectra for different compounds are obtained due to unsaturated linkages in the molecule, known as chromophores, which are double or multiple molecular bonds. Greater detail on the electrochemical aspects of spectroscopy is available in chapter two of Rao (1975). The factors that govern the wavelength maxima of different compounds are the differences in the electronegativities of the elements forming the multiple bond and the ease at which this bond is formed. A spectrophotometer consists of basically three
parts a light source, a monochromameter and a detecting device (Robertson, 1967).

When standard compounds are not available tentative identification of pigments can be achieved using UV-visible spectroscopy by comparing sample wavelength maxima with spectra reported in the literature (Mabry et al., 1970, Markham and Mabry 1975, DeLeenher and Neils, 1992). Absorption spectroscopy may also be used for the quantification of both pigments by preparation of a standard curve enabling calculation of an unknown compound’s concentration. Though not as frequently used by itself, the coupling of UV-visible detectors to HPLC systems has revolutionised the way in which non-volatile compounds can be quantified and analysed.

2.8.1 Identification of carotenoids using UV-visible spectroscopy

There is ample literature available on the UV-visible spectra of carotenoids. Carotenoids have been successfully identified after extraction by spectroscopic analysis (Heinonen et al., 1989; Food and Nutrition Board, 1980). A detailed description of the spectroscopic properties of carotenoids is given by Lea (1988).

In addition to identification using UV-visible spectroscopy, carotenoids may also be quantified by measurement of the optical densities of known concentration of standard solutions to determine the concentrations of unknown samples (Seikel, 1962). Simpson et al., (1985) described a means for establishing the concentration of a pure carotenoid using the extinction coefficient of a carotenoid when its molecular weight and, therefore, its identity was known.

2.8.2. Identification of flavonoids using UV-visible spectroscopy

A traditional method for the structural elucidation of flavonoids involves the use of shift reagents and UV-visible spectroscopy. A comprehensive review of the influence reagents have on the flavonoid structure and the many different
flavonoid compounds in given by Mabry and his colleagues (1970). Methods of flavonoid glycoside identification rely upon hydrolysis and their spectroscopic detection as an aluminium chloride chelate complex.

Feng and his colleagues (1988) successfully identified two flavonoid compounds in the bran of Len variety wheat, apigenin-6-arabinoside-8-C-hexoside and apigenin-6-C-hexoside-8-C-pentoside using shift reagents to facilitate the identification of hydroxyl groups and their location on the flavonoid nucleus. Ferreres et al., (1991) also employed this technique for the identification of honey flavonoids. Blouin et al., (1981) successfully isolated and identified isoquercetin rutin, quercetin 3-0-robinoside, quercetin 3-0-glucoglycoside and kaempferol 3-0-glucoglucoside from cottonseed flour by examination of their absorption spectra.

This method is considered limited in terms of identification of individual flavonoid compounds in a mixture of flavonoids (Haslar et al., 1989). Haslar and his colleagues (1989) developed a simpler method of glycoside identification by RP-HPLC with PAD of aglycones that included kaempferol, morin, myricetin, rutin and quercetin.

2.9 Mass spectral analysis of pigments

Mass spectrometry enables the determination of a compound’s molecular weight facilitating the identification of that compound. A sample is subjected to ionisation and a quantitative recording of the ion fragments is obtained (Silverstein, et al., 1974). There are several different mass spectral techniques available to facilitate the analysis of both volatile and non-volatile compounds. The type of compound to be analysed and the level of information required determine the type of process selected. The distinguishing feature between each process is the way in which the compounds are atomized to produce individual molecular ions.

MS involves the ionisation of a compound using an electric or magnetic field to produce molecular ions into a gaseous phase, under vacuum. The type of
ionisation is determined by the sample to be analysed and the information required. Traditional methods involve gas-phase ionisation, which includes electron ionisation, chemical ionisation, desorption chemical ionisation, and negative ion chemical ionisation. Other methods of ionisation involve field desorption ionisation that involves using an emitter to tunnel electrons. This method includes field desorption and field ionisation techniques. Particle bombardment may also be used to ionise compounds. This process includes techniques such as fast atom bombardment (FAB) and secondary ion mass spectrometry. Other methods involve using atmospheric pressure to ionize an analyte such as electrospray ionisation, electrospray mass spectrometry (EMS) and atmospheric pressure chemical ionisation (CI). Laser desorption or matrix-assisted laser desorption ionisation (MALDI) involves dissolving the analyte in a solution that absorbs at a laser wavelength. This matrix is subjected to a laser pulse, and after vaporisation it is able to be detected.

Traditionally, the most common form of mass spectroscopy has involved derivatisation of the non-volatile compound such as flavonoids with perdeuteriomethylation (PDM) and trimethylsilylation (TMS) for analysis by GC-MS (Donovan et al., 1999). The mass spectra of rutin derivatized with TMS and its fragmentation pattern is displayed in Figure 2.8. The main disadvantage of the derivatisation methods is that it increases the molecular weight of the compound significantly.

![Figure 2.8](image)

**Figure 2.8.** Scheme of the fragmentation pattern and mass spectra of TMS derivatized rutin. $S = C + II, OS + II + III, R + Si(Me)_3$ [5].
Underivatized flavonoid glycosides may be analysed by soft ionisation techniques using field desorption (FD), fast atom bombardment (FAB). Fast Atom bombardment involves dissolving the sample in a polar matrix (i.e. thioglycerol) and application of the sample onto the end of a probe that is inserted into the mass spectrometer. The sample is bombarded with a fast stream of either xenon atoms or caesium ions. Both positive and negative ions are produced and are analysed by the mass spectrometer (Mellon, 1991).

With the development instrumentation selectivity and sensitivity, it is possible to analyses compounds immediately after liquid chromatographic separation, by electrospary mass spectroscopy (Careri et al., 1999). Operating in the positive ion mode good results were obtained. Barley polyphenols were examined using HPLC-MS and with the use of principle component analysis, the dimers and trimers of proanthocyanidins together with phenolic compounds were used to distinguish different beer types (Whittle et al., 1999). Phenylflavonoids were analysed using a similar approach with detection achieved by means of a triple-quadrupole mass spectrometer in the positive ion mode using HPLC-tandem mass spectrometry (Stevens et al., 1999).

These methods have the advantage of only requiring a small amount of compound that requires minimal sample preparation. Good information on the molecular ion and sugar fragmentation patterns can be obtained.

Electrospray mass spectroscopy offers another non derivatisation method of analysis. The technique involves transference of the ions in solution phase. Droplets of the compound are ionised resulting in protonation or deprotonisation of the molecule depending on the nature of the compound. For example amino acids will be protonated and alcohols and acids deprotonated.
2.9.1 Mass spectral analysis of carotenoids

Velter and Meister (1985) examined carotenoids using Fast Atom Bombardment (FAB) in the positive ion mode. In the positive ion mode protonated and cationated molecules were produced. They cautioned that for the identification of unknowns and in particular β-carotene under these conditions, a radical cation is formed and hence more information regarding the ionisation process is required to prevent structural misinterpretation. Using 1,2,4-trichlorobenzene and 3-nitrobenzylalcohol with 0.1 % of β-carotene the spectrum obtained gave incredibly high relative intensity of the molecular ion peak. Stratton et al., (1993) used dichloromethane-acetonitrile and 10 mM of ammonium acetate (25:75:2.5, v/v/v) at a flow rate of 50 µl.min⁻¹, for the analysis of Singlet oxygen oxidation products of β-carotene using a ion spray/Sciex API 111 MS.

2.9.2 Mass spectral analysis of flavonoids

Mass spectral analysis of flavonoids enables the detection and determination of the aglycone, substituents on the flavonoid nucleus, the location of sugar groups on the flavonoid nucleus (Markham, 1982) and interglycosidic linkages (Schmid, 1972). A comprehensive description of flavonoid mass spectra using electron impact MS is given (Markham, 1982). Fragment ions represent parts of the parent ion and provide information on the parent ion structure. Most aglycones are sufficiently volatile at 100-230°C. The more polar flavonoids such as the glycosides require higher temperatures to volatilise. Fragmentation patterns, molecular ion information for each class of flavonoid and the loss of specific groups of compounds are explained. All identification has involved the analysis of purified compounds which are made sufficiently volatile to enable GC-MS by derivatisation.

In general, the molecular ion of the flavonoid is always an intense ion. Ions of moderate intensity are shown by flavones with less than four OH groups due to retro-Diels-Alder (RDA) reactions. The typical fragmentation of a flavonoid involves removal M-1, M-CO, A-CO, aryl cation C from the C ring and a carbonyl
group from the B ring, (Figure 2.10). Flavonoids are highly aromatic compounds and large peaks are observed due to doubly charged ions. With simple flavones the fragmentation proceeds by defined pathways and spectra may be interpreted to identify the number of substituents on the A, B and C rings. With more complex flavones such as those containing four or more OH groups, less information on the substitution pattern is obtained and the spectra are dominated by the molecular ion (M-15), the M-28 ion and the M-43 ion.

![Proposed fragmentation of alkaline extracts via the Retro-Diels-Alder scheme.](image)

Figure 2.9. Proposed fragmentation of alkaline extracts via the Retro-Diels-Alder scheme.

The literature available on mass spectral analysis of flavonoids describes methods for MS analysis of derivatised flavonoids (Mabry and Ulubelen, 1980; Schmid et al., 1973). Mabry and Ulubelen (1980) produced an excellent review on flavonoid and phenolic mass spectra. Discussed are methods for PM and PDM derivatisation and analysis of flavonoids using electron impact mass spectrometry (EIMS). Great detail on the fragmentation pathways for all classes of flavonoid is given. Considerable advancements in the mass spectrometry of flavonoid disaccharides were made using CD$_3$I and NaH in dimethylformamide (DMF) for PDM, a method of hydrolysis that involves fewer steps with much less handling. An intense aglycone and molecular ion peak, in addition to information on the mass sequence of sugars was readily achieved. Compound resolution was improved using an OV17 GC column rather than the conventional SE30, OV1 or OV101columns. After PDM, the samples mixtures were subjected to acid hydrolysis and ethylation to provide information on the sugar sequences as well as interglycosidic linkages.
In the 1970's advances in instrumentation and derivatising agents facilitated the development of new mass spectral techniques (Kingston (1971). For non-volatile compounds derivatisation is required to enable volatilisation of the analyte, prior to entry into the mass spectrometer. Permethylated or perdeuteromethylated derivatives are preferred, as low molecular weights compared to other derivatising agents are produced (Mabry and Ulubelen, 1980; Domon and Hostettmann, 1985). New derivatising agents include O-bis-trimethylsilylacetamide and trimethylchlorosilane.

Schels and his colleagues (1977) reported for the first time the results of MS silylated flavonol O-glycosides. Trimethylsilylation was achieved by dissolving the flavonoid in KOH dried pyridine and N, O-bis-trimethylsilylacetamide then leaving the mixture to react at room temperature for 6 hours. Over 19 different flavonols were examined using electron impact MS. The mass spectra showed high intensity molecular ion peaks and standard fragmentation patterns. An additional sugar fragmentation was observed in the lower mass range. This method of derivatisation was considered to be advantageous over the PDM method. No further purification of the compound after derivatisation was required which is often the case of PDM or PM derivatized compounds where incomplete derivatisation and the production of additional products require further purification (Boullant et al., 1975). The method was much easier to perform and is able to be used on all classes of flavonoid. This silylation technique was latter applied to the analysis of flavone and flavanone glycosides (Schels et al., 1977). Furuya (1965) published a note on the successful derivatisation of 18 flavonoids with trimethylsilyl ethers. Using 1-2 mg of purified flavonoids the samples were dissolved in pyridine, hexamethyldisilazane and trimethylchlorosilane, left to react then injected into a GC.

The development of fast atom bombardment and electrospray mass spectroscopy enabled underivatized, non-volatile sample analysis (Domon and Hostettmann, 1985).
FAB-MS is currently the most popular method for the analysis of C-glycosylflavonoids and anthocyanins (Jay, 1993). This method of analysis is preferable to gas phase ionisation methods such as EMS, which has the disadvantage of increasing compound molecular weight and may produce artefacts hindering interpretation of structures. Domon and Hostettmann (1985) compared the results of 6 underivatised flavonoids using three different soft ionisation techniques, desorption chemical ionisation (DCI), FAB and field desorption (FD). Information on the molecular ion, the sugar sequence and aglycone fragmentation pathways were obtained using both DCI and FAB. They found that DCI gave good ionisation intensity in both the negative and positive mode but was best in the negative mode. Fast atom bombardment mass spectroscopy was found to be more appropriate for the analysis of high molecular weight compounds (>900 MW), however, the need to use a sample matrix caused signal interference and complicated structural elucidation.

2.10 Thin layer chromatography for the identification and separation of pigments

Thin layer chromatography is one of the simplest ways to purify and compare extracts. It is interesting to note that carotenoids were one of the first compounds to be separated by column chromatography in 1906 by Tswett (Stahl, 1962). Even though other separation techniques are available, TLC still offers a fast and effective method, for the separation and tentative identification of compounds, by comparison with authentic standards as well as offering the ready collection of separated compounds. Unfortunately, the separation ability is not, however, as effective as HPLC and some compounds with similar structural arrangements will not separate (Simpson et al., 1985) regardless of the solvents and plate resins used.

Compounds are separated following the basic principals of chromatography with application of an extract onto a stationary phase and elution by either ascending or descending chromatography, most commonly ascending, with a developing solvent whilst contained in a sealed tank. There are many different stationary phases available such as silica, polyamide and cellulose gel. The phase used is
dependent on the sample polarity characteristics, which also influence the type of developing solvents used. Solvent developing systems range from polar to non-polar combinations such as methanol-water to ethyl acetate-chloroform. Samples may be readily detected under visible or UV light. To enhance detection, agents may be applied to the plates.

2.10.1 TLC of carotenoids

Thin layer chromatography was traditionally used for the qualitative analysis and separation of carotenoids (Simpson et al., 1985). Points to consider when using TLC as an analysis tool are that carotenoids are readily oxidised by heat and light, therefore making it essential to conduct analysis under nitrogen away from direct light. Common solvent systems employed for separation on Silica gel plates include: acetone and petroleum ether (6:1, v/v); Hexane-isopropyl alcohol-ethyl ether-acetone-acetic acid (85:12:1:4:1, v/v); hexane-ethyl acetate-ethanol-acetone (95:3:2:2, v/v).

2.10.2 TLC of flavonoids

TLC may still be used for the purification of flavonoids for later structural identification and for quantitative analysis (Mabry et al., 1970). In addition TLC is still used for the rapid identification of medicinal flavonoids (Wagner et al., 1983).

Feng and McDonald (1989) used TLC for the identification of flavonoids extracted from Hard Red winter wheat by comparison with previously identified wheat extracts. Using polyamide TLC with a solvent system of water/methanol/butanone (4:3:3, v/v) they confirmed the identity of the extracts. Ferreres and his colleagues (1991) used preparative scale TLC for the purification of phenolic compounds extracted from honey. Non-polar samples were separated on silica gel with toluene-acetic acid (4:1, v/v) and the more polar compounds with toluene/acetic acid (5:2, v/v). Blouin et al., (1981) used TLC to monitor the elution of flavonoids from an open column and Thick layer chromatography for the separation of flavonoid pigments.
2.11 Identification of flavonoids by chemical methods.

Chemical tests may be utilised to obtain structural information on carotenoid and flavonoid compounds. These tests rely on a sound knowledge of the pigment's chemical and physical properties and their characteristic behavioural responses under certain conditions. Chemical tests often enable rapid detection of pigment types. For example, flavonoids may be readily identified in a sample by increasing the alkalinity of a solution. If flavonoids are present they respond to the increase in pH by producing a strong yellow colour, which is removed with acidification of the solution.

To distinguish between flavonoid mono-C-glycosylflavones, di-C-glycosylflavones and O-glycosyl-C-glycosylflavones acid hydrolysis may be performed (Jay, 1993). Sugar groups that are attached by oxygen linkages will hydrolyse and sugars attached by carbon linkages will not. Basically hydrolysis reactions occur due to the presence of hydroxyl ions in water and are catalysed by the addition of an acid or a base. Flavonoids are weakly acidic polyphenolics (Feng et al., 1988) and classified as acid labile hemeacetal compounds. In the presence of HCL and methanol, the hemeacetal acting as an alcohol reacts with more of the solvent to form an acetal by either nucleophilic addition to a carbonyl group or by formation by a carbo cation respectively.
CHAPTER 3. METHODS AND MATERIALS

3.1 GENERAL PROCEDURES

3.1.1 Storage

Extracts were stored in a at -20° C freezer to reduce the occurrence of product change.

3.1.2 Glassware

Prior to any experimentation a glassware cleaning agent, chromosulphuric acid chromium trioxide (NAY Code 1676), was used to ensure that no other compounds could contaminate samples. After reaction with the cleaning agent (overnight) the glassware was carefully rinsed clean with tap water prior to finally rinsing with deionised water.

3.1.3 Solvent degassing

All solvents used for the RP-HPLC analysis of compounds were degassed to reduce fluctuations in baseline that may be attributed to air bubble variation within solvents. Solvents used for HPLC were all of HPLC grade. Solvents were degassed using nitrogen.

3.2 MATERIALS

3.2.1 Characteristics and source of chemicals

Analytical grade chemicals were obtained from Sigma chemicals, flavonoid and carotenoid standards were obtained from Sigma chemicals or Fluka chemicals. Greater detail on these compounds is provided in Table 3.1.
Table 3.1 Origin and properties of standards.

<table>
<thead>
<tr>
<th>Standard name</th>
<th>Origin</th>
<th>Product number</th>
<th>MW</th>
<th>mass</th>
<th>Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Flavonoids and phenolic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apigenin</td>
<td>Sigma</td>
<td>A-3145</td>
<td>C_{15}H_{10}O_{5}</td>
<td>270.2</td>
<td>95 %</td>
</tr>
<tr>
<td>Caffaic acid</td>
<td>Sigma</td>
<td>C-0650</td>
<td>C_{6}H_{8}O_{4}</td>
<td>180.2</td>
<td>95 %</td>
</tr>
<tr>
<td>Catechin</td>
<td>Sigma</td>
<td>C-1788</td>
<td>C_{15}H_{14}O_{6}</td>
<td>290.3</td>
<td>95 %</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>Sigma</td>
<td>C-3878</td>
<td>C_{16}H_{18}O_{9}</td>
<td>354.3</td>
<td>95 %</td>
</tr>
<tr>
<td>Flavone</td>
<td>Sigma</td>
<td>F2003</td>
<td>C_{15}H_{10}O_{6}</td>
<td>286.2</td>
<td>95 %</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>Sigma</td>
<td>F-3500</td>
<td>C_{10}H_{10}O_{4}</td>
<td>194.2</td>
<td>95 %</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>Sigma</td>
<td>K-0133</td>
<td>C_{15}H_{10}O_{6}</td>
<td>286.2</td>
<td>95 %</td>
</tr>
<tr>
<td>Morin</td>
<td>Sigma</td>
<td>M-4008</td>
<td>C_{15}H_{10}O_{7}</td>
<td>302.2</td>
<td>95 %</td>
</tr>
<tr>
<td>Myricetin</td>
<td>Sigma</td>
<td>M-6760</td>
<td>C_{15}H_{10}O_{8}</td>
<td>318.2</td>
<td>95 %</td>
</tr>
<tr>
<td>Quercetin</td>
<td>Sigma</td>
<td>Q-0125</td>
<td>C_{15}H_{10}O_{7}.H_{2}O</td>
<td>338.3</td>
<td>95 %</td>
</tr>
<tr>
<td>Rutin</td>
<td>Sigma</td>
<td>R-5143</td>
<td>C_{27}H_{40}O_{16}</td>
<td>610.5</td>
<td>95 %</td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>Sigma</td>
<td>V-2250</td>
<td>C_{8}H_{6}O_{4}</td>
<td>168.1</td>
<td>95 %</td>
</tr>
<tr>
<td>Xanthone</td>
<td>Sigma</td>
<td>X-6125</td>
<td>C_{13}H_{8}O_{2}</td>
<td>196.2</td>
<td>97 %</td>
</tr>
<tr>
<td><strong>Carotenoids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trans-β-carotene</td>
<td>Sigma</td>
<td>C-9750</td>
<td>C_{40}H_{56}</td>
<td>536.9</td>
<td>95 %</td>
</tr>
<tr>
<td>β-apo-8'-carotenal</td>
<td>Fluka</td>
<td></td>
<td>C_{30}H_{40}O</td>
<td>416.65</td>
<td>&gt;20 %</td>
</tr>
<tr>
<td>All-trans lutein</td>
<td>Sigma</td>
<td>X-6250</td>
<td></td>
<td>568</td>
<td>95 %</td>
</tr>
</tbody>
</table>

3.2.2. Solvents

For all RP-HPLC chromatography and for mass spectral and NMR analysis, HPLC grade solvents were used (Table 3.2).

Table 3.2 HPLC grade solvents used for HPLC, mass spectral and NMR analysis.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Origin</th>
<th>Code</th>
<th>Formula</th>
<th>Mw</th>
<th>Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>BDH</td>
<td>15250</td>
<td>H_{3}OH</td>
<td>32.04</td>
<td>99.8 %</td>
</tr>
<tr>
<td>Formic acid</td>
<td>Sigma</td>
<td>F-0507</td>
<td>CH_{2}O_{2}</td>
<td>46.03</td>
<td>99 %</td>
</tr>
<tr>
<td>Butanol</td>
<td>Sigma</td>
<td>BT-105</td>
<td>C_{4}H_{10}O</td>
<td>74.12</td>
<td>99 %</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>BDH</td>
<td>152856K</td>
<td>CH_{3}Cn</td>
<td>41.03</td>
<td>99.7 %</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>BDH</td>
<td>152486E</td>
<td>CH_{3}COOC_{2}H_{5}</td>
<td>88.11</td>
<td>99.8</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>BDH</td>
<td>152456V</td>
<td>CH_{2}Cl_{2}</td>
<td>84.93</td>
<td>99.8 %</td>
</tr>
<tr>
<td>n-hexane</td>
<td>BDH</td>
<td>104446C</td>
<td>CH_{3}(CH_{2})<em>{4}CH</em>{3}</td>
<td>86.18</td>
<td>95 %</td>
</tr>
<tr>
<td>Isopropyl alcohol</td>
<td>Sigma</td>
<td>19030</td>
<td>(CH_{3})_{2}CHOH</td>
<td>60.10</td>
<td>99 %</td>
</tr>
</tbody>
</table>

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All other reagents used for compound extraction including acetone, petroleum ether, chloroform were of analytical grade.

3.2.3 Thin layer chromatography materials

Silica gel 60 TLC plates 20 cm x 20 cm from Fluka (Cat No. 99570) were used along with cellulose 20 x 20 cm 250 µ from Fluka (Cat No. 95382) and Polyaminde TLC plates 20 cm x 20 cm from Fluka (Cat No. 17286).

An Alltech glass chromatography tank (20 X20 cm) was used to develop the plates. A purpose built multi-plate holder was designed and made from stainless steel to hold 10 plates and to allow the development of this number of plates or less at the one time.

3.2.4 Open column chromatography materials

For the separation of phenols from antibiotics, pesticides various aromatics and nitrogenous compounds, Amberlite XAD-2 (Sigma Aldrich, Catalogue No. 21,646-1), was used.

Sephadex G-15 size exclusion resin (< 1500 MW) (Sigma Aldrich) Catalogue No. G15120 was used to separate out polar compounds with a molecular weight less than 1500.

3.2.5 RP-HPLC materials

In all instances where RP-HPLC analysis was conducted, amber HPLC vials (Cat No. 95194), black caps with silicone liners (Cat No. 980611) and vial inserts from Alltech were used.
3.3 PREPARATION OF SOLUTIONS AND STANDARDS

3.3.1 Sodium hydroxide.

Using a top loading analytical scale a 1 M solution of sodium hydroxide was prepared under the fume hood by adding 4 g to a 1000 mL deionised water in a volumetric flask. This amount was derived following the formula:

\[ 1 \text{M NaOH}\]

\[ \text{MW(NaOH)} = 40 \]

No moles, \( n = CV \) or \( V/1000 \times M \) (in litres) \( (M = \text{molarity}) \)

\( C = \text{concentration} \)
\( V = \text{volume} \)

\[ \text{No moles}, \ n = m/\text{MW} \]

Unknown is how much NaOH to weigh out to make a 1M solution

\[ V/1000 \times 1 \text{M} = m(\text{unknown})/\text{MW(NaOH)} \]

Assume you make up 100 mL

\[ 100/1000 \times 1 = m/40 \]

Therefore \( m = 4 \) g

Dissolve 4g NaOH in 100 ml of water.

The mixture was agitated with a magnetic stirrer till dissolved on an electromantle. The solution was stored in a bottle and correctly and clearly labeled.

3.3.2 Hydrogen chloride solution

A 1 M solution of HCl was prepared from a 10 M solution by following the formula \( M_1 V_1 = M_2 V_2 \). Where \( M_1 \) represent the initial molarity, \( V_1 \) the initial volume, \( M_2 \) the final molarity and \( V_2 \) the final volume.

To find the volume of the 10 M solution required to make a 1 M solution.

\[ M_1 = 10 \text{ M} \]
V1 = x
M2 = 1 M
V2 = 1 L

Transposing the equation to find \( V1 = \frac{M2 \times V2}{M1} \)

= 0.1 L

3.3.3 Alkaline salt solution for kan sui noodles

Kansui solution for the preparation of alkaline noodles was prepared by adding 1 L of solution comprising 6 g K\(_2\)CO\(_3\) and 4 g Na\(_2\)CO\(_3\) with 10 g NaCl in 1 L water. Solubilisation was achieved by mixing in an Erlenmeyer flask with a magnetic stirrer on an electro mantle at room temperature (Moss et al., 1985).

3.3.4 Salt noodles

For salt noodles a solution of 20 g NaCl in 1L water was prepared by mixing in an Erlenmeyer flask with a magnetic stirrer on an electro mantle at room temperature.

3.3.5 Preparation of standard solutions

3.3.5.1 Carotenoids

Stock solutions of lutein, \( \beta \)-carotene and \( \beta \)-apo-8'-Carotenal were prepared to a concentration of 1000 \( \mu \text{g.mL}^{-1} \) in 10 mL McCartney bottles. The mass of substance (0.01 g) was determined on an analytical balance to which 10 mL of hexane was added. The concentration in micrograms per millilitre was determined as follows.

\[ 0.01 \text{ g/10 mL} = 0.0001 \text{ g.mL}^{-1} \text{ converted to } \mu \text{g.mL}^{-1} \text{ by multiplying by } 10^{-6} \]
= 1000 µg.mL⁻¹ of substance in solution or 1mg/mL.

Stock solutions of β-carotene and β-apo-8'-carotenal were kept at –20 °C after the McCartney bottle was flushed with nitrogen, sealed with parafilm and wrapped in aluminium foil, to reduced oxidative and photo oxidation. Solutions were generally prepared fresh for each experimental trial conducted.

The lutein standard was prepared by adding hexane (1000 µL) directly by injection into the vial in which it was purchased. The sample was gently mixed and split into four (250 µL) portions, which were stored in amber HPLC vials flushed with nitrogen and sealed with parafilm at –80 °C, until required.

Standards used for the HPLC quantification of carotenoids were prepared prior to analysis, at required concentrations using the following equation:

\[ C_1 V_1 = C_2 V_2 \]

Where \( C_1 \) represents the initial concentration, \( V_1 \) the initial volume, \( C_2 \) the final concentration and \( V_2 \) the final volume.

**3.3.5.2 Flavonoids and phenolic compounds**

Flavonoid standards were prepared in HPLC grade methanol following the procedure described for stock solution preparation of carotenoids 3.3.5.1. Stock solutions were stored at –20 °C until required.
3.4 WHEAT SAMPLES

3.4.1 Principle trial wheat

The Australian Standard White wheat Rosella bran and flour variety was used for all initial experimentation to facilitate the development of flavonoid and carotenoid extraction and analysis methods. The characteristics of this wheat are outlined in Table 3.3. This wheat was obtained from Agrifood Technology (formally the Academy of Grain Technology), Hoppers Crossing, Victoria, where the detailed analytical profile was performed.
Table 3.3. Rosella wheat profile. Report of analytical results.

<table>
<thead>
<tr>
<th>Analysis performed</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grain sample number</td>
<td>V105583</td>
</tr>
<tr>
<td>Test weight (kg/hi)</td>
<td>83.5</td>
</tr>
<tr>
<td>1000 kernel Weight (g)</td>
<td>38.8</td>
</tr>
<tr>
<td>Moisture (%)</td>
<td>10.1</td>
</tr>
<tr>
<td>Protein (%) (as is)</td>
<td>10.9</td>
</tr>
<tr>
<td>Protein (%) 13.5 % m.b.</td>
<td>10.8</td>
</tr>
<tr>
<td>Ash (%) (as is m.b.)</td>
<td>1.26</td>
</tr>
<tr>
<td>Ash (%) (13.5 % m.b.)</td>
<td>1.25</td>
</tr>
<tr>
<td>Falling number (sec)</td>
<td>426</td>
</tr>
<tr>
<td>Hardness (PSI scale)</td>
<td>22</td>
</tr>
<tr>
<td>Flour sample number</td>
<td>F105584</td>
</tr>
<tr>
<td>Extraction rate (%)</td>
<td>60</td>
</tr>
<tr>
<td>Moisture (%)</td>
<td>12.3</td>
</tr>
<tr>
<td>Protein (%) (as is)</td>
<td>9.40</td>
</tr>
<tr>
<td>Protein (%) (13.5 % m.b.)</td>
<td>9.30</td>
</tr>
<tr>
<td>Diastatic Activity (mg)</td>
<td>80</td>
</tr>
<tr>
<td>Colour grade</td>
<td>-2.1</td>
</tr>
<tr>
<td>Ash (%) (as is)</td>
<td>0.41</td>
</tr>
<tr>
<td>Ash (%) 913.5 % m.b.</td>
<td>0.40</td>
</tr>
<tr>
<td>Farinograph</td>
<td></td>
</tr>
<tr>
<td>Water absorptivity (%)</td>
<td>56.3</td>
</tr>
<tr>
<td>Development time (min)</td>
<td>2.0</td>
</tr>
<tr>
<td>Stability (min)</td>
<td>4.3</td>
</tr>
<tr>
<td>Breakdown (B.U.)</td>
<td>90</td>
</tr>
<tr>
<td>Extensograph 45 min</td>
<td></td>
</tr>
<tr>
<td>Extensibility (cm)</td>
<td>19.6</td>
</tr>
<tr>
<td>Maximum height (B.U)</td>
<td>290</td>
</tr>
<tr>
<td>Area (sq. cm)</td>
<td>83</td>
</tr>
<tr>
<td>Vasography max height (B.U.)</td>
<td>1090</td>
</tr>
</tbody>
</table>
3.4.2 Trial wheat two

The Australian Standard White wheat Rosella flour variety was used for extraction efficiency analysis and for the development of carotenoid semi quantification methods. The characteristics of this wheat are outlined in Table 3.4. This wheat was obtained from Department of Agriculture Wagga Wagga.

Table 3.4 Wheat flour characteristics

<table>
<thead>
<tr>
<th>Variety</th>
<th>Pedigree</th>
<th>Test weight (kg/hl)</th>
<th>Hardness (PSI scale)</th>
<th>Kernel weight (g)</th>
<th>Protein (%)</th>
<th>Flour extraction rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rosella</td>
<td></td>
<td>77.7</td>
<td>31</td>
<td>40</td>
<td>11.5</td>
<td>72</td>
</tr>
<tr>
<td>JB 2967</td>
<td>DK/4Hartog//2QT3</td>
<td>75.4</td>
<td>69.5</td>
<td>35.5</td>
<td>12.3</td>
<td>74.7</td>
</tr>
<tr>
<td></td>
<td>765</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JD 1221</td>
<td>WW725-A/SkuaN1601.95</td>
<td>77.6</td>
<td>70.5</td>
<td>35.3</td>
<td>11.95</td>
<td>76.3</td>
</tr>
</tbody>
</table>

3.4.3 Wheat variety comparison samples

For the comparison of different wheat variety flavonoid and carotenoid compounds, Australian Standard White flour from South Australia was obtained from Agrifood. The wheats were characterised by a 70-75 % extraction rate, protein content between 8-10.5 %, with a moisture base content of 14 %. Varieties included: Meering, Machete, Vectis, Janz, Eradu, Hartog, Yanac, Suneca, Tammin, Katunga, Goldmark, Rosella B, Trident and Cadaux. Batavia wheat flour was also used throughout these experiments and was characterised by a 60 % extraction rate, 13.0 % moisture content and a protein content of 11.4 %. The original rosella wheat (Section 3.2.4.1) was compared to these samples.
3.5 EQUIPMENT AND INSTRUMENTATION

3.5.1 RP-HPLC equipment

3.5.1.1 RP-HPLC System 1

A Varian 9100 auto sampler, 9010 Varian solvent delivery system and a Varian 9050 Ultra violet-visible (UV-vis) detector interfaced to 9021 Varian liquid chromatography (LC) Star workstation was used for the bulk of the flavonoid and carotenoid separation and semi-quantification work.

3.5.1.2. RP-HPLC System 2

For the UV visible characterisation of flavonoids, a Varian 9100 auto sampler, 9010 Varian solvent delivery system and a Varian 9050 Ultra violet-visible (UV-vis) detector and 9065 Photodiode array detector (PAD) interfaced to 9021 Varian liquid chromatography (LC) Star Workstation were used. A Varian PolyviewYM spectral processing application interfaced with the PAD provided post run analysis of data, in the time, absorbency and wavelength domain.

3.5.1.3. RP-HPLC System 3

For carotenoid separation and characterisation using UV-visible detection, a Hewlett Packard 1040 A HPLC detection system with PAD (220- 550 nm) and software with a Biorad HPLC (model 1330) were employed.

3.5.1.4 RP-HPLC columns used

Several different RP-HPLC columns were used through this research, to facilitate the separation of flavonoids and carotenoid compounds.
3.5.1.5 Column 1

A Waters, 3.9 x 150 mm, 5 mm, RP C18 column (serial No. t4208p21) was used for compound separation.

3.5.1.6 Column 2

A Varian 150 X 460 mm, 5 µm reverse phase C18 column (cat No. 92758) was used for compound separation.

3.5.1.7 Column 3

A Phenominex Ultracarb C18, 5 ODS column, 250 x 460 mm, (Cat No. 113599)

3.5.1.8 Guard column

To protect the columns from possible blockage from unfiltered material a Waters C18 pre column (Part No. WAT085824) was employed.

3.5.1.9 Fraction collector

A Varian Foxy Junior automated fraction collector was used to collect separated compounds eluated from the HPLC column.

3.5.2 UV-visible spectroscopy

Flavonoid standards with purified and un-purified wheat flour and bran extracts were analysed with a Cary 1E UV-vis spectrophotometer. Quartz crystal cuvettes with a 1 cm path length were used.
3.5.3 Mass Spectroscopy

3.5.3.1 Carotenoid Electrospray Mass Spectroscopy (EMS)

Carotenoid samples were analysed in the positive ion mode with a VG Bio Q quadropole EMS with an acetonitrile-isoamyl alcohol solvent system.

3.5.3.2 Fast Atom Bombardment (FAB) for flavonoid identification

A JEOL DX303 FAB mass spectrometer was used for analysis with a xenon atom beam (energy of 1 Kv) and a source of $10^{-5}$ Torr. The gun current was 10 mA and emission scanning was made at 100-800 Amu per 3 seconds. The sample matrix was thioglycerol. CSIRO North Ryde provided the instrumentation and technical expertise.

3.5.3.3. Electrospray mass spectroscopy (EMS) for flavonoid identification

Mass spectra were obtained with a VG Bio Q triple-quadropole MS. Nitrogen gas was used to nebulise and evaporate the solvent. Samples were analyzed in the negative ion mode. La Trobe University Chemistry Department provided the instrumentation and technical expertise.

3.5.4 GC-MS

3.5.4.1 GC-MS instrumentation

For structural elucidation underivatised and derivatised flavonoid standards were analysed using a Varian Star 3400 GC equipped with a Varian Saturn II mass spectrometer. The MS was operated in the electron impact mode with electron energy of 70 eV over a scan range of 45-400 Da.
3.5.4.2 GC columns

3.5.4.1 Column 1

Column type: DB 1701 (J and W Scientific Cat. No. 123-0732), 30 m X 0.32 mm ID. Film 0.25 microns. Temperature limits -20 °C to 280°C.

3.5.4.2 Column 2

Column type: BPX70 capillary column (SGE) 25 m x 0.22 mm ID. Film 0.33 mm fused silica. Stationary phase 70 % cyanopropl (equiv.) polysilphenylene-siloxane. Highly polar. Temperature limits, 50°C-260°C.

3.5.4.3 Column 3

Column type: BPX5 (SGE). 25 m X 0.22 mm I.D., 0.25 micron film thickness. Composed of 5 % phenyl (equiv) 95% dimethyl polysiloxane. Non-polar. Temperature limits -40°C to 370 °C.

3.5.4.4 Column 4


3.5.5 Nuclear magnetic resonance spectroscopy

Proton 1H MR spectra was performed for the assessment of product alteration by derivatising agents. A Bruker advance DPX 300 NMR with a 300MHZ/52 mm capacity was used for the attainment of proton spectra. Samples were analysed in glass NMR tubes.
3.5.6 COLOUR ANALYSIS

Flour, bran, noodle and noodle sheets colour was measured using a Minolta Chroma Meter (CR300) following the Judd-Hunter L*, a*, b* system.

3.6 METHODS

The following methods were used in more than one experiment during this study, or where standard methods or methods that did not require adaptation and were developed by other researches, as described in the literature. Methods that required adaptation or that were optimised or developed to improve compound detection and separation, for example, are described in the relevant chapters.

3.6.1 RP-HPLC

3.6.1.1 Flavonoid elution systems for the chromatographic separation of compounds

The predominant method used for the separation and semi-quantification of flavonoid and phenolic compound was a gradient elution system using formic acid-water and methanol. Methanol (solvent A) and formic acid-water (5:95,v/v) (solvent B) were employed. The elution system was 0-2 minutes 7% B in A (isocratic), 2-8 minutes 7-15 % B in A (linear gradient), 8-25 minutes, 15-75 % B in A (linear gradient), 25-27 minutes, 75-80 % B in A (linear gradient), 27-29 minutes, 80 % B in A (isocratic) 30 minutes. The column temperature was ambient with a the flow rate of 0.7 ml/min, to suit the chromatographic system and to maintain pressure within the optimum column pressure range (<300 atm).

Other solvent elution systems were trialed and developed; these are described in Chapter 4.
3.6.1.2 Elution systems for the separation of carotenoid compounds

Pigment separation was achieved using a linear gradient from 60 % acetonitrile- water (90:1, v/v) and 40 % ethyl at time zero to 100 % ethyl acetate over 25 minutes and held for 15 minutes for a total 40 minute run time. Compounds were detected at 450 nm when the solvent flow rate was 0.7 mL.min⁻¹.

Other solvent elution systems were trialed and developed; these are described in Chapter 5.

3.6.1.3 Calculation of gradient increase per minute

Gradient increase per minute was established by calculation of the slope of solvent A, between 17-22 minutes of each of the trialed runs.

Calculation example – slope = \frac{\text{rise}}{\text{Run time (minutes)}} = \frac{\text{Percentage of solvent increase}}{5} = 1.6 \text{ % per minutes}

3.6.1.4 Carotenoid semi-quantification methods

3.6.1.4.1 Carotenoid quantification using RP-HPLC and relative response factors

Samples were separated by RP-HPLC using a C18 column and guard column with peak detection by UV-visible spectrophotometer. Quantification was achieved for the individual carotenoid compounds using \( \beta \)-apo-8' -carotenal as an internal standard. The IS was added to the flour prior to extraction. Quantification of hydrocarbon carotenoids was achieved by calculation of the concentration (\( \mu \text{g.mL}^{-1} \)) of \( \beta \)-carotene versus the peak area ratio of \( \beta \)-
carotene: β-apo-8′-carotenal (Khachick and Beecher (1987). The oxygenated carotenoids (xanthophylls) were quantified by calculation of lutein versus the peak area ratio of lutein: β-apo-8′-carotenal. This was designed to enable accurate quantification of both carotenoid classes. The relative response factor (RRF) was calculated by determining the peak area ratios of β-carotene to β-apo-8′-carotenal at different concentrations of β-carotene (25, 50, 75, 100 and 125 µg.mL⁻¹) against β-apo-8′-carotenal at 50 µg.mL⁻¹.

\[
RRF = \frac{\text{amount standard} \times \text{area IS}}{\text{Area standard} \times \text{amount IS}}
\]

\[
\text{Amount Unknown} = \frac{\text{Area (unknown) \times amount (IS) \times RRF (unknown)}}{\text{Area (IS)}}
\]

The peak area ratios of β-carotene to β-apo-8′-carotenal were plotted against the increasing concentrations of β-carotene. A line of best fit was drawn then the concentrations of the unknown compounds in the wheat extracts were determined, after calculation the peak area ratio of the individual peaks against the internal standard.

The reproducibility of peak area in the samples was determined, to establish accuracy by calculation of the standard deviation of the peak area counts of the internal standard used for development of the peak area versus concentration standard curves.

3.6.1.4.2 Pigment semi-quantification using RP-HPLC

Semi-quantification was achieved by adding standard to the extract either prior to extraction or after extraction analysis.

Calculations were adjusted to consider the injection volume, amount of initial sample used and the amount of wheat extracted as follows:

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Total peak area average of sample x concentration of standard
   Peak Area of standard

= concentration mg.mL\(^{-1}\) or \(\mu g.mL^{-1}\)

Multiplied by the injection volume

Divided by the amount of sample extracted

Multiplied by the dilution factor

3.6.1.4.3 Relative Standard Deviation (RSD)

The relative standard deviation was calculated by determining the standard deviation of the peak areas of the standards used in analysis and dividing this by the average peak area. The percentage relative standard deviation was obtained by multiplying the result by one hundred.

Standard deviation of IS/ average peak area of IS \(\times 100\)

The peak area average of the IS or ES was calculated from the chromatograms of standards in each analysis.

3.6.1.4.4 Carotenoid recovery to determine extraction and analysis efficiency

Carotenoid recovery was calculated using the following:

\[
\frac{\text{Peak area of IS in sample}}{\text{Peak area average of IS} \times \text{dilution factor}} \times 100 = \times \%
\]

The peak area average of the IS was calculated from the chromatograms of the standards used to prepare calibration curves of lutein and \(\beta\)-carotene.
3.6.2 Colour analysis

Flour, bran, noodle and noodle sheets colour was measured using a Minolta meter (CR300). Flour and bran colour was measured in a 3 x 1.5 cm diameter disk placed onto a white weighing plate, to reduce possible colour influence from the bench, lightly packed to reduce the incidence of shadowing. The Chroma Meter was calibrated against a white tile. Three readings of the same sample at three different points were taken then the mean value recorded.

Flour, bran, noodle and noodle sheets colours were measured using a Minolta meter (CR300) following the Judd-Hunter L*, a*, b* system. Flour and bran colour was measured in a 3 x 1.5 cm diameter disk placed onto a white weighing plate to reduce possible colour influence from the bench, lightly packed to reduce the incidence of shadowing. The Minolta meter was calibrated with a white tile. Three readings of the same sample at three different points were taken and the mean value recorded.

3.6.3 WHEAT PIGMENT EXTRACTION METHODS FOR FLAVONOIDS

3.6.3.1 Large-scale alkaline extraction

Rosella bran samples (100 g) were extracted twice with 4 L of water (pH 11, with sodium hydroxide (NaOH)) following a procedure described by Feng et al., 1988. Due to lack of appropriate glass ware 12.5 g lots of the 100 g sample were separated into 500 mL Erlenmeyer flasks and were extracted with 250 mL of deionised water. The solutions were shaken at room temperature (20 - 24°C) in an Orbital shaker incubator at 100 RPM overnight. The extracts were decanted from the settled insolubles, combined and adjusted to pH 5 with hydrogen chloride (HCl). The white precipitate that resulted with removal of proteins was separated by centrifugation (Bechmann) at 500 rpm, 20°C, for 20 minutes. Samples were purified on a glass column (8.5 cm x 1 m) packed with Amberlite XAD-2 as described in 3.4.3.
3.6.3.2 Large-scale methanol extraction

Rosella bran (100 g) was extracted then placed in Whatman 30 mm x 100 mm extraction thimbles (Aldrich Cat No. z271063) and extracted with methanol (150 mL) in a Soxhlet apparatus with methanol for 2 hours. The solution was reduced to dryness under rotary evaporation, with the water bath set at 70°C at 300 rpm. Samples were purified as for the large-scale purification process described in 3.4.2.

3.6.3.3 Medium-scale methanol extraction

Wheat bran (50 g) was placed in Whatman 30 mm x 100 mm extraction thimbles then extracted with methanol (250 mL) in a Soxhlet apparatus at 70°C for 2 hours. The solvent was condensed by rotary evaporation in a 70°C water bath to 50 mL or until no more solvent was evaporated. The solution was left to cool (3°C) and settle. Settled insolubles that formed with the addition of methanol (used for recovery of all solutions) were centrifuged for 5 minutes. The supernatant (up to 20 mL) was purified by open column chromatography, as described in 3.4.2.

3.6.3.4 Methanol extraction

This method was an adaptation of the method developed by Wootton (1994), Section 3.4.3.2. To a soxhlet extraction thimble (Whatman 30 x 100 cm), 25 g of bran was wet packed with methanol (20 ml) into the thimble. Boiling chips were added to a round-bottomed flask containing the extraction solvent, methanol (200 ml) and the bran was refluxed for two hours. The soxhlet equipment was heated with a thermostatically controlled electromantle, in a Soxhlet chamber, fill rate of 1 - 3 minutes. The fill rate was determined by taking an average of three cycles, the time it took to fill and empty the Soxhlet chamber.

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3.6.3.5 Small-scale methanol extraction

The bran, (10 g), was placed in Whatman 30 mm x 100 mm extraction thimbles and extracted with methanol (150 ml), by Soxhlet extraction for 2 hours (at approximately 70°C). After extraction, the solution was filtered through No. 1 Whatman filter paper using a Büchner funnel, to remove particulate matter such as flour and small bran particles. The solution was rotary evaporated at 70°C, 50 rpm, until approximately 5 ml of solution remained.

3.6.3.6 Small-scale alkaline extraction

Rosella flour and bran (2g) was extracted in an Erlenmeyer flask (125 mL) with alkaline water, pH 11. The alkaline water was prepared with the addition of sodium hydroxide (10 M then 5 M) to 800 mL deionised water (Feng and McDonald, 1989). The flasks were shaken and left at room temperature for 13 hours. The solution was decanted from the flour or bran, into 200 mL bottles and stored at 3°C. The remaining bran and flour was re-extracted with additional 80 mL alkaline water for 6 hours.

After the second extraction the solutions were decanted. The first and second extracts were combined. The solutions were acidified with acetic acid to pH 5. The denatured protein and particulate matter were removed by centrifugation in 200 mL tubes, for 10 minutes at 4000 rpm, in a Beckman J2-H5 centrifuge with a JA 14 rotor at 20°C. The extract was purified as described in Section 3.5.1.

3.6.3.7 Petroleum ether and acetone extraction method (King 1962)

This simple and fast method for examination of wheat flavonoids was derived from a method described by King (1962). Rosella wheat bran (16.5 g) held in a paper thimble (Whatman, 30 x 30 mm) was extracted in a Soxhlet apparatus with 150 ml petroleum ether (40-60°C) for two hours. After extraction the bran was removed from the thimble and added directly to the solvent flask for overnight extraction. The bran was separated from the solvent by filtration in
a Buchner funnel (No. 1 Whatman filter paper.) The bran was air dried prior to a second phase extraction involving percolation, at room temperature in 80% acetone (150 ml). The acetone extract was condensed by rotary evaporation for removal of the non-aqueous fraction, which was later reduced, by evaporation on a boiling water bath. The residue was redissolved in deionised water (16.5 ml) and extracted three times with n-butanol (8 ml). The extracts were combined and reduced in a water bath to between 2-5 ml.

3.6.4 EXTRACT PURIFICATION METHODS

3.6.4.1 Large-scale open-column flavonoid purification

Samples were purified on Amberlite XAD-2 resin as described by Feng et al., 1988. The Amberlite XAD-2 (500 g) was applied dry to a glass column (8.5 x 100 cm). The Amberlite XAD-2 column was prepared by soaking in deionised water for approximately 1 ½ hours and applied as a slurry to the column. The column resin was backwashed with deionised water to remove any air bubbles. The resin was then washed with 4 L of deionised water, prior to the application of the sample to further settle the resin. The sample was applied via a Pasteur pipette to the column. Extracts were then applied to the column and the adsorbed flavonoid fraction was washed with 8 L of DiH2O to remove non-phenolic water-soluble impurities. The flavonoid and phenolic fraction was then eluted from the column with 2 L of methanol.

The methanol extract was reduced by rotary evaporation to dryness with the water bath set at 70 °C, 300 rpm, until it was concentrated to approximately 10 mL. Methanol was used to redissolve dried sample from the round bottom flask. Approximately 12 mL of solution was applied to the column. The residue was then applied to the top of a column (2.5 cm x 50 cm) Sephadex G-15 (Sigma-Aldrick G15120). The G-15 column was prepared by soaking the 10 g resin in 0.2 M NaOH for approximately 1 1/2 hours to facilitate expansion. This was applied as slurry to the column packed with glass wool. The resin was left to settle by gravity and washed with 20 mL water. The flavonoid sample was eluted with 400 mL DiH2O at a flow rate of 120 mL per hour.
Fractions were collected from the column, at 0 - 60 mL, 60 - 200 mL, 200 - 450 mL. The flavonoid fraction eluted at 60 - 200 mL as determined by its yellow colouration. The fractions were reduced on a boiling water bath to dryness then re-dissolved in a minimal amount of methanol for storage.

Pack column (8.5 X 100 cm) with 500 g preconditioned Amberlite XAD-2 (deionised water for 1 ½ hours)
- let packing settle by gravity
- back wash with a pump
- wash the column packing with 4 L DIH20
- apply extract (10-75 mL)
- wash absorbed flavonoids with 8 L water
- collect the flavonoid fraction by eluting with 2 L methanol
- condense the solution by rotary evaporation

**Purification on Sephadex G-15**

Applied as a slurry 10 g of Sephadex G-15 conditioned in n 0.2 M NaOH
- For 1 ½ hours
- apply sample
- elute 450 mL water
- collect 0-60 mL
- elute 450 mL water
- collect 0-60 mL
- elute 50 mL methanol
- collect 50 mL fraction

**Figure 3.1 Summary of the flavonoid purification process**

### 3.6.4.2 Small scale purification process.

Purification was achieved with open column chromatography in two stages. The first stage involved purification using Amberlite XAD-2 followed by the second stage using Sephadex G-15 resin.
Stage 1. The Amberlite (40 g) was applied as slurry to a column (2.5 x 50 cm): the resin was prepared by washing with water (100 ml, deionised). The extracted sample was added to the column then washed with deionised water (300 ml). Methanol (100 ml) was used to elute the flavonoid fraction from the column. This eluate was collected and reduced to 10 mL by either rotary evaporation or evaporation on a boiling water bath.

Stage 2. The Amberlite purified extract was subjected to particle size purification using Sephadex G-15 size exclusion resin. The resin was applied as slurry to a glass chromatography column (2.5 x 50 cm) after the resin had been hydrated in 0.2 M NaOH for more than 2 hours. Prior to application of the sample, the resin was washed with 400 ml deionised water. The sample was applied to the column and eluted with methanol (120 ml), at a flow rate of approximately 2 mL.min⁻¹ then the eluate was collected in fractions. This fraction was collected and reduced under RE to approximately 3 mL, and stored at 3°C in the refrigerator until analysis.

3.6.5 CAROTENOID EXTRACTION FROM WHEAT FLOUR

All solvents used were of HPLC grade. All glassware was cleaned and rinsed prior to extraction, as described in Section 3.1.2. The extraction vessel (Soxhlet apparatus or Erlenmeyer flask) containing the flour and solvents was flushed with nitrogen. All experiments were conducted away from direct light to reduce carotenoid oxidation.

3.6.5.1 Hexane and diethyl ether extraction

3.6.5.1.1 Small scale extraction (20 g)

Samples of flour (20 g) were extracted with hexane (70 mL) and diethyl ether (30 mL) (Wright and Shearer, 1984) for 1 hour, in an Erlenmeyer flask (250 mL), at room temperature. The sample was continuously stirred using a magnetic stirring bar placed in the flask on an electro mantle at room temperature.
After extraction, the solution was filtered through No. 1 Whatman filter paper under vacuum then reduced to dryness in an evaporative water bath (temperature setting 40 °C). The samples were re-dissolved in hexane (3 mL) and centrifuged to remove remaining flour particles. For HPLC analysis and quantification, the samples were reduced to dryness under nitrogen gas then dissolved in acetone (10 mL), in a volumetric flask.

3.6.5.1.2 Large-scale extraction (100g)

Carotenoids were extracted from Batavia flour (100 g) in a 1L Erlenmeyer flask with 350 mL hexane and 150 mL diethyl ether for 1 hour at room temperature. For HPLC quantification of the carotenoids β-apo-8'-carotenal was added (50 µg.mL⁻¹). The flask was flushed with the inert gas, nitrogen, covered with parafilm and foil to reduce oxidation as well as loss of solvent. After extraction the solution was filtered from the flour through Whatman No. 1 filter paper under vacuum. The solution was reduced under rotary evaporation with the water bath at 40 °C until approximately 2 mL volume was obtained (about 40 min). The sample was re-dissolved in acetone (5 mL in a volumetric flask). A white precipitate formed. An aliquot (500 µL) was centrifuged and used for HPLC injection.

3.6.5.2 Petroleum ether extraction

Flour extraction with 50 mL petroleum ether (40-60°C) was done in a Soxhlet apparatus at 40°C for 30 minutes (Wagner et. al., 1983).

3.6.5.3 Acetone and petroleum ether

Flour was extracted with acetone (40 mL) and petroleum ether (40 mL) with anti-oxidants 0.1 MgCO₃ and ascorbic acid (1 g) in a 500 mL Erlenmeyer flask (Wills, et.al., 1988).

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3.6.5.4 Total carotenoid content determination

Wheat flour was extracted following a method for total carotenoid content (AACC 14-50, 1983). Duplicate samples of each variety were extracted routinely. Flour (8 g) was extracted with water-saturated n-butanol (40 mL), in an Erlenmeyer (50 mL) flask overnight, at room temperature for 18-20 hours. The solutions were filtered through No. 1 Whatman filter paper in a Buchner funnel under vacuum. All experiments were conducted away from direct light, and sample vessels were flushed with nitrogen to reduce carotenoid degradation.

An aliquot (2 mL) was measured in a 1 cm path length quartz cuvette at 436 nm with a Pharmacia Biotech Novaspec II spectrophotometer (LKB Biocrhom, England).

Calculation of concentration was achieved using the following equation:

\[
\text{Pigment} \text{1mg.100 mL}^{-1} = \frac{\text{absorbance of sample} \times \text{volume of extract solution} \times 1000}{100 \times \text{std. Abs. } \beta\text{-carotene}}
\]

\[
\text{Dry weight of sample} = \frac{\gamma \times 40 \text{ mL} \times 1000 \text{ mL}}{1.663 \times 100 \text{ mL} \times 8 \text{ g}} = \chi \text{ ppm or } \mu\text{g.g}^{-1}
\]

3.6.6 Mass Spectroscopy

3.6.6.1 Carotenoid Electrospray Mass Spectroscopy

Carotenoid samples were analysed in the positive ion mode with a VG Bio Q quadropole E/MS with an acetonitrile-isoamyl alcohol solvent system.
3.6.6.2 Fast Atom Bombardment (FAB) for flavonoid identification

A JEOL DX303 FAB mass spectrometer was used for analysis with a xenon atom beam (energy of 1 Kv) and a source of $10^{-6}$ Torr. The gun current was 10 mA and emission scanning was made at 100-800 Amu per 3 seconds. The sample matrix was thioglycerol.

3.6.6.3. Electrospray mass spectroscopy for flavonoid identification

3.6.6.3.1 Method 1.

Mass spectra were obtained with a VG Bio Q triple-quadrapole MS. Sample (300µM) were dissolved in methanol:water, (50/50 v/v) with 1 % acetic acid. Samples (10-20 µL) were injected via a 10 µL injection loop via a rheodyne injector. The mobile phase was methanol:water (50/50 v/v) at a flow rate of 5 µL. Nitrogen gas was used to nebulise and evaporate the solvent. Samples were analysed in the negative ion mode.

3.6.6.3.2 Method 2

Mass spectra were obtained with a VG Bio Q triple quadrupole mass spectrophotometer. The isopropyl water solvent flow rate was set at 5 mL per minute. The B1 voltage varied according to each sample fragmentation pattern requirements. Samples (10-20 µL) were injected via a 10 µL injection loop via a rheodyne injector. Nitrogen gas was used to evaporate the solvents.

3.6.7 GC-MS

For structural elucidation underivatised and derivatised flavonoid standards were analysed using a Varian Star 3400 GC equipped with a Varian Saturn II mass spectrometer. The MS was operated in the electron impact mode with electron energy of 70 eV over a scan range of 45-400 Da.
Column types, conditions and temperatures are described in more detail in chapter 11. Spectra were analysed using Star Chromatography software (Varian). GC analysis for the development of a flavonoid mass spectral method was performed on a Varian Star 3400 gas chromatograph equipped with a Flame ionisation detector (FID). Oven temperatures and conditions varied according to methods employed as discussed in Chapter 11.

3.6.7.1 GC-MS sample preparation methods

3.6.7.1.1 Method 1 permethylation

1 mg of flavonoid was weighed in a HPLC vial and to this 200 µl of dimethylformamide (DMF) followed by 100 µL N,O-bis(Trimethylsilyl)acetamide (BSA) (Pierce code 38836) was added to the compound under nitrogen the sample was vortexed for 30 minutes and heated for 15 minutes at 70°C (Peirce, 1996).

3.6.7.1.2 Method 2 silation

The flavonoid sample (15 mg) was combined in an HPLC screw capped vial with 1 mL Tri-sil ‘Z’, vortexed for 30 seconds then heated for 15 minutes at 70°C (Peirce, 1996). Later to ensure no oxidation was occurring, the samples were derivatized in reaction vials with nitrogen flowing through the vial during the addition of the derivatizing agent (Figure 3.2 ).
Figure 3.2. Nitrogen flow through a HPLC autosampler vial. The system is closed to reduce oxidation of the derivatising agents with air and moisture.

3.6.7.1.3 Method 3 silation

The flavonoid sample (1-2 mg) was dissolved in 0.1 mL anhydrous pyridine after which 0.1 mL hexamethyldisilazane and 0.03 mL of trimethylchlorosilane are added. The solution was left to stand for 10 minutes after vortexing for more than 30 seconds (Furuya, 1965). The solution was pipetted into a eppendorf tube and the supernatant’s solution was removed after centrifugation for 3 minutes, in a bench-top centrifuge. Two samples that were prepared in this manner include one with the derivatising agent by itself and one with the standard rutin together with the derivatising agent.

3.6.7.2 Gas chromatography conditions

A Varian GC star 3400CX Gas Chromatograph interfaced with Varian Star software version 4.2 windows was used for the analysis of flavonoids.

1 µL of the derivatised samples was injected onto the column via a Varian 8200CX autosampler. Latter 0.1 uL samples were injected to ensure that the derivatising agent was not swamping the samples, as occurred in the test mix analysis of the BP20 column.
3.6.7.3 Flow rate calculations

To ensure that the GC system was operating effectively, the flow rate was calculated following the method described in section 3.

Calculation of average linear velocity (u)

\[ u = \frac{L}{tr} \]

\( u \) = average linear velocity \( (\text{cm} . \text{sec}^{-1}) \)

\( L \) = column length \( (\text{cm}) \)

\( tr \) = Retention time of non-retained peak \( (\text{seconds}) \)

Methane is injected manually and the resulting peak time is converted from minutes \((100\text{ths})\) to seconds

non retained peak retention time = 2.069 minutes \( \times \) 60 seconds = 124.1 seconds

\[ u = \frac{3000 \text{ cm column length}}{124.14 \text{ seconds}} \]

\[ = 24.16 \text{ cm/second} \]

(J&W Scientific 1996)

The average linear velocity is converted to flow rate in ml/min using the following equation:

\[ \text{mL.min}^{-1} = (u)(0.47 \times d^2) \]

\( u \) = linear velocity \( (\text{cm} . \text{sec}^{-1}) \)

0.47 is a constant

\( d^2 \) = the square of the internal diameter of the column in cm

therefore the flow rate = 24.16 \((0.47 \times 0.32^2)\)
= 1.16 mL.min\(^{-1}\)

(SGE capillary column installation validation procedure. Publication No. 0047-C Rev0012/95)

3.6.7.4 Determination of split ratio

Using a bubble flow meter the time it takes for the bubble to move 10 mL is determined. The time is converted into minutes and multiplied by 10 mL.

22 seconds in 10 mL

\[
\frac{22}{60} \times 10 = 27.27 \text{ mL.min}^{-1}
\]

the ratio between the column flow rate and the split flow is determined

27.3 mL.min\(^{-1}\) split flow: 2.6 mL.min\(^{-1}\) flow rate which is approximately a 10:1 split ratio.

3.6.8 Nuclear magnetic resonance spectroscopy

3.6.8.1 Method 1.

Catechin (100 mg) was dissolved in 500 mL dimethylformamide (DMF) and derivatized with 300 mL of N,O-bis(Trimethylsilyl)acetamide (BSA). The entire reaction was performed under nitrogen to prevent oxidation of the derivatising agent. The solution was shaken for 30 seconds prior to heating at temperatures between 70-80°C for 15 minutes. To prevent spectroscopic analysis interference, the DMF was removed from the solution using nitrogen. To the reduced solution approximately 2 mL of deuterated acetone (d\(_6\)) was used to dissolve the sample. A sample of morin was also prepared in the same manner.
3.6.8.2 Method 2.

To a 50 mg sample of catechin, 1 mL of dichloromethane (DCM) and 0.5 mL of trifluoroacetice anhydride (CF3) was combined. The sample was left to dissolve for 45 minutes or until no solid was observed, occasionally the sample was stirred. The solution was reduced under nitrogen until dryness and was redissolved in 2-3 mL d₆ acetone prior to analysis.

3.6.8.3 Unmodified sample preparation

Approximately 50 -100 mg of the flavonoids catechin and morin were dissolved in 1-3 mL d₆ acetone.

3.6.9 PREPARATION AND TESTING OF NOODLES

3.6.9.1 Preparation of alkaline salted noodles

Noodles were prepared by mixing the flour (50 g) in a Kenwood mixer, fitted with a paddle beater. The flour was added and mixed at low speed (setting 1). Kansui: salt solution (1:1, v/v) (17.5 mL) was added gradually to reduce flour clumping. The combination was mixed at medium speed (setting 2) for 30 seconds. The flour dough forming was roughly kneaded to remove uneven clumping prior to the crumbly dough being mixed at a high speed (setting 3) for 2 minutes.

The dough was gently hand pressed in preparation for sheeting, in order to assist the crumbly mixture to stick together. Next the dough was sheeted using a noodle machine (Attas Pasta Machine OMC Marcato) by passing through rollers (spaghetti alla chitarra 2 mm accessory). Initially the dough was passed through rollers setting 1 approximately 6 mm apart. The sheet was folded after passing through the rollers every time for a maximum of 10 times. The roller setting was adjusted to 3, approximately 2 mm apart, and passed through and folded 3 times. The dough was collected on a plastic plate and placed in a plastic bag to rest for 30 minutes.
After resting the sheets for $\frac{1}{2}$ hour, each sheet was passed through rollers set at a gap of 1.1 mm and cut into 1.5 mm wide strands by a set of no. 20 cutting rollers (Avanti). The noodle pieces were collected and placed in a plastic bag.

3.6.9.2 Preparation of white salted noodles.

Noodles were prepared as for the alkaline salted noodles though only salt solution was added in the mixing process.

3.6.9.3 Testing pH of noodle sheets

A sample of dough sheet (10 g) was homogenised with water (100 mL) and the pH measured after 30 minutes by placing the pH electrode in the slurry.

3.6.9.4 Determining optimum cooking time

Optimum cooking time is defined as the time it takes for the white core of the noodle strand to disappear. This was established by placing a noodle strand between glass slides at 1 minute intervals after 3 minutes of cooking. Noodles (25 g) were added to previously boiled water (250 mL) and cooking time was determined from the time the noodles were placed into the water. At intervals of approximately 20 seconds a strand of noodle was removed, placed between two small glass plates and pressed. This step was performed to establish optimum cooking time, which was determined by the length of time it took for the opaque centre of the noodle core to disappear.

3.6.9.5 Determining water uptake of noodles

Following a method described by Hong (1992), noodles (25 g) were added to water 250 mL that was previously brought to boil. The noodles were cooked for the optimum cooking time, as measured in section 3.4.9.4, and then drained through a sieve. The noodles were cooled under running tap water for
approximately 1 minute and further drained in the sieve by gentle agitation for a further minute prior to being weighed. The cooked noodle weight was determined using the following calculation:

\[
\text{Water uptake (\%)} = \frac{\text{weight of cooked noodle (g)} - \text{dry weight of noodle (g)}}{\text{dry weight of noodle}} \times 100
\]
CHAPTER FOUR.
EXTRACTION AND SEPARATION METHOD IDENTIFICATION AND DEVELOPMENT FOR THE ISOLATION AND CHARACTERISATION OF FLAVONOID AND PHENOLIC COMPOUNDS IN WHEAT

4.0 Introduction

Work in this chapter focused on applying elution systems reported in the literature to find a successful separation method for flavonoid and phenolic compounds extracted from wheat bran and flour. This research enabled wheat flavonoid and phenolic compounds to be identified and characterised in later experiments. Initial experimentation focused on identification of an RP-HPLC method and elution system, to facilitate the separation of flavonoids together with the phenolic compounds from wheat. The method that was identified as providing the best separation of compounds was optimised to obtain maximum compound resolution. Different extraction methods for recovery of flavonoids were examined then selected on the basis of high extraction rates, compound type extraction, as well as ease of characterisation and simplicity of the process. To assist in establishing the presence of flavonoids, an alkaline and acid test was applied to the extracts for there fast and simple identification. The outcomes of these experiments are defined and discussed.

4.1 RP-HPLC method development for the separation and tentative identification of flavonoid and phenolic compounds from wheat.

A method for the separation and characterisation of flavonoids is required to facilitate and enable the comparison of the pigments of different wheat varieties. Ample literature available on the separation of flavonoid and phenolic compounds from plant matrices, but none on the extraction and analysis of wheat extracts using RP-HPLC analysis. Several of the different solvent elution systems utilised for the analysis of flavonoid and phenolic compounds obtained from other plant materials were trialed to identify a method that gave superior resolution of compounds in the wheat extracts.
These methods differed in the solvents used but were similar in the fact that all employed C18 columns for compound separation. Solvent polarity, gradient or isocratic elution and column type will all influence flavonoid compound resolution.

The overall objective of these trials was to identify a chromatographic system that would enable good resolution of pigments. This would then enable optimisation of chromatographic conditions to facilitate the:

1) Selection of the most effective pigment extraction method.
2) Characterisation and quantification of pigments; using UV-visible spectroscopy with photodiode array detection.
3) Purification of pigment extracts for conclusive structural identification.
4) Enable rapid analysis and identification of wheat pigments by comparison with authentic standards.

An effective separation method was defined as providing good baseline-to-baseline separation of wheat extracts and standards with good peak resolution, without any peak tailing. Method development in RP-HPLC usually involves comparison of chromatographic conditions on the basis of the separation of individual compounds. Good separation is considered to have been achieved when: A) Compounds elute greater than 0.4-0.5 minutes apart (Vande Casteele et al, 1982); B) Good retention time reproducibility with less than 10% relative standard deviation is obtained; C) Compounds elute after the solvent front and D) Compounds can be clearly distinguish from background interference. In addition to these requirements, a method with a minimal run-time that did not compromise compound separation was preferred to minimise solvent consumption and sample analysis time.

To this day RP-HPLC analysis has not been used for the quantification and characterisation of flavonoid compounds extracted from wheat. The objective of this part of the research was to establish if RP-HPLC could be used for the routine processing of large numbers of samples for semi-quantification of flavonoids and tentative identification.
4.1.1 Method and Materials

4.1.1.1 Samples analysed

A wheat bran sample (Section 3.4.1) was extracted following an alkali water technique (Section 3.6.3.1) to assist in the detection of the most appropriate elution system. Initial investigations focused on peak separation using the flavonoid standards, morin and rutin, an aglycone and a sugar aglycone respectively, to access peak retention time and resolution effectiveness of the different solvent elution systems.

4.1.1.2 Chromatographic conditions

Compound separation and detection was achieved as described in Section 3.5.1.1. A reverse phase C18 column (Section 3.5.1.6) was used for compound separation with a C18 Guard Pak (Section 3.5.1.8) pre column was used.

4.1.1.3 Elution systems

A number of different chromatographic conditions and solvent elution systems were employed to assist in establishing the best approach to good separation:

4.1.1.3.1 Methanol- formic acid-water

An adapted method described by Vande Casteele et al., (1982) was trialed. The gradient elution system involving methanol and formic acid –water (5:95, v/v) was not altered however the flow rate was reduced from 2.5 mL.min⁻¹ to 0.7 mL.min⁻¹ to suit the chromatographic system by maintaining system pressure below 300 atmospheres and within the optimum column pressure range. A gradient elution system of methanol (solvent A) and formic acid-water (5:95,v/v) (solvent B) was employed. The elution system was 0 - 2 minutes 7 % solvent A in B; 2 - 8 minutes, 7 - 15 % solvent A in B; 8 - 25
minutes, 15-75 % solvent A in B; 25-27 minutes, 75-80 % solvent A in B; held to 29 minutes. Detection was achieved at 280 nm.

4.1.1.3.2 Acetonitrile- water- acetic acid

An isocratic mobile phase of acetonitrile: water: 3 % acetic acid (33:64:3, v/v) was used (Novijanto, 1992). The flow rate was 1.0 mL.min⁻¹, run time 15 minutes and the column was operated at room temperature. Detection was achieved at 280 nm.

4.1.1.3.3 Methanol- 1 % acetic acid

Koupai-Abyazani, et al., (1992) described this method as suitable for the separation of flavonoid compounds from sainfoin. A mobile phase of methanol (solvent A) and 1 % acetic acid (solvent B) was employed with compounds separated firstly by gradient elution then isocratic elution. The elution system was 0-30 minutes, 15 - 60 % solvent A in B; 30 – 50 minutes, 60 % solvent A in B isocratic elution. Solvent was eluted onto the column at a flow rate of 1 mL.min⁻¹. Compounds were detected at a wavelength of 280 nm.

4.1.1.3.4 Acetonitrile- NaHPO₄- triethylamine acetate

This method was an adapted version described in the Phenomenex catalogue (1994) p.1.124 where a mobile phase of A 0.05 M NaH₂PO₄ and 0.01 M TEA, pH 2.6 and B: acetonitrile was employed. However instead of using TEA, a solvent of a similar nature triethylamine acetate was employed. No run time was given so an arbitrary time expected, to allow the wheat extract compounds to elute from the column, of 40 minutes was trialed. A gradient elution system of 30 % solvent B to 70 % solvent B within the run time was employed. The solvent was delivered at a flow rate of 1.0 ml/min that was later adjusted to 0.7 mL.min⁻¹ to reduce the operating pressure that was causing problems with leakages. Compounds were detected at 254 nm.
4.1.1.3.5 Methanol- chloroform- acetonitrile

Isocratic elution of chloroform, methanol and acetonitrile (10:30:60, v/v). Conducted with a flow rate of 1 mL.min⁻¹, with detection at 280 nm.

4.1.1.3.6 Methanol: chloroform: acetonitrile

Isocratic elution of chloroform, methanol and acetonitrile (10:30:60, v/v). Conditions as described in 4.1.1.3.5 but at a lower flow rate of 0.7 mL.min⁻¹.

4.1.2 Results and discussion

Method 4.1.1.3.1 with methanol: formic acid-water (5:95, v/v) was initially run under the conditions specified in the literature (Vande Casteele et al., 1982) at a flow rate of 2.5 mL.min⁻¹. Using the Varian chromatographic system this resulted in a system pressure of 320 atmospheres, which with the gradient elution program, would have exceeded the column and system pressure limit of 400 atmospheres. For this reason, the flow rate was reduced to 1.5 mL.min⁻¹ and the system pressure was reduced to 215-225 atmospheres. The standards morin and rutin did not elute within the runtime. The solvent flow rate was decreased further to 0.7 mL.min⁻¹ and the run time extended by 4 minutes. This resulted in the morin and rutin standards producing well resolved peaks at 21.67 and 19.71 minutes respectively (Figure 4.1 a and b respectively). A wheat extract was injected (Figure 4.1 c). A number of different compounds were detected with a range in retention times from 13 to 33 minutes suggesting differences in compound structures with different polarity characteristics.
Figure 4. 1. RP-HPLC chromatogram of morin standard (A), rutin standard (B) and an alkaline-water Rosella wheat bran extract (C). Sample separated using a gradient elution system of formic acid and methanol with compound detection at 280 nm.
Method 4.1.1.3.2 employed isocratic elution using acetonitrile: water: 3% acetic acid (33:64:3). The elution of the standard compound using this solvent system was not clearly evident (Figure 4.2). The standards eluted very early in the run, resolving from the column after 1.18 minutes. Compounds with these retention times were also observed in the alkaline bran extract. The compounds detected after 3 minutes were poorly resolved with broad peaks. Poor standard resolution and compound detection coupled with broad peak resolution of the alkaline bran extract resulted in this method being rejected. No further optimisation of this method was conducted.
Figure 4.2. RP-HPLC chromatogram of Morin standard (A), Rutin standard (B) and an alkaline-water bran extract (C). Separation was achieved by isocratic elution of acetonitrile/water/3 % acetic acid (33:64:3, v/v) across a RP C-18 column with compound detection at 280 nm.
Method 4.1.1.3.3 involved compound separation using methanol and 1 % acetic acid following a gradient elution program. This method was initially run as described in the literature with the solvent system of 15 % methanol in 85 % aqueous acetic acid (1 %). This did not facilitate the elution of the standard compounds within the run time. Rather than extending the run time it was thought that increasing the percentage of methanol earlier on in the run may enable the standard compounds to elute within the 50 minute run time.

This adaptation enabled the standards to elute within the run time with rutin and morin showing well-resolved peaks appearing late in the run (Figure 4.3).
Figure 4.3. RP-HPLC chromatogram of Morin standard (A), Rutin standard (B) and alkaline-water Rosella bran extract (C). Separation was achieved by using methanol: 1 % acetic acid gradient elution across a RP C-18 column with compound detection at 280 nm.
The use of the solvent systems described in Method 4.1.1.3.4. employing 0.05 M NaH₂PO₄ and 0.01 M triethylamine acetate, pH 2.6 and B: acetonitrile, resulted in the early elution of morin at 7.8 minutes (Figure 4.4 a). In previous methods, morin had been found to elute after the majority of compounds in the alkaline bran extract, using this elution system, however, morin eluted earlier. In order to enable the separation of the alkaline bran extract compounds, the elution profile was modified to ensure that morin appeared at a later retention time. The solvent delivery system run time was adjusted from 1.0 mL.min⁻¹ to 0.7 mL.min⁻¹. As expected, a longer retention time for the standard was observed (Figure 4.4 B).

Figure 4.4. Morin standard separated using a trimethyl acetate:NaH₂PO₄: acetonitrile gradient elution at a flow rate of 1 mL.min⁻¹ (A) and 0.7 mL.min⁻¹ (B), with compounds detected at 254nm.
Using this method, separation of the compounds in the alkaline bran extract was found to be poor. Compounds eluted early from the column between 3 and 5 minutes with no baseline-to-baseline separation observed by a broad band of compounds observed between these times (Figure 4.5). A major peak at 6.9 minutes with peak tailing and poor resolution, blending with another compound at 8.2 minutes was observed. Additional compounds after 10 minutes were also evident suggesting that a number of different compounds with distinctly different polarity characteristics are within the bran extract.

Figure 4.5. RP-HPLC trace of an alkaline-water bran extract separated using acetonitrile:sodium phosphate:trimethyl acetate gradient elution at 0.7 mL.min⁻¹ across a RP C-18 column with compound detection at 280 nm.

One of the challenges the flavonoid researcher faces is that there are many types of flavonoid compounds. Over 3000 flavonoid compounds have been previously identified (Mabry et al., 1970), many very similar in their molecular formula, differing only in the position of OH or methoxyl groups on the aglycone structure. Another challenge is that it is difficult to quantify flavonoids, as reference compounds are unavailable (Hasler, et al 1989), or
have retention times that are the same as the flavonoid and phenolic compounds being analysed in the wheat samples.

4.1.3 Conclusion

Different solvent elution systems using polar and non-polar solvent combination resulted in different separation profiles. Primarily the success of an elution system was determined by the ability of the solvents and the solvent elution program to resolve compounds in the alkaline bran extract. From these experiments, it was established that two of the solvent delivery systems were appropriate for the separation of the flavonoid and phenolic compounds extracted from wheat. The formic acid and methanol elution system and the methanol and acetic acid gradient elution system may be used for the characterisation of flavonoid and phenolic compounds within Australian Standard White wheats. Compound separated and detected by the formic acid and methanol gradient elution system, tended to elute earlier than for the acetic acid and methanol system. For this reason this system was considered to be most appropriate to further develop and optimise.

4.2 OPTIMISATION OF THE CHROMATOGRAPHIC CONDITIONS FOR THE METHANOL-FORMIC ACID GRADIENT ELUTION SYSTEM TO MAXIMISE THE COMPOUND RESOLUTION OF WHEAT EXTRACTS.

4.2.1 Introduction

As was observed in Section 4.1, the chromatographic profiles of flavonoid bran extracts are very complex, comprising a number of closely eluting compounds with little baseline-to-baseline separation. To improve the RP-HPLC separation of the major flavonoid compounds characteristic of rosella bran, solvent optimisation trials were performed using formic acid and methanol eluants. The trials involved increasing the gradient increment times, and therefore decreasing the gradient slope, to better facilitate baseline-to-baseline peak separation.
If baseline-to-baseline separation could be achieved it may be possible using fraction collection methods after chromatographic separation of compounds, to collect individual compounds. This would assist in the comprehensive identification of these compounds using mass spectroscopy or NMR. More defined, better separation is also desirable for accurate quantification of the individual flavonoid and phenolic components in the wheat extract.

The objective of the following experiment was to establish if decreasing the gradient increment time and therefore the solvent percentage change over time would facilitate baseline-to-baseline separation of the flavonoid extract.

4.2.2 Methods and Materials

4.2.2.1 Extraction and Purification.

Rosella wheat bran was extracted with methanol in a soxhlet apparatus and purified as described in Section 3.6.3.2.

4.2.2.2 RP-HPLC.

The flavonoid sample was analysed and separated using the same chromatographic system as described in Section 4.1.1.3.1. The gradient elution system was examined in detail to optimise sample separation, reduce run-time and improve solvent usage efficiency by reducing solvent consumption. A number of trials were conducted and these are outlined in Table 4.1. The gradient increase was calculated as described in section 3.6.1.3.
Table 4.1. Optimisation of the formic acid – water (5:95, v/v): methanol gradient elution system.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Time (minutes)</th>
<th>Gradient increase (%/min)</th>
<th>Solvent composition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Solvent A %</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>2.57</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>15</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>25</td>
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<td>85</td>
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<tr>
<td></td>
<td>30</td>
<td>80</td>
<td>25</td>
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<tr>
<td></td>
<td>35</td>
<td>80</td>
<td>20</td>
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<tr>
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<td>2</td>
<td>1.67</td>
<td>7</td>
</tr>
<tr>
<td></td>
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<td>35</td>
<td>40</td>
<td>60</td>
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<td>40</td>
<td>75</td>
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<td>80</td>
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<td>0.9</td>
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<td>0.5</td>
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<td></td>
<td>60</td>
<td>7</td>
<td>93</td>
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</tbody>
</table>
4.2.3 Results and Discussion

The chromatograms of each of the different methods are displayed in Figure 4.6. Co-elution of compounds became evident during this examination with two peaks at approximately 16.8 and 17.0 minutes, method 1 and 5. Evidence of co-elution was also apparent for the first peak for methods 2, 3, 4 and 6. These chromatograms displayed a shoulder prior to a peak at approximately 17.0 minutes. Previous analysis of flavonoid extracts separated by RP-HPLC with methanol and 0.6 % formic acid, by gradient elution has shown peak co-elution. It is thought that this occurs because of the similar polarity of the compounds. Some compounds have been reported to be stereoisomers such as apigenin, which may differ in configuration by the position of the OH groups on the flavonoid nucleus, making separation of stereoisomers very difficult.
Figure 4.6 RP-HPLC profiles of a flavonoid extract subjected to 6 different gradient elution methods (Trials 1-6, Table 4.1), with detection at 280 nm.
To facilitate comparison of the different solvent systems, gradient increases per minute, for each of the methods are displayed in Figure 4.7.

![Graphs of solvent percentage changes over time for RP-HPLC methods 1-6.](image)

**Figure 4.7.** Solvent percentage changes over time of RP-HPLC methods 1-6.

Peak separation was improved with the reduction in gradient slope increase. A visual comparison of the trial 1 chromatogram with trial 4 chromatogram highlights this effect (Figure 4.6). The trial 1 chromatogram displays valley to
valley separation between 17 and 22 minutes and the trial 4 chromatogram predominantly displays baseline-to-baseline separation during the same period.

TABLE 4.2. Comparison of peak separation between 17 and 22 minutes for the method with the highest gradient increase rate, method 1 and the lowest gradient increase rate, method 6.

<table>
<thead>
<tr>
<th>METHOD 1</th>
<th>METHOD 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak retention time</td>
<td>Separation</td>
</tr>
<tr>
<td>17.912</td>
<td>BV</td>
</tr>
<tr>
<td>18.280</td>
<td>VV</td>
</tr>
<tr>
<td>18.773</td>
<td>VV</td>
</tr>
<tr>
<td>19.380</td>
<td>VV</td>
</tr>
<tr>
<td>20.091</td>
<td>VV</td>
</tr>
<tr>
<td>20.725</td>
<td>VV</td>
</tr>
<tr>
<td>21.221</td>
<td>VV</td>
</tr>
<tr>
<td>21.459</td>
<td>VV</td>
</tr>
</tbody>
</table>

With the higher gradient increase more peaks were eluted during the 17-22 minute time frame for samples achieved using trial 1. The retention time of the first peak also eluted slightly later than for that of the method 6 first peak. This can be explained by analysis of the solvent combination percentage difference of the two different methods, the later facilitating better separation (Table 4.3).
TABLE 4.3. Solvent A and B percentage increases during 17-22 minutes of chromatographic analysis.

<table>
<thead>
<tr>
<th>Trials</th>
<th>SOLVENT A (%)</th>
<th>SOLVENT B (%)</th>
<th>% INCREASE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>46-64</td>
<td>35-53</td>
<td>18</td>
</tr>
<tr>
<td>2</td>
<td>44-52</td>
<td>47.5-56</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>41-45.5</td>
<td>54-58</td>
<td>4.5</td>
</tr>
<tr>
<td>4</td>
<td>41-44.5</td>
<td>59-56</td>
<td>3.5</td>
</tr>
<tr>
<td>5</td>
<td>40-43</td>
<td>60-57</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>40.2-42.2</td>
<td>57-59</td>
<td>2</td>
</tr>
</tbody>
</table>

The solvent percentage increase during 17-22 minutes for the trial 1 sample was the highest and this may account for more peaks being resolved during that time period. It is apparent that as the solvent percentage differential decreases so do the number of peaks eluting as trial 1 displays seven peaks; trial 3, five compounds; trial 5, six compounds and trial 4, four compounds. Baseline-to-baseline peak separation is achieved by reducing the solvent percentage increase and the gradient increase over time. This is clearly shown by the elution profile of the flavonoid subjected to the trial 4 program.

4.2.4 Conclusion

Some improvements in compound resolution with optimisation of the gradient elution system were achieved. The best separation was achieved when the solvent gradient was reduced to 0.5 % increase per minute. The elution system was solvent A, methanol and solvent B, formic acid-water (5:95 v/v). At 0 - 2 minutes 7 % solvent A in B; 2 - 8 minutes, 7 - 15 % solvent A in B; 8 - 15 minutes, 15-40 % solvent A in B; 15-40 minutes, 40-50 % solvent A in B; 40-45 minutes, 50-75 % solvent A in B; 45-50 minutes, 75-80 % solvent A in B and 50-60 minutes 80 to 7 % solvent A in B.
Baseline-to-baseline separation of two of the peaks of methanol extract analysed using methanol and formic acid-water was achieved. Further separation may be achieved if gradient increases of 0.4, 0.3, 0.2 and 0.1 % per minute are conducted; it is also recommended that further work to optimise this elution system be carried out.

4.3 COMPARISON OF DIFFERENT EXTRACTION METHODS

4.3.1 Introduction

With the potential for RP-HPLC to provide a rapid analysis and quantification technique for flavonoid characterisation in wheat, a number of extraction and purification methods were employed. Ultimately an extraction method that enabled the rapid isolation of flavonoid compounds with the ready separation of these compounds for characterisation and quantification was sought.

Several different methods for the extraction of flavonoids were trialed to examine the impact of extraction on the types and numbers of compounds extracted, as well as to examine the impact purification has on compound detection and elution (Table 4.4). The objective of the following experiments was to determine the most appropriate method for the analysis of flavonoid extracts from wheat, using RP- HPLC with UV-visible detection at 280 nm. This wavelength was selected from literature as it was found to be the most commonly used wavelength for flavonoid analysis.

Assessment and comparison of extraction techniques was based on:
1) Sample extraction repeatability;
2) Compound number and polarity comparison between all the different extracts;
3) Relative abundance of major peaks;
4) The number of different compounds extracted;
5) Compound resolution.
Four different extraction techniques were examined with purification of the extracts as summaries in Table 4.4.

Table 4.4. Methods employed for the extraction of flavonoids from wheat flour and bran

<table>
<thead>
<tr>
<th>Method</th>
<th>Summary of extraction procedure</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2 g of alkali water (pH 11) extracted wheat bran, purified on Amberlite XAD-2 and Sephadex G-15</td>
<td>Feng &amp; McDonald, 1989.</td>
</tr>
<tr>
<td>2</td>
<td>16.5 g wheat bran soxhlet extracted with petroleum ether (40-60), filtered and percolated with 80 % acetone, condensed and extracted with n-butanol. Purified on Sephadex G-15 eluted with methanol then Sephadex LH-20.</td>
<td>King, 1962.</td>
</tr>
<tr>
<td>3</td>
<td>Extracted following method 2 however no purification was performed.</td>
<td>King, 1962</td>
</tr>
<tr>
<td>4</td>
<td>Methanol soxhlet extracted wheat bran (25 g), purified on Amberlite XAD-2 and Sephadex G-15</td>
<td>Wootton, 1994</td>
</tr>
</tbody>
</table>

The methods differed in the amounts of bran extracted, the solvent systems and purification techniques. As no information on the chromatographic profiles of these wheat extracts was available internal or external standards were not added. Methods were compared on the basis of the number of different compounds extracted and the resolution of the compounds using the same chromatographic system. The overall objective was to find, then develop a method of extraction that was fast and simple which would facilitate the detection and total concentration determination of flavonoid compounds in wheat.
4.3.2 Materials and methods

4.3.2.1 Extraction techniques employed.

In all instances the principle trial wheat Rosella (Section 3.4.1) was used to assess extraction method impact.

4.3.2.1.1 Small-scale alkaline extraction

The small-scale alkaline extraction method is described in Section 3.6.3.6. After extraction the sample was purified following the procedure described in Section 3.6.4.2.

4.3.2.1.2 Methanol extraction and purification

This method was an adaptation of the method developed by Wootton (1994), Section 3.6.3.2. To a Soxhlet extraction thimble, 25 g of bran is extracted following the procedure described in Section 3.6.3.4. After extraction the solution was concentrated by rotary evaporation (40 - 50°C at 30 RPM) to 6 mL, then purified following the procedure described in Section 3.6.4.2.

4.3.2.1.3 Petroleum ether and acetone extraction with purification

This method is described in detail in Section 3.6.3.7. After extraction the sample was purified following the procedure described in Section 3.6.4.2

4.3.2.1.3 Petroleum ether and acetone extraction without purification

This method is described in detail in Section 3.6.3.7 and is the same method applied above for method three however no sample purification after extraction was employed.
4.3.2.2 RP-HPLC analysis

A Varian chromatographic system as described in Section 3.5.1.1 was used to separate and visual extract components. Extracts were examined using an acetonitrile-water- acetic acid (3 %) isocratic elution system, at a flow rate of 1 mL.min$^{-1}$. Detection was at 280 nm. Compounds were separated using a Waters C 18 column and guard column as described in section 3.5.1.5 and 3.3.18 respectively. Extract was injected via a 50 µL injection loop at an injection volume of 20 µL.

4.3.3 Results and discussion

Using the different extraction processors differences in the number of peaks and in the relative abundance of peaks at the same retention times were observed. Differences may be related to the concentration of the sample with less sample facilitating better resolution. Differences may be attributed to the solvent extraction ability with regard to extraction of polar and less polar compounds within the wheat bran. The sample extracted by method 1 with alkali water showed better separation and lower peak heights at the same attenuation. Over ten compounds were observed with six predominant compounds (Figure 4.8). The chromatographic profile of the bran extracted with methanol (Figure 4.9) was different to that of the alkaline extract. This suggests that different solvents extract different concentrations of compounds within the bran as well as different compounds. Wheat bran extracted with petroleum ether and acetone with and without sample purification (Figures 4.10 and 4.11 respectively) showed very similar chromatographic profiles. This suggests that for this extraction method conducting compound purification by open column chromatographic methods is of no benefit.

Extraction with either alkaline water or methanol resulted in a greater number of compounds being observed and slight better compound resolution than those compounds extracted with petroleum ether and acetone.
Figure 4.8. Method 1 - alkaline extraction. The RP-HPLC chromatogram was obtained using an acetonitrile-water-acetic acid (3%) isocratic elution system at a flow rate of 1 mL.min$^{-1}$ with detection at 280 nm.

Figure 4.9. Method 2 - methanol extraction. The RP-HPLC trace was obtained using an acetonitrile-water-acetic acid (3%) isocratic elution system at a flow rate of 1 mL.min$^{-1}$ with detection at 280 nm.
Figure 4.10. Method 3 - petroleum ether and acetone extraction with purification. The RP-HPLC trace was obtained using an acetonitrile-water-acetic acid (3%) isocratic elution system at a flow rate of 1 mL.min\(^{-1}\) with detection at 280 nm.

Figure 4.11. Method 4 - petroleum ether and acetone extraction with no further compound purification. The RP-HPLC trace was obtained using an acetonitrile-water-acetic acid (3%) isocratic elution system at a flow rate of 1 mL.min\(^{-1}\) with detection at 280 nm.
It has been reported that the retention times of flavonoids is influenced by the hydrogen bond interaction with the stationary and mobile phase (Casteele, et al., 1982). In general terms, under the operating conditions of this experiment the more polar compounds are eluted first. The more polar compounds are generally likely to contain for example, 3-OH groups rather than two and the less polar glycosylated hydroxide groups causing increased retention times due to hydrophobic moiety formation with the shielding action by hydrogen bonds and steric hindrance.

4.3.4 Conclusion

It was of no surprise that different chromatographic profiles were observed for compounds extracted with solvents of differing polarities. When selecting the most appropriate extraction method the extraction efficiency should be taken into account. This was not at first possible, until a standard, which eluted separately from the wheat extract was found, later identified as catechin. Without the extraction efficiency as a guide in selection of the best extraction method, the number of compounds detected and their resolution was used to measure method success. For this reason, the alkaline water extraction and the methanol extraction method were examined further.

4.4 FURTHER COMPARISON OF METHANOL AND ALKALINE WATER FLAVONOID EXTRACTIONS

4.1 Introduction

Two different large-scale extraction techniques for flavonoid isolation and identification were compared, a methanol extraction and an alkaline water extraction procedure. Assessment and comparison of extraction techniques was based on;

1) Sample extraction repeatability;
2) Compound retention time comparison between all the different extracts;
3) Relative abundance of major peaks;
4) The number of different compounds extracted;
5) Compound resolution.

4.4.2 Method and materials

4.4.2.1 Extraction procedure

Bran from the wheat variety Rosella (100 g) Section 3.4.1 was extracted by the alkaline methods described Section 3.6.3.6. Bran was extracted with methanol Section 3.6.3.4. Both extracts were purified following the procedure described in Section 3.6.4.2.

4.4.2.2 Analysis

After purification the flavonoid eluate collected from the G-15 column was analysed using RP-HPLC with UV-visible detection at 280 nm. A gradient elution system using formic acid methanol gradient, at a flow rate of 0.7 mL.min-1 was used to separate the different compounds within the extracts as described in Section 3.6.1.1

4.4.3 Results and discussion

An alkaline and methanol extraction method was compared. At a glance visual comparison of the chromatographic profiles of the two extraction methods indicate that similar compounds have been extracted. The two different extraction techniques showed similar peak retention time information for major peaks, with comparable peaks being detected at 19, 20, 25 and 28 minutes (Figure 4.9). Differences in the number of compounds identified were observed.

113
A) Methanol extract, 26 peaks.

B) Alkaline water extract, 44 peaks.

Figure 4.12. Chromatographic comparison of the alkaline water extract with the methanol extract. A gradient elution system using formic acid methanol gradient, at a flow rate of 0.7 mL.min\(^{-1}\) was used to separate the different compounds detected at 280 nm.

In the alkaline extract, a minor peak at 20.20 minutes was observed whereas at this retention time the methanol extract contained a much larger peak area compound. A major peak was observed in the methanol extract at 21.04 minutes, which was not observed in the alkaline extract.

Closer examination revealed 12 major peaks (classified according to peak areas being greater than 7000 peak area counts) observed in the alkaline
extract and the eight major peaks observed in the methanol extract (Table 4.5).

Table 4.5. Retention times and the relative abundance (%) of the major compounds identified in the alkaline and methanol extracted wheat bran samples

<table>
<thead>
<tr>
<th></th>
<th>Methanol extract</th>
<th>Alkaline water extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retention time</td>
<td>Retention peak</td>
<td>Retention peak</td>
</tr>
<tr>
<td>(min)</td>
<td>area (counts)</td>
<td>area (counts)</td>
</tr>
<tr>
<td>19.84</td>
<td>19669</td>
<td>19.89</td>
</tr>
<tr>
<td>20.47</td>
<td>32095</td>
<td>20.52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21.37</td>
</tr>
<tr>
<td>21.04</td>
<td>27740</td>
<td>22.04</td>
</tr>
<tr>
<td>23.11</td>
<td>9175</td>
<td>22.45</td>
</tr>
<tr>
<td>23.71</td>
<td>7545</td>
<td>23.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>23.82</td>
</tr>
<tr>
<td>24.93</td>
<td>7413</td>
<td>25.13</td>
</tr>
<tr>
<td>25.60</td>
<td>17411</td>
<td>25.60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>26.81</td>
</tr>
<tr>
<td>28.79</td>
<td>23008</td>
<td>28.74</td>
</tr>
</tbody>
</table>

4.4.4 Conclusion

The extraction of flavonoid compounds with alkali and methanol yielded eight major compounds of comparable retention times and peak areas. Compound differences were observed between the extraction methods with a larger number of compounds detected in the alkaline extract. If focus is to be on the major compounds the methanol extraction method has significant time advantages over the alkaline extraction process.
4.5 ACID AND ALKALINE TEST FOR FAST AND SIMPLE FLAVONOID IDENTIFICATION

4.5.1 Introduction

Flavonoids are classified as weakly acidic polyphenolic compounds with acid labile hemiacetal bonds. Their presence in solution may be readily detected by changing the pH of solution to acidic or alkaline. Changes in the pH of solution result in the dissociation of the OH group which influences the colour of the solution. If flavonoid compounds are present in solution under alkaline conditions, they will turn the solution colour to a bright yellow barley sugar colour, dependant on concentration, and in acid conditions revert to a clear solution.

4.5.2 Method and materials

4.5.2.1 pH modification

The initial pH of the sample was determined with pH indicator strips to be neutral at pH 7 and made either acidic with HCl (section 3.3.1) or alkaline with NaOH (section 3.3.2). End solution pH was tested to ensure acidity (pH 4) or alkalinity (pH 12).

4.5.2.2 Sample preparation and analysis

The samples extracted with the various methods 1-4, as described in section 4.3.2.1 were used in this experiment.

4.5.3 Results and discussion

Flavonoid compounds were detected in each of the differently extracted samples. In alkaline solutions, the sample containing a flavonoid compound or compounds is yellow but in acid solutions the flavonoid colour becomes clear.
This approach offers a very simple method to confirm the extraction or otherwise of flavonoid compounds.

TABLE 4.6. Results of acid and alkaline assessment of wheat bran for the ready identification of flavonoids by the action of acid labile hemiacetal bonds.

<table>
<thead>
<tr>
<th>Extraction method</th>
<th>initial pH</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8</td>
<td>colour change, pH 4 clear, pH 12 yellow</td>
</tr>
<tr>
<td>2</td>
<td>13</td>
<td>precipitate formed</td>
</tr>
<tr>
<td>3</td>
<td>9</td>
<td>very slight yellow with precipitation at pH &gt;12</td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td>colour change, pH 4 clear, pH 12 yellow</td>
</tr>
</tbody>
</table>

A precipitate was formed with the conversion of the solution to a pH of greater than or equal to pH 11. With the addition of an acid solution the sample became clear. Formation of a precipitate is not indicative of flavonoid compounds only colour (Wootton, personal comm. 1994).

4.5.4 Conclusion

Using this method of identification provided a rapid means for establishing if flavonoids had been extracted. This approach was later applied to help identify when flavonoids were eluted from the open column. This enabled the elimination of 26 elutes collected at 50 ml increments, from open column purification on Amberlite XAD-2 and Sephadex G-15. Another possible application could have been to identify flavonoid concentrations obtained using different extraction and purification method using UV-Visible
spectroscopy similar to the practice of estimating total carotenoid content following the AACC, 1983). This method could be further refined to estimate total flavonoid content which may be beneficial for the detection and segregation of wheat for Chinese (alkaline) noodle production for the Japanese market. It is assumed that wheat with a high flavonoid content would produce a good yellow colouration ideal for Chinese noodles.
5.0 Introduction

This chapter focuses on the identification and development of appropriate extraction and analysis methods for the quantification and characterisation of carotenoid pigments in wheat. A number of different RP-HPLC methods reported in literature were trialed and the results of these investigations discussed. With the identification of a suitable carotenoid compound separation technique, optimisation of this method was conducted to maximise chromatographic quality, run-time and solvent efficiency. For the semi quantification of carotenoids, an internal standard method was investigated using the standard β-apo-8'carotenal. Finally, an extraction method with high efficiency was sought by examining several different carotenoid extraction methods. The culmination of each phase enabled the trial semi-quantification of carotenoids in three different wheat varieties.

5.1 RP-HPLC METHOD SELECTION FOR THE HPLC SEPARATION OF CAROTENOID COMPOUNDS

5.1.1 Introduction

To facilitate the identification and quantification of carotenoid compounds extracted from wheat, using RP-HPLC with UV-visible detection, several different chromatographic methods were examined. A reproducible method that facilitated well-defined compound resolution with separation of the individual compounds, that would also enable the quantification of carotenoids using an internal or external standard was sought. HPLC accuracy and precision is effected by the reproducibility of peak retention time, peak height and peak area. These are all functions of chromatographic variables that include column type, elution conditions such as flow rate and mobile phase (Bakalyar and Henry 1976). In this study, the mobile phase of several different solvent systems both gradient and isocratic elution systems were examined. To assist comparison of methods the flow rates, column type and detection wavelength were held
constant.

Methods were selected or rejected according to the resolution of oxygenated and hydrocarbon carotenoids, using lutein (oxygenated) and β-carotene (hydrocarbon) standards and accessed for the ability to resolve individual compounds present in the wheat flour extract.

5.1.2 Method and materials
5.1.2.1 Sample preparation of standards

Standard solutions of lutein and β-carotene standards were prepared in acetone under nitrogen gas as for Section 3.3.5.1 to provide solutions of 10 µg.g⁻¹.

5.1.2.2 Carotenoids extraction from wheat flour.

Carotenoids were extracted from the principal trial wheat (Section 3.4.1) Rosella flour (10 g), by refluxing 10 g Rosella flour with acetone (50 mL) for 1 hour (Wootton, 1994). The sample was filtered through Whatman No. 3 filter paper then reduced to dryness by rotary evaporation. The sample was redissolved in acetone (2 mL) flushed with nitrogen to remove air, prior to analysis to assist compound stability.

5.1.2.2 RP HPLC conditions

To assist in the identification and optimisation of a method, the most commonly used column for carotenoid separation reported in the literature, a C18, was used for comparison of the effectiveness of the different solvent systems. Samples were analysed by RP-HPLC with a Varian system as described in Section 3.5.1.1. Separation was performed using a C18 column (Section 3.5.1.7). To protect the column a Waters C 18 pre-column (section 3.5.1.8) was used. Various solvent elution systems were employed. Solvents were degassed as described in Section 3.1.3. Detection was performed at
450 nm, the flow rate kept constant at 0.7 mL.min⁻¹ and the sample volume injected, in all instances, was 50 µL via a 60 µL loop.

5.1.2.3 Elution systems

5.1.2.3.1 Acetone-water

A gradient elution program employing acetone (a) and water (b) was used (Minguez-Mosqueara and Hornero-Mendez, 1993) as follows:

<table>
<thead>
<tr>
<th>time (minutes)</th>
<th>Solvent A</th>
<th>Solvent B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>75</td>
<td>25</td>
</tr>
<tr>
<td>5</td>
<td>75</td>
<td>25</td>
</tr>
<tr>
<td>10</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>12</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>17</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>22</td>
<td>75</td>
<td>25</td>
</tr>
</tbody>
</table>

5.1.2.3.2 Methanol-dichloromethane and acetonitrile

An isocratic elution mixture of methanol-dichloromethane and acetonitrile (10:20:70 v/v) was employed (Heinonen et. al.,1989). Flow rate 0.7ml.min⁻¹, detection at 450 nm. End time 30 minutes.

5.1.2.3.3 Acetonitrile- water and ethyl acetate

Pigment separation was investigated using a linear gradient from 100 % acetonitrile- water (90:1, v/v) to 100 % ethyl acetate over 37 minutes (Wright and Shearer, 1984). Detection 450 nm, flow rate 0.7 ml.min⁻¹, end time 37 minutes.
5.1.2.3.4 Methanol-acetonitrile-chloroform

An isocratic elution system employing methanol-acetonitrile-chloroform (30:60:10) was trialed for separation of compounds over 30 minutes.

5.1.2.3.5 Methanol-acetonitrile-chloroform

The solvent composition of Section 5.1.2.3.4 above was altered to methanol-acetonitrile-chloroform (10:60:30, v/v) to increase the non polar solvent composition, in order to improve the retention of polar compound and to facilitate better separation.

5.1.2.3.6 Methanol and methanol-chloroform

Methanol (solvent A) and methanol-chloroform (94:6, v/v) (solvent B) gradient elution from 100 % A to 100 % b in 30 minutes (Spanos et al., 1993).

5.1.3 Results and discussion

RP-HPLC separation of lutein and β-carotene using a gradient elution system of acetone and water method (Section 5.1.1.3.1) was achieved (Figure 5.1 A). The resolution of the β-carotene standard was not as good as the lutein standards and showed a broader peak eluting over 1 minute. Separation of the wheat flour extract (Figure 5.1 B) resulted in detection of three major peaks at 15.6, 19.5 and 23.2 minutes. The compound at 19.5 minutes displayed poor resolution as well as a broad peak area, due to the long retention on the column, stationary phase. This would suggest that compounds of similar polarity were not separated using this solvent system.
Separation of lutein and β-carotene using a mobile phase of methanol-dichloromethane-chloroform (Section 5.1.2.3.2) was not achieved (Figure 5.2 C). To ensure that the solvent system did not facilitate separation, lutein and β-carotene standard were examined in separate runs (Figure 5.2 A and B). This solvent system used did not facilitate separation of the more polar oxygenated lutein standard from the non-polar β-carotene standard. Not surprisingly, the separation of individual compounds in the carotenoid extract was also poor. It was assumed that as standard of different structure and polarity could not be resolved, then many of the potential carotenoid pigments in the wheat extract could not be and would therefore be co-eluting.
Figure 5.2. RP-HPLC chromatograms of standards β-carotene and lutein separated using an isocratic elution system of methanol-dichloromethane-acetonitrile (10:20:70, v/v), with compounds detected at 280 nm.

The ethyl acetate and acetonitrile-water gradient elution (Section 5.1.2.3.3) system was able to separate the standards and wheat extract pigments. Peak resolution of standards was excellent, observed by narrow based well resolved peaks (Figure 5.3). Similarly, good separation of the carotenoid extract was achieved with detection of three major peaks at 21.2, 32.7 and 34.3 minutes together with several other smaller peaks (Figure 5.3 B).
Figure 5.3. RP-HPLC chromatogram of lutein and β-carotene standards and an acetone extraction of Rosella wheat flour. Compound separation was achieved using a gradient elution separation with ethyl acetate and acetonitrile-water (9:1, v/v), with compounds detected at 280 nm.

Resolution of lutein using the isocratic elution system of methanol-acetonitrile-chloroform (Section 5.1.2.3.4) was not achieved. Lutein eluted immediately from the column and was not detected. The β-carotene standard displayed a very short retention time of 2.6 minutes. The lutein standard, therefore, probably was not retained on the column for long enough to allow detection. The solvent composition was altered to attempt retention of the standards (Section 5.1.2.3.5), however, there was no improvement. Analysis of the wheat extract was not conducted.

To establish which of the two elution systems previously identified as being able to separate the standard and the wheat extracts, solvent elution systems Sections 5.1.2.3.1 and 5.1.2.3.4 were compared. Criteria for acceptance were
based on the ability of the method to maximize baseline-to-baseline separation of compounds hence facilitate semi-quantification and tentative compound identification. Different chromatographic profiles were observed (Figure 5.4) with greater separation observed using the ethyl acetate-acetonitrile gradient elution system. The run time of this method was later increased to ensure that all compounds had sufficient time to elute from the column and to be detected.

Figure 5.4. Comparisons of an acetone extracted Rosella wheat separated by a gradient elution system with ethyl acetate and 90 % acetonitrile compared to the same extract separated with a gradient elution system of acetone and water. Compounds were detected at 280 nm.

5.1.4 Conclusion

The acetonitrile-water and ethyl acetate gradient elution system achieved the best separation for both reference compounds as well as for the wheat extract
carotenoid pigments. The run time of this method did not appear to maximize the elution of all carotenoids as compounds were observed at the end of the run time. This method required optimisation for carotenoid characterisation and quantification.

5.2 ACETONITRILE-WATER AND ETHYL ACETATE GRADIENT ELUTION SYSTEM METHOD DEVELOPMENT FOR THE QUANTIFICATION OF WHEAT FLOUR CAROTENOID COMPOUNDS.

5.2.1 Introduction

Previous experimentation identified a superior carotenoid RP-HPLC chromatographic separation system using an ethyl acetate and acetonitrile/water (90:10, v/v) gradient elution system. It was observed, however, that both reference standards and wheat extract compounds did not start eluting until around 21 minutes. To reduce solvent waste and run time for the analysis of different wheat flour carotenoid extracts, optimisation of this method was investigated.

The method for the separation of carotenoid pigments in wheat was improved by reducing compound retention times and by altering the gradient elution system.

5.2.2 Method and materials

5.2.2.1 Sample preparation

Standard solutions (10 μg.mL⁻¹) of lutein and β-carotene standards were prepared in acetone under nitrogen gas as for Section 3.3.5.1
5.2.2.2 Chromatographic conditions

Samples were analysed by RP-HPLC with a Varian system as described in Section 3.5.1.1. Separation was performed using a C18 column (Section 3.5.1.7) and pre-column (Section 3.5.1.8). Detection was performed at 450 nm, the flow rate kept constant at 0.7 mL.min\(^{-1}\) and the sample volume injected, in all instances, was 50 µL via a 60 µL loop.

5.2.2.3 Sample elution system

The acetonitrile-water and ethyl acetate (Section 5.1.1.3.4) solvent elution system was further developed.

5.2.3 Results and discussion

The gradient elution method Section 5.1.2.3.3 was modified after plotting the solvent combination percentages over time (Figure 5.5), to identify at what solvent combination, the lutein and \(\beta\)-carotene compounds eluted from the column. This solvent system, as it was first trialed, resulted in the elution of the oxygenated carotenoid compound, lutein at 21.4 minutes and the hydrocarbon reference carotenoid, \(\beta\)-carotene at 35.4 minutes that corresponded with a solvent combination of approximately 60 % acetonitrile-water in 40 % ethyl acetate.

![Gradient elution system for carotenoid separation with 90 % acetonitrile (A) and ethyl acetate (B)](image)

Figure 5.5. Gradient elution system with acetonitrile-water and ethyl acetate before.
From this information, it was decided to start the gradient elution from 100 % acetonitrile-water (90:10 v/v) at time zero, to 60 % acetonitrile-water (90:10 v/v). The two gradient systems are summarized in Table 5.1.

**Table 5.1. Comparison of gradient elution systems**

<table>
<thead>
<tr>
<th>Gradient elution system 1</th>
<th>Gradient elution system 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (min)</td>
<td>Solvent A (%)</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>50</td>
<td>0</td>
</tr>
</tbody>
</table>

A standard containing lutein, β-apo-8'-carotenal and β-carotene and a Rosella flour carotenoid extract was analysed before alteration of the method, then retention times compared with the chromatographic analysis of these compounds following the second systems methodology.

Comparison of standard solutions analysed, before modification of the gradient elution and after, resulted in comparable chromatographic profiles, in terms of compound separation profile but with a considerable reduction in the overall run-time. The run time was reduced by 14 minutes. Compound resolution was not compromised.

**5.2.4 Conclusion**

By examining the gradient elution solvent composition over time, it was identified that the separation of compound occurred at of 60 % acetonitrile. This facilitated the development of a more time efficient elution system, with excellent compound resolution.
5.3. APPLICATION OF β-APO-8-CAROTENAL AS AN INTERNAL STANDARD.

5.3.1 Introduction

To facilitate the accurate quantification of carotenoid compounds by RP-HPLC, it is essential to use a standard. Generally HPLC quantification employs an IS or ES for the quantification of individual pigments. The IS method is considered superior to ES method. This is because the standard is applied prior to extraction of the sample and is therefore subjected to the same conditions as the extract, allowing for calculation of and identification of losses and other changes that may occur during the extraction process (Khachik and Beecher, 1985).

Use of the IS method enables high accuracy and precision measurement, it is especially useful in instances where absolute amounts or concentrations are required (Gordon, 1994). This is because the relative response factor (RRF) used in calculations compensates for variations in injection volume, extraction efficiencies and in detection sensitivity. When an IS cannot be added because available standards elute at the same time as compounds in the extract, an ES may be employed.

The use of an IS for the accurate quantification of carotenoids from wheat is essential, as the extraction procedures generally comprise many steps through which sample loss may occur. Loss can be accounted for by calculation of pigment recovery rates. Ideally, the internal standard used should elute separately from the other compound in the extract but have similar chromophoric characteristics, solubility and chromatographic behaviour (Macrea, 1988). In addition to this, it should also be readily available and stable (Khachik and Beecher, 1987).

For the analysis of oxygenated and hydrocarbon carotenoids several different IS have been used including: C-45-β-carotene (Khackick and Beecher, 1987),
β-apo-8’-carotenal (Khachik et al, 1986) and isozeaxanthin. The use of lutein and β-carotene as IS was rejected, as both carotenoids have been reported to be present in wheat extracts and were tentatively identified in wheat extracts in a previous trial (Section 5.1), by comparison of retention times of authentic lutein and β-carotene standards. After some investigation into the commercial availability of potential internal standards, it was decided that β-apo-8’-carotenal would be most appropriate as it was readily available and had a different molecular composition and degree of oxygenation to lutein and β-carotene.

An internal standard method described by Khachik and Beecher, (1987) involved determination of the RRF of a selected standard, in this case β-carotene, to that of an internal standard, β-apo-8’-carotenal. The peak area of the unknown compound may be quantified by relating the peak area ratio and the area of the internal standard peak, to the area ratios obtained from calibration curves. These calibration curves are obtained by plotting the area ratio of known concentrations of standards, versus the concentrations of the individual carotene.

The above method was trialed for the quantification of carotenoid pigments in wheat using β-apo-8’-carotenal as an internal standard.

5.3.2 Method and materials

5.3.2.1 Sample extraction and preparation method

To establish if β-apo-8’-carotenal could be added to wheat extracts, a sample of Rosella flour (Section 3.6.5.1.1) was extracted and compared to a sample extracted with the addition of β-apo-8’-carotenal (50 µg.mL⁻¹). Duplicate samples were extracted as described in Section 3.3.5.1.
5.3.2.2 Chromatographic conditions

Samples were analysed by RP-HPLC with a Varian system as described in Section 3.5.1.1. Separation was performed using a C18 column (Section 3.5.1.7) and pre-column (Section 3.5.1.8). Detection was performed at 450 nm, the flow rate kept constant at 0.7 mL.min⁻¹ and the sample volume injected, in all instances, was 50 µL via a 60 µL loop.

5.3.2.3 Sample elution system

The elution system as described in Section 3.6.1.2 was employed with gradient elution of acetonitrile-water and ethyl acetate.

5.3.2.4 Chromatographic and injection reproducibility

To ensure injection volume reproducibility, a standard solution was injected three times, from the IS peak area count and retention times, the coefficient of variation was calculated (Section 3.6.1.3.3). Injection reproducibility was considered excellent if less than 5 % variation occurred. Poor injection reproducibility was corrected by purging of the auto-sampler injection tubing and syringe to remove trapped air.

5.3.2.5 Extraction efficiency

The extraction efficiency of the wheat flour extraction method together with the recovery percentage using this chromatographic system and method was calculated following the procedure described in Section 3.6.1.3.4.

5.3.3 Results and discussion

Excellent peak retention and injection volume reproducibility was obtained 1.68 % RSD (Table 5.2).
Table 5.2. Raw data for calculation of the relative standard deviation between the IS peak area and retention times within two duplicate sample extracts.

<table>
<thead>
<tr>
<th>Retention time</th>
<th>Peak Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>13.06</td>
<td>9039547</td>
</tr>
<tr>
<td>13.11</td>
<td>9004203</td>
</tr>
<tr>
<td>13</td>
<td>8762957</td>
</tr>
<tr>
<td>Average</td>
<td>8935569</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>150527.3</td>
</tr>
<tr>
<td>Relative standard deviation</td>
<td>1.68%</td>
</tr>
</tbody>
</table>

β-apo-8-carotenal was found to elute at approximately 13 minutes well away from carotenoids isolated from wheat (Figure 5.6 A) and B) respectively.
Figure 5.6. A carotenoid extract without the addition of the IS (A) and with the inclusion of the IS added prior to extraction (B). The IS eluted at 13 minutes separately from all other wheat carotenoid compounds. Separation was achieved with a RP – C18 guard column and column with an acetonitrile-water (90:1 v/v) and ethyl acetate gradient elution system with compounds detected at 450 nm.
There are several advantages for using β-apo-8-carotenal as an internal standard. It elutes separately from all other compounds in the wheat extract between lutein and β-carotene standard on the C18 Reversed-phase HPLC column. It does not naturally occur in the wheat extracts investigated. It can be added to the wheat extract at the extraction stage having similar solubility to other carotenoids. It is stable with no apparent alteration, as shown by no alteration in its retention time or chromatographic profile in the wheat extracts. A good recovery of β-apo-8’-carotenal at 87 % from wheat extracts was achieved. This suggests that β-apo-8’-carotenal has similar solubility behaviour to other carotenoids in the sample.

5.3.4 Conclusion

It has been shown that β-apo-8’-carotenal has the best requirements of an internal standard for the quantitative determination of both hydrocarbon and oxygenated carotenoids.

5.4 ADAPTATION OF CAROTENOID EXTRACTION METHODS FROM OTHER FOODSTUFFS FOR THE EXTRACTION AND QUANTIFICATION OF WHEAT PIGMENTS

5.4 Introduction

Information on the extraction efficiency of methods for the separation of carotenoids from wheat flour and bran matrices has not been reported in the literature. The following is an account of trials conducted to develop a superior extraction and carotenoid analysis procedure, to enable reliable quantitative determination of these pigments in wheat.

General methods for carotenoid extraction involve extraction with organic non-polar solvents (Kruger and Reed, 1988; Fallon et. al., 1987; Khachik et. al., 1986; Ball, 1988; Goodwin, 1994). The addition of anti-oxidants to prevent oxidation and a saponification step to hydrolyse carotenoid esters, in order to assist in
the separation of carotenoids from other components such as chlorophyll and proteins (Khachik and Beecher, 1985) may also be used. Wheat flour contains little if any chlorophyll thus it has carotenoid levels much lower than fruits and vegetables. Because simpler chromatographic profiles were expected it was thought that a saponification step might not be necessary, so that the removal of this step would increase compound recovery.

Four different extraction procedures using saponification, anti-oxidant addition and different solvent systems were compared. For this work, extraction rates of > 95% were sought to ensure result reliability. A method that was fast and simple that did not increase the incidence of oxidative and heat degradation, determined by extraction rate together with chromatographic profile analysis was sought.

5.4.2 Method and materials

5.4.2.1 Sample extraction

Flour (Section 3.4.1) was used in all pigment extraction trials. Duplicate extractions of 20 g of flour were performed routinely. Extraction efficiency was determined by adding β-apo-8-carotenal, (50 μg.mL⁻¹), prior to the extraction of flour and calculated as described in Section 3.6.1.3.4. The concentration of lutein was calculated using the external standard method (Section 3.6.1.3.2).

A summary of the extraction methods is provided below (Table 5.4). Details are available in Section 3.6.5.1. All extracts were filtered through Whatman No. 1 filter paper, reduced to dryness by rotary evaporation using a water bath set at 40°C, then redissolved in a minimal amount of extraction solvent. This solution was then centrifuged in a bench top centrifuge to remove remaining flour particles, reduced to dryness and redissolved in 1 mL HPLC solvent. The extraction efficiencies were calculated as described in Section 3.6.1.3.3.
Table 5.3. Carotenoid extraction procedures examined.

<table>
<thead>
<tr>
<th>Sample Trial</th>
<th>Extraction Methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Flour extraction with 50 mL petroleum ether (40-60°C) in a Soxhlet apparatus at 40°C for 30 minutes (Wagner e. al., 1983. 11). (Section 3.6.5.2) After filtration the sample was redissolved in a minimal amount of diethyl ether (1 mL) and divided into two</td>
</tr>
<tr>
<td>2</td>
<td>Flour was extracted with acetone (40 mL) and petroleum ether (40 mL) with anti-oxidants 0.1 MgCO₃ and ascorbic acid (1 g) in a 500 mL Erlenmeyer flask (Wills, et.al.,1988) (Section 3.6.5.3). The solution was stirred for one hour at room temperature and saponified.</td>
</tr>
<tr>
<td>3</td>
<td>Flour (20 g) was extracted as for Trial 3, however a second extraction was performed after the extracted flour was filtered and air-dried. The flour was re-extracted for 1/2 hour with the same solvents, no anti-oxidants were added. Both filtrates were combined and reduced</td>
</tr>
<tr>
<td>4</td>
<td>Flour (20 g) was extracted with hexane-diethyl ether (70:30, v/v) in an Erlenmeyer flask, for 1 hour at room temperature with stirring (Wright and Shearer, 1984). Section 3.6.5.1.</td>
</tr>
</tbody>
</table>

5.4.2.2 Saponification

Saponification involved dilution of the purified carotenoid extract to 25 mL with diethyl ether and saponification with 25 mL of 20 % potassium hydroxide in methanol (Macindoe, 1975) overnight at room temperature.

5.4.2.3 RP-HPLC method

Samples were analysed by RP-HPLC with a Varian system as described in Section 3.5.1.1. Separation was performed using a C18 column (Section 3.5.1.7) and pre-column (Section 3.5.1.8). Detection was performed at 450
nm, the flow rate kept constant at 0.7 mL.min\(^{-1}\), and the sample volume injected, in all instances, was 50 µL via a 60 µL loop.

Concentration results were calculated (Section 3.6.1.3.2) from duplicate samples from which the mean, standard deviation and RSD were calculated (Section 3.6.1.3.3). Analyses were repeated if the RSD was greater than 5 %.

5.4.3 Results and discussion

Significant differences in the extraction efficiencies of methods trialed were observed (Figure 5.7, trials 1, 2, 3 and 4). Chromatographic profiles became more complex as the extraction efficiency improved, supporting the need for maximum extraction efficiency for total carotenoid characterisation of wheat flour. The lowest carotenoid recovery method (Trial 1) with a rate of 17 % showed the simplest carotenoid profile with the lowest lutein concentration (0.11 µg.ml\(^{-1}\)). The most efficient recovery system was the hexane-diethyl ether extraction method giving an extraction of 100.1 % (Trial 4).
Figure 5.7. RP-HPLC analysis of carotenoid extraction methods. Peaks 1, 2 and 3 represent lutein, β-apo-8’carotenal and β-carotene respectively. Separation was achieved with a RP – C18 guard column and column with an acetonitrile-water (90:1 v/v) and ethyl acetate gradient elution system with compounds detected at 450 nm.
The principle reason for the low extraction efficiencies of some of the methods was thought to be due to poor carotenoid solubility in the solvents used, together with poor percolation of the solvent through the flour with loss of sample through the many stages of the extraction and recovery process. In trial 1, petroleum ether was used which resulted in low compound recovery and little to no extraction of hydrocarbon carotenoids eluting between 35 and 45 minutes. In trial 2, the use of petroleum ether and acetone with stirring of the solvent through the flour greatly increased compound recovery, 39 %. The influence of a second extraction conducted in trial 3 with the same solvent, in conjunction with removal of the saponification step, resulted in a much greater carotenoid recovery rate, 74 %.

The addition of anti-oxidants and a saponification step did not improve carotenoid recovery, trial 2, although an improvement in peak resolution was observed. The chromatographic profile of this sample showed greater peak resolution of the non-polar carotenoids eluting after the internal standard (peak 2). The results to date, do not substantiate whether the addition of anti-oxidants or the saponification of the flour resulted in the improved chromatographic resolution. Further studies using the trial 4, extraction procedures, which compare the effects of anti-oxidants and saponification, must be conducted to confirm their influence.

The superior influence on the solubility of carotenoids with hexane, and diethyl ether was clearly observed. It is believed that improved interface with stirring, carotenoid solubility, in this solvent, and removal of the saponification step influenced extraction efficiency more than any other factor. This coupled with the fact that extraction was conducted at room temperature may have also eliminated any incidence of thermal oxidation. It is important to mention that unlike acetone and petroleum ether, these solvents have not been reported to cause carotenoid oxidation, thus their use instead of oxidative solvents may also have improved compound recovery.
5.4.4 Conclusion

The different extraction methods showed varied extraction efficiencies and different RP-HPLC profiles, this was thought to be consistent with the total concentrations of carotenoids extracted.

Extraction with hexane and diethyl-ether achieved a carotenoid extraction rate of 100% which will enable the quantification and characterisation of these compounds from wheat flour. It is would appear that the improved solvent interface together with carotenoid solubility and the removal of the saponification step markedly improved the extraction efficiency.

Optimisation of the RP-HPLC method resulted in excellent separation of carotenoids enabling both quantitative and qualitative studies.

5.5 THE SEMI QUANTIFICATION OF CAROTENOID COMPOUNDS PRESENT IN THREE DIFFERENT AUSTRALIAN WHEAT VARIETIES

5.5.1 Introduction

This section describes improved carotenoid analysis in wheat using RP-HPLC analysis and semi-quantification with β-apo-8′-carotenal. Traditionally total carotenoid content is calculated using β-carotene equivalents (AACC method 14-50. 1983). Lutein is the predominant pigment in wheat (Markley and Bailey, 1935) not β-carotene and therefore concentrations based on a β-carotene standard can only be considered estimates. Comparison of methods available for the quantification of carotenoids were made
5.5.2 Methods and materials

5.5.2.1 Colour analysis

The colour characteristics of the three flours described in section 3.4.2 were examined using a Minolta chromometer. The lightness, (L), yellowness (b*) and hue or redness (a*) was measured. The flour was transferred to a small petrie dish and lightly packed for analysis, five readings were taken then the mean values calculated.

5.5.2.2 Pigment profile analysis.

Extraction procedure. Flour (20 g) was extracted by stirring at room temperature with hexane and diethyl ether (section 3.6.5.1.1). The solution was filtered and reduced to dryness by rotary evaporation. The samples were re-dissolved in hexane (2 mL) then centrifuged to remove fine flour particles that remained after filtration. After drying the samples under nitrogen, they were dissolved in acetone (5 mL) for analysis.

5.5.2.3 RP-HPLC analysis

Samples were analysed by RP-HPLC with a Varian system as described in Section 3.5.1.1. Separation was performed using a C18 column (Section 3.5.1.7) and pre-column (Section 3.5.1.8). Detection was performed at 450 nm, the flow rate kept constant at 0.7 mL.min\(^{-1}\) and the sample volume injected, in all instances, was 50 µL via a 60 µL loop.

5.5.2.4 Sample elution system

The elution system as described in Section 3.6.1.2 was employed with gradient elution of acetonitrile-water and ethyl acetate.
5.5.2.5 Standard solutions and quantitative evaluation

Quantification was achieved using the relative response factor method (Section 3.6.1.3.1). Three stock solutions were prepared by dissolving 0.29 mg of β-apo-8'-carotenal, 0.32 mg β-carotene and 1 mg of lutein in 10 mL of acetone, for final concentrations of 290, 320 and 1000 µg.mL⁻¹ respectively. HPLC standard solutions were prepared by combining each of the standards at specific concentrations in, 2 mL amber HPLC vials, in acetone (Table 5.2).

Table 5.4. Concentrations of different standard mixtures used to prepare the peak area ratio calibration curves of β-carotene to β-apo-8-Carotenal following the IS method and the calibration curves of β-carotene and lutein following the ES method.

<table>
<thead>
<tr>
<th>Standard</th>
<th>β-apo-8'-carotenal</th>
<th>β-carotene</th>
<th>lutein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration (µg/ml)</td>
<td>Concentration (µg/ml)</td>
<td>Concentration (µg/ml)</td>
</tr>
<tr>
<td>ISCL 1</td>
<td>50</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>ISCL 2</td>
<td>50</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>ISCL 3</td>
<td>50</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>ISCL 4</td>
<td>50</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>ISCL 5</td>
<td>50</td>
<td>25</td>
<td>25</td>
</tr>
</tbody>
</table>

5.5.2.6 Quantification method

The internal standard method of quantification was employed as described in (Section 3.6.1.3.1). HPLC peak area ratios of β-carotene to β-apo-8-carotenal were obtained by plotting the peak area ratio of β-carotene at different concentrations (5, 10, 15, 20 and 25 µg.mL⁻¹) to that of a constant amount of β-apo-8'-carotenal (50 µg.mL⁻¹). A line of best fit was drawn then the total carotenoid content was determined from the slope of the graph, by calculating
the ratio of total peak area in the sample, against the standard peak area in the sample.

A gradient elution system using acetonitrile-water (90:10, v/v.) and ethyl was employed. Separation was performed using a C 18 column and guard column with detection at 450 nm.

The reproducibility of the peak area and retention times of the standards was determined to establish accuracy, by calculation of the coefficient of variation (Section 3.6.1.3.2). Extraction efficiency was calculated from the peak area of the internal standard in the flour extracts, against the average peak area of the internal standard established in the calibration runs.

5.5.3 Results and discussion

To examine the influence of pigment content on Minolta b* values and hence yellow colouration, three wheat samples were assessed. These wheat samples were obtained from NSW Agriculture. A Rosella flour sample was used as a control to examine two wheat samples, JB 2967 and JD 1221 characterised by high yellowness. Average colour values of duplicate samples are displayed below, Table 5.3. The JB 2967 sample exhibited the highest Minolta b* value and the lowest L value reading.

Table 5.5. Colour Characteristics of the wheat flours examined.

<table>
<thead>
<tr>
<th>Sample identification</th>
<th>Minolta colour value</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L</td>
<td>a*</td>
<td>b*</td>
</tr>
<tr>
<td>Rosella (B)</td>
<td>95.73</td>
<td>-0.96</td>
<td>+8.28</td>
</tr>
<tr>
<td>JD 1221</td>
<td>95.07</td>
<td>-1.90</td>
<td>+13.70</td>
</tr>
<tr>
<td>JB 2967</td>
<td>93.90</td>
<td>-2.03</td>
<td>+15.29</td>
</tr>
</tbody>
</table>
The flours of JD 1221 and JB 2967, were visibly yellow and both displayed high Minolta b* value readings.

The calibration curves obtained from a plot of the area ratios of lutein and β-carotene peaks to that of the IS against the concentration of these carotenoids (Figure 5.8 and 5.9) gave good linearity over the range of concentrations.

Figure 5.8. Plot of standard curves for lutein concentration versus peak area ratio of β-apo-8'-carotenal internal standard. Equation $y = 18.574x - 2.6956$.

Figure 5.9. Plot of standard curves for β-carotene concentration versus peak area ratio of β-apo-8'-carotenal internal standard. Equation $y = 64.861x + 1.4421$. 
For the quantification of total carotenoids in wheat the hydrocarbon carotenoid amounts were calculated using β-carotene: they were considered to be those compounds which eluted after 15 minutes. The oxygenated carotenoids, such as lutein, were those that eluted before 13 minutes, this compensated for the different chromophoric activity of the two classes of carotenoids. Differences in chromophoric activity were identified by comparison of the peak areas of lutein and β-carotene prepared at the same concentrations. Lutein displayed a much greater peak area than the β-carotene standard at the same concentration.

An average extraction efficiency of 97% was obtained for the three flour samples. Both standard and sample reproducibility was high (< 5% RSD).

Chromatograms show (Figure 5.10), that the flour extracts contained peaks 1-5, however JB 2967 and JD 1221 displayed two smaller peaks, eluting shortly after peak 5.

Oxygenated and hydrocarbon carotenoids were identified by their elution characteristics. The oxygenated carotenoids eluted from the column first followed by the hydrocarbon carotenoids (Kachick et. al., 1986). The majority of compounds were distinguished by their polarity characteristics as hydrocarbons, they eluted after the internal standard. The first major pigment was identified as the oxygenated carotenoid lutein, (peak 1) by comparison of its retention time with an authentic standard.

In general, the pigment profiles for all the compounds were remarkably similar. β-carotene was present in the samples at a very low concentration eluting just after to peak 3.
Figure 5.10. RP-HPLC profiles of carotenoids extracted from three different flours. Separation was achieved with a RP – C18 guard column and column with an acetonitrile-water (90:1 v/v) and ethyl acetate gradient elution system with compounds detected at 450 nm.
The amount of pigment varied between wheats. The Rosella flour exhibited the lowest concentration of carotenoids, while JB 2967 flour contained the highest concentrations (Figure 5.11). The JB 2967 flour also displayed the highest yellow (b*) value (Table 5.3). Pigment concentration, therefore, influences yellow colouration. This supports the findings of other researchers who established that a high correlation exists between yellow pigment content and Minolta b* values (AWB, 1995-96).

![Figure 5.11. Total carotenoid content of flours examined.](image)

The five major pigments identified were compared (Figure 5.12). JB 2967 flour exhibited the highest concentrations of compound in peaks 1 - 4 and a slightly lower concentration of peak 5. The Rosella flour showed lower pigment concentrations of all peaks. How each individual compound actually influences colour has yet to be determined.
Figure 5.12. Differences in pigment concentrations between three wheat flour varieties.

The concentrations obtained were compared, with values between 2.5 and 2.7 µg.g\(^{-1}\) reported by the Australian Wheat Board, for yellow pigment content of Australian Prime Hard wheats, (AACC 14-50. 1983). These concentrations are much less than those established in this study. As RP-HPLC is a more sensitive method of analysis, however, it is not surprising that quantification using RP-HPLC showed higher concentrations of pigments. As the chromophoric activity of β-carotene is quite different to that of lutein and β-apo-8'-carotenal the use of this standards relative response factor may be resulting in exaggerated concentrations. Before any conclusions can be drawn, further experimentation must be conducted to establish the relationship between the two methods of quantification.

5.5.4 Conclusion

RP-HPLC analysis of carotenoids extracted from three different wheat flours showed five pigments were common to them all. Two of the flours had two additional pigments, which may have been observable simply because of their presence in higher concentration. Of the pigments extracted, lutein was the only xanthophyll with the others being non-oxygenated carotenoids. These results indicate the effectiveness of the extraction and analytical procedures demonstrating the presence of more carotenoids in wheat flour than previously acknowledged.
CHAPTER 6. WHEAT BRAN FLAVONOID EXTRACT PURIFICATION FOR INDIVIDUAL COMPOUND IDENTIFICATION.

6.0 Introduction.

During the RP-HPLC method development stage for the semi-quantification and tentative identification of wheat pigment extracts, it was established that the flavonoid extracts from wheat are complex. The extract of Rosella wheat bran has displayed numerous co-eluting compounds and several major pigments. For the conclusive identification of these major compounds, structural identification methods must be applied. For this to occur, the major pigments will need to be separated from the mixture of compounds.

In this chapter, a number of different methods were employed to identify a process that would enable the separation of the major pigments and the concentration of these compound for mass spectral analysis. The first approach involved examination of the open column purification method devised by Feng and his colleagues (1988). Other methods included thin layer chromatography to identify a development system that could separate the compounds using different solvent systems and adsorbents. Sample purification success was examined by HPLC, to assist in establishing if individual compounds could be obtained. Finally, an automated sample collection system was examined that enabled the collection of compounds after separation by HPLC.

6.1 EXAMINATION OF OPEN COLUMN PURIFICATION.

6.1.1 Introduction

The following experiments were conducted to establish the benefit of open column purification for the separation of flavonoid compounds in wheat from other water soluble components. Open column purification of Rosella wheat bran extracts was conducted using Amberlite XAD-2 resin and Sephadex G-15. Purified and un-purified samples were compared. As solvent was eluted
from this column, it was collected in 2 mL fractions. To assist in establishing
the abundance of phenolic and flavonoid compounds that were eluting from
the column UV-visible absorbance was recorded at 254 nm. This also
assisted in helping to gain an understanding of when the majority of flavonoid
compounds eluted and with what solvent.

RP-HPLC analysis of the fractions was recorded to compare the purified
fraction with the purified fraction and to understand, at what stage of the
solution collection process, the majority of flavonoid compounds were eluted.
Of particular interest was the collection of compounds eluting at approximately
18 and 22 minutes, thought to be ferulic acid and apigenin compounds
respectively.

6.1.2 Method and materials

6.1.2.1 Sample extraction, purification and collection

Bran from Rosella wheat (Section 3.4.1) was extracted as a higher
concentration of flavonoids is contained in bran. This could facilitate the
collection of an increased concentration of individual compounds. To remove
non-phenolic compounds from the flavonoid extract, open column size
exclusion chromatography was employed using Amberlite XAD-2 and
Sephadex G-15 resin as described in Section 3.6.4.1.

To gain an understanding of the type and relative concentrations of
compounds eluting from the column 2 mL fractions were collected into HPLC
vials with the aid of tubing attached to the end of the column.

Colour remained on the column after washing with water so to facilitate
pigment collection, the column was eluted with 10 ml methanol with the elate
collected (from vial 29 onward). This ran until vial 36. To allow collection of
all pigment, the column was eluted with 10 ml 0.2 M NaOH prior to the
collection of the elute.
In addition to the samples collected after elution from the G-15 column, an unpurified methanol extract, a XAD-2 purified extract and another different XAD-2 purified extract were analysed then compared.

6.1.2.3 UV-visible spectroscopy analysis

Samples were analysed at 254 nm with a Cary 1E spectrophotometer (Section 3.3.2) to establish at which stage the flavonoids were eluting from the G-15 column. This wavelength was selected to distinguish flavonoids from phenolic compounds.

6.1.2.4 RP-HPLC analysis

The collected fractions were examined by RP-HPLC following the formic acid - water and methanol gradient elution system Section 3.6.1. Identification was conducted at 280 nm using a UV-visible and a photodiode array detector (Section 3.5.1.2) to obtain spectra between 190-367 nm. Flow rate 0.7 mL.min⁻¹.

6.1.3 Results and discussion

The sample extract after Soxhlet extraction with methanol and concentration was a dark brown viscous solution. After application onto the Amberlite column, colour could be observed at the top of the resin, after washing of the column with water, light brown orange and yellow colour descended down through the column resin (Figure 6.1).
Figure 6.1. Amberlite XAD-2 open column purification of methanol extracted Rosella bran.

With the washing of the non-flavonoid and phenolic compounds with water the column eluate from 0-100 mL was a pale cloudy yellow but after 100 mL became clear. The flavonoid and phenolic compounds were collected after elution from the column with methanol and where observed as a yellow eluate.

Comparison of the unpurified methanol extract with the purified methanol eluate from the columns showed a much simpler HPLC compound profile (Figure 6.2). Further comparison of these samples with the NaOH extract highlights that different compounds do elute with different elution solvents. Common to all fractions collected were compounds between 13.92 and 27 minutes. The unpurified extract exhibited two major groups of compounds between 2- 8 minutes and 13-22 minutes (Figure 6.3). Major peaks were identified at 3.35 and 16.57 minutes with a very broad peak at 18.57 minutes. The XAD-2 methanol extract also contained a multitude of peaks but these compounds were better resolved with improved baseline to baseline
separation although co-elution of compounds was still common to all samples (Figure 6.4). The sample elute from 0.2 M NaOH exhibited compounds between 15 and 27 minutes with two major peaks at 22.34 and 24.01 minutes (Figure 6.5).

Figure 6.2. Chromatographic profile comparison of methanol extract before purification on XAD-2 (Blue), after purification on XAD-2 (red) and of the 0.2 NaOH eluate (black). Separation was achieved with a C18 guard column and column with a formic acid - water and methanol gradient elution system at 0.7 ml.min\(^{-1}\). Identification was conducted at 280 nm using a UV-visible and a photodiode array detector.
Figure 6.3. Unpurified methanol extract. Separation was achieved with a C18 guard column and column with a formic acid - water and methanol gradient elution system at 0.7 mL.min\(^{-1}\). Identification was conducted at 280 nm using a UV-visible and a photodiode array detector.
Figure 6.4. Amberlite XAD-2 purified methanol extract. Separation was achieved with a C18 guard column and column with a formic acid - water and methanol gradient elution system at 0.7 mL.min⁻¹. Identification was conducted at 280 nm using a UV-visible and a photodiode array detector.
Figure 6.5. NaOH elute from the Amberlite XAD-2 purification of the methanol extract. Separation was achieved with a C18 guard column and column with a formic acid - water and methanol gradient elution system at 0.7 mL.min\(^{-1}\). Identification was conducted at 280 nm using a UV-visible and a photodiode array detector.

From this comparison it appears that purification on XAD-2 is more effective as chromatographic quality was improved.

The impact of open column purification on Sephadex G-15 can be observed by examination of the UV-visible absorbency profiles of the different fractions collected (Figure 6.6).
Figure 6.6 UV-visible profile of G-15 elutes collected

High absorbency readings were observed from 2 to 22 mL of elute, with the majority of compounds washing from the column by 44 mL. A reduction in flavonoids eluting from the column was evident from 22 mL onward, as shown by an absorbency reading of analysed fractions of approximately 0.2 nm. After 60 mL of samples were collected with elution by water, methanol was used to remove retained flavonoids. Compounds with a high absorbance at 254 nm were again observed and continued to elute with the application of NaOH, used to ensure removal of all compounds. Collection of eluate after 100 mL was not conducted but it is evident from the UV-visible profile of the eluates that further collection of flavonoids could have been done, as high absorbance readings at 70 mL were recorded. The absorbency of compounds gradually decreased from 26-40 mL, until the column was eluted with methanol and 0.2 M NaOH, which promoted a sharp increase in absorbency from 58mL and 88 mL. A yellow colour remained on the G-15 column despite application of methanol and NaOH.
Similar HPLC profiles of elutes 2 to 30 mL were observed. Figure 6.7 shows a representation of sample eluted from 2 to 14 mL. The primary differences observed were in the peak area counts of the compounds. For example, at 6 mL, a major peak was identified at 6.3 minutes with an area count of 94662. A compound with a similar retention time was observed in the 12 mL eluate with a peak area of 88466. The 2 mL water eluates were characterized by closely eluting peaks starting from 11 minutes and ending at approximately 22 minutes. (Figure 6.8). Other major peaks occurred at 17.7 (peak area 327510) and 18.25 minutes (peak area 446421). Similar retention times were observed within the sample elute of the 12 mL elute (Figure 6.9) although a greater peak area of compounds at 17 and 18 minutes of 942345 and 335218 was observed. The major peak profile changed as the compounds eluted from the column. The majority of flavonoid and phenolic compounds appeared to have been eluted from the column with water by 26 mL. Collection of flavonoids can be achieved from 0-26 mL by elution with water.

![Figure 6.7](image)

Figure 6.7 Representation of the change in the methanol extract elution profile as samples elute from a Sephadex G-15 column. Elutes 2 to 14 mL from near to far.
Figure 6.8. Sephadex G-15 water eluate at 6 mL. Separation was achieved with a C18 guard column and column with a formic acid - water and methanol gradient elution system at 0.7 mL.min⁻¹. Identification was conducted at 280 nm using a UV-visible and a photodiode array detector.
Figure 6.9. Sephadex G-15 water eluate at 12 mL. Separation was achieved with a C18 guard column and column with a formic acid - water and methanol gradient elution system at 0.7 mL.min⁻¹. Identification was conducted at 280 nm using a UV-visible and a photodiode array detector.

Elution of the column with methanol altered the composition of the sample with an increase in less polar compounds, characterized by a longer retention time (Figure 6.10). Compounds eluted between 12-30 minutes, two major peaks at 25.45 and 25.92 minutes were observed. The methanol elutes showed a large poorly resolved broad band at 14 minutes. Compounds eluted after 29 minutes and it is expected that more would have eluted form the
column, if the run time had been increased. This is not surprising, as the methanol washing of the column would have removed less polar compounds from the column resin, which were not able to be solubilised in the water, elute.

Fig 6.10. Sephadex G-15 methanol eluate. Compounds were separation using a C18 guard column and column with a formic acid - water and methanol gradient elution system at 0.7 mL.min⁻¹. Identification was conducted at 280 nm using a UV-visible and a photodiode array detector.
6.1.4 Conclusion

Sample purification on XAD-2 improved compound detection. The profiles of XAD-2 purified samples when compared to G-15 water elutes were very similar. The use of Sephadex G-15 did not facilitate the simplification of compound profiles. As the concentration of the flavonoids decreased, observed in samples eluted after 20 mL, the chromatographic quantity increased. The use of methanol to elute compounds from the G-15 column resulted in the elution of less polar compounds with longer retention times.

6.2 THIN LAYER CHROMATOGRAPHIC SEPARATION OF FLAVONOID COMPOUNDS

6.2.1 Introduction

Thin layer chromatography has been used for the rapid separation of flavonoid compound from plant extracts. A summary of the many different extraction solvents and plate resins is available in Table 6.1. This section of research focused on identifying a TLC method that would assist in the separation of the complex flavonoid compounds identified in wheat, for the purification of individual wheat flavonoids, for mass spectral analysis. A separation method that would facilitate the isolation of standards from a test solution and clear separation of a sample extract was sought with no banding or streaking.

The different development solvents can also be used to characterize the flavonoids and help to distinguish between flavonoid glycosides and aglycones.
Table 6.1. TLC methods for the separation of flavonoids.

<table>
<thead>
<tr>
<th>DEVELOPING SOLVENTS</th>
<th>TLC PLATE TYPE</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform: hexane (1:1, v/v)</td>
<td>Silica</td>
<td>Feng et al., 1988</td>
</tr>
<tr>
<td>Ethyl acetate: ethyl methyl ketone: formic acid :</td>
<td>Silica</td>
<td>Santos et al.,</td>
</tr>
<tr>
<td>water (5:3:1:1, v/v/)</td>
<td></td>
<td>1995</td>
</tr>
<tr>
<td>Ethyl acetate; petroleum ether (3:1, v/v)</td>
<td>Silica</td>
<td>Markam, 1982</td>
</tr>
<tr>
<td>Methanol: acetic acid: water (9:1:1, v/v)</td>
<td>Polyamide</td>
<td>Markam, 1982</td>
</tr>
<tr>
<td>Chloroform: methanol (15:1, v/v)</td>
<td>Silica</td>
<td>Markam, 1982</td>
</tr>
<tr>
<td>Ethyl acetate; pyridine: water: methanol</td>
<td>Silica</td>
<td>Markam, 1982</td>
</tr>
<tr>
<td>(80:20:10:5, v/v)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chloroform: acetic acid: water (50:45:5, v/v)</td>
<td>Silica</td>
<td>Blouin, 1981</td>
</tr>
<tr>
<td>Ethyl acetate: formic acid: water (66:14:20, v/v)</td>
<td>Silica</td>
<td>Feng et al., 1988</td>
</tr>
<tr>
<td>Ethanol: formic acid: water</td>
<td>Silica</td>
<td>Feng et al., 1988</td>
</tr>
<tr>
<td>Methanol: water (3:2, v/v)</td>
<td>Silica</td>
<td>Markam, 1982</td>
</tr>
<tr>
<td>Isopropyl alcohol: water (3:2, v/v)</td>
<td>Silica</td>
<td>Markam, 1982</td>
</tr>
<tr>
<td>n-butanol: acetic acid: water (4:1:5, v/v)</td>
<td>Silica</td>
<td>Wagner et al., 1983</td>
</tr>
</tbody>
</table>

2 dimensional chromatography

(B) 1st dimension n-butanol: acetic acid:water (4:1:5, v/v upper phase) 2nd dimension 15% acetic acid.

Cellulose Blouin, 1981
6.2.2 Method and materials

The TLC methods applied are summarised in Table 6.1 were trialed.

Alkaline extracted bran samples were used to test the different TLC methods. The extraction and preparation methods are described in Section 3.6.3.2. No compound purification for the removal of non-phenolic compounds was conducted.

6.2.3 Results and discussion

The majority of methods employed did not facilitate good clear separation of compounds. Streaking of compounds up the plate was a common indicative of the complexity of the flavonoid and phenolic extracts as previously identified. Differences in compound types could be observed with differences in colour florescence under UV light at 254 and 365 nm, in some instances. A summary of the methods trialed and their success for separation is outlined in Table 6.2.

During assessment, it was observed that the bran extract contained compounds different to the standards rutin and apigenin aglycone. Compounds showed similar Rf values and colours under UV light to ferulic acid and chlorogenic acid standards.
Table 6.2. Efficacy of TLC in the separation of Rosella wheat flavonoids and phenolic compounds.

<table>
<thead>
<tr>
<th>DEVELOPING SOLVENTS</th>
<th>Plate type</th>
<th>Separation</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform: hexane (1:1, v/v)</td>
<td>Silica</td>
<td>Yes</td>
<td>Some</td>
</tr>
<tr>
<td>Ethyl acetate: methyl ethyl ketone: formic acid : water (5:3:1:1, v/v)</td>
<td>Silica</td>
<td>No</td>
<td>Streaking</td>
</tr>
<tr>
<td>Ethyl acetate: petroleum ether (3:1, v:v:0)</td>
<td>Silica</td>
<td>No</td>
<td>Streaking. No clearly different compounds</td>
</tr>
<tr>
<td>Methanol: acetic acid: water (9: 1: 1, v/v)</td>
<td>Polyamide</td>
<td>No</td>
<td>Compounds remained at application point</td>
</tr>
<tr>
<td>Chloroform: ethanol: butane-2-one (12:2: 1, v/v)</td>
<td>Polyamide</td>
<td>No</td>
<td>Compounds remained at application point</td>
</tr>
<tr>
<td>Chloroform: methanol: butanol (12:2:1, v/v)</td>
<td>Polyamide</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Chloroform: methanol (15:1, v/v)</td>
<td>Silica</td>
<td>Yes</td>
<td>4 bands identified</td>
</tr>
<tr>
<td>Ethyl acetate: pyridine: water: methanol (80:20:10:5, v/v)</td>
<td>Silica</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Chloroform: acetic acid: water (50:45:5, v/v)</td>
<td>Silica</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>n-butanol: acetic acid: water (40:10:50, v/v)</td>
<td>Silica</td>
<td>Some</td>
<td>Yes some mostly streaking</td>
</tr>
<tr>
<td>Ethanol: formic acid: water</td>
<td>Silica</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Methanol : water (3:2, v/v)</td>
<td>Polyamide</td>
<td>No</td>
<td>Streaking</td>
</tr>
<tr>
<td>Isopropyl alcohol: water (3:2, v/v)</td>
<td>Polyamide</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td><strong>2 dimensional chromatography</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st dimension n-butanol: acetic acid:water (4:1:5, v/v), 2nd dimension</td>
<td>Cellulose</td>
<td>Yes</td>
<td>Clearly distinguished compounds</td>
</tr>
<tr>
<td>15 % acetic acid:water</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
By far the most successful method was the two-dimensional method employing the upper phase of n-butanol: acetic acid: water (4:1:5, v/v) in the first dimension and 15% acetic acid in water in the second (Figure 6.11).

Depiction of TLC separation

Figure 6.11. Diagrammatic representation of the separation of a bran flavonoid extract by two-dimensional TLC and their colours under UV light at 365 nm.

A number of different compounds were observed under UV light at 365 nm. This method facilitated the greatest number and the clearest separation of compounds and warranted further investigation.

6.2.4 Conclusion

A number of different solvents and combinations of solvents were examined, in conjunction with different polarity plates, to identify a method that could separate individual compounds within the wheat extracts, or at the very least separate groups of compounds. In most instances, flavonoid separation using one-dimensional TLC was not achieved as compound streaking up the plate was frequently observed. Compounds were partially separated using
chloroform and methanol (15:1, v/v) on silica plates and with n-butanol:acetic acid: water (40:10:50 v/v).

By far the most successful method involved separation using two-dimensional methods. Distinct groups of compounds were separated using n-butanol:acetic acid: water (4:1:5 v/v) in the first dimension, then after drying of the plate, further compound separation with 15 % aqueous acetic acid in the second dimension.

6.3 TWO DIMENSIONAL CHROMATOGRAPHY

6.3.1 Introduction

Two dimensional TLC was further investigated and applied owing to the success observed in separating flavonoid compounds identified in Section 6.2, using n-butanol: acetic acid: water, in the first dimension on cellulose, and 15 % acetic acid in the second dimension. Using this method, seven different compound groups were clearly separated from each other. The purity of these seven compounds was established by collection of the separated compounds and characterisation by RP-HPLC.

6.3.2 Method and materials

6.3.2.1 Pigment extraction

10 g of Rosella bran (3.4.1) was defatted in a 250 mL round bottomed flask with 50 ml light petroleum ether (40-60°C) for ½ an hour at 40°C following an adaptation of a method described by Wagner et al., (1983). Boiling chips were added to the flask that was heated on an electromantle. The solution was filtered through a Büchner funnel with No. 1 Whatman filter paper. Flavonoids were then extracted from the dried flour with 50 mL methanol for 10 minutes at 100°C. The solution is filtered from the flour as before and rotary
evaporated to dryness, at 70°C under vacuum. The sample was redissolved in 1 mL methanol for TLC separation.

6.3.2.2 TLC

The sample solution (30 µl aliquot) was applied to the left hand corner of the TLC plate. The plates were developed in a glass TLC tank in a multi-plate holder. Prior to insertion of plates, the TLC tank atmosphere was equilibrated for ½ hour with the developing solvent. Enough solvent was added to enable plates to stand in approximately 8 mm of solution. The developing solvent was using n-butanol: acetic acid: water (4:1:5, v/v) in the first dimension on cellulose, and 15 % acetic acid in water in the second dimension.

6.3.2.3 Sample scale up and collection

To facilitate the collection of enough samples, 14 plates were run of the bran extract. Separated samples were scrapped from the TLC plate and transferred to McCartney bottles, once the separated compounds had been identified under UV light at 254 and 365 nm. Similar compounds with similar Rf values and positions and colours were combined. The sample was separated from the cellulose resin by washing with methanol until no visible colour remained on the resin. The solution was transferred to HPLC vials and reduced under nitrogen, with heating on an elecromantle at 100°C, to dryness.

6.3.2.4 RP- HPLC analysis of samples

A Varian system (Section 3.3.1.1) with a C18 column (Section 3.3.1.6) and guard column (Section 3.3.1.8) was used to separate the compounds following the formic acid methanol gradient elution system. 50 µl volumes of sample were injected onto the column.
6.3.3. Results and discussion.

Two dimensional TLC of methanol extracted Rosella bran resulted in the observation of six different compounds (Figure 6.12).

![Figure 6.12. Representation of a two-dimensional TLC separated bran extract.](image)

Collection of the individual spots and analysis by RP-HPLC showed that each sample collected contained a number of different compounds (Figure 6.12). Two-dimensional chromatography despite providing clearly separated spots, did not facilitate the separation of individual compounds.

Differences in the pigment profiles and in the elution time of compounds for each of the different samples were observed. In sample 1, most of the compounds eluted from 14 minutes with major pigments observed at 20.3, 22.2, 22.7, 24.3 and 29.3 minutes (Figure 6.13). Sample 2 displayed improved
separation of compounds, with baseline-to-baseline separation of compounds. A major pigment identified at 9.12 minutes (Figure 6.14).

Despite the clear differences in colour under UV-light between samples 2 and 3 (Figure 6.15), similar RP-HPLC profiles were observed. Predominate compounds at 9.12 minutes, 12.1, 22.3, 22.8 and 29.3 minutes were detected. Sample 6 displayed compounds from 14 minutes (Figure 6.17), similar in profile to sample 1. Samples 4 and 5 displayed major compounds at 22.2, 22.7, 24.5, 29.3 and 31.7 minutes.
Figure 6.13. RP-HPLC chromatogram of TLC separated compound 1. Compounds were separated with a C18 guard column and column with a formic acid - water and methanol gradient elution system at 0.7 mL.min\(^{-1}\). Detection with UV-visible spectroscopy at 280 nm.
Figure 6.14. RP-HPLC chromatogram of TLC separated compound 2. Compounds were separated with a C18 guard column and column with a formic acid - water and methanol gradient elution system at 0.7 mL.min⁻¹. Detection with UV-visible spectroscopy at 280 nm.
Figure 6.15. RP-HPLC chromatogram of TLC separated compound 3. Compounds were separated with a C18 guard column and column with a formic acid - water and methanol gradient elution system at 0.7 mL.min\(^{-1}\). Detection with UV-visible spectroscopy at 280 nm.
Figure 6.16. RP-HPLC chromatogram of TLC separated compound 4. Compounds were separated with a C18 guard column and column with a formic acid - water and methanol gradient elution system at 0.7 mL.min⁻¹. Detection with UV-visible spectroscopy at 280 nm.
Figure 6.17. RP-HPLC chromatogram of TLC separated compound 5. Compounds were separated with a C18 guard column and column with a formic acid - water and methanol gradient elution system at 0.7 mL.min⁻¹. Detection with UV-visible spectroscopy at 280 nm.
Figure 6.18. RP-HPLC chromatogram of TLC separated compound 6. Compounds were separated with a C18 guard column and column with a formic acid - water and methanol gradient elution system at 0.7 mL.min⁻¹. Detection with UV-visible spectroscopy at 280 nm.
6.3.4 Conclusion

Whilst good TLC separation of compounds in the wheat bran extract was achieved, it was established that within these samples, a number of different compounds were identified. TLC separation did not facilitate the simplification of the wheat bran extract or enable individual sample separation.

6.4 FRACTION COLLECTION PURIFICATION OF WHEAT BRAN EXTRACTS

6.4.1 Introduction

In Sections 6.1 to 6.3 traditional methods for the purification of plant extracts were employed to assist in the simplification of the wheat bran flavonoid fractions. Neither open column chromatography nor TLC chromatography assisted in the attainment of individual wheat bran extract compounds, although open column purification did improve HPLC chromatographic quality for observation of pigments.

To separate and collect the major compounds identified in wheat bran, a Foxy Junior Fraction Collector connected to a Varian HPLC chromatographic system was employed. The following experiments were designed to find the optimum fraction collection program for the separation of the major flavonoid compounds.

6.4.2 Methods and materials

6.4.2.1 Sample preparation

An alkaline bran extracted sample was used in this experiment obtained as described in Section 3.6.3.1 using trial wheat Section 3.4.1.
6.4.2.2 RP- HPLC analysis of samples

A Varian system (Section 3.5.1.1) with a C18 column (Section 3.5.1.6) and guard column (Section 3.5.1.8) was used to separate the compounds following the formic acid methanol gradient elution system. 50 µl volumes of sample were injected onto the column.

6.4.2.3 Post column fraction collection

A Varian Foxy Junior automated fraction collector was used to collect separated compounds eluted from the HPLC column (Section 3.5.1.9). Samples were collected after detection. A number of different collection programs were investigated, as outlined in more detail below. After fraction collection, the success of the different programs in separating out individual compounds was examined by HPLC. Prior to HPLC analysis, as the chromatographic solution was predominately water elute, the samples collected were concentrated by freeze-drying. A number of different fraction collection programs were trialed to develop a method for the separation of individual flavonoid compounds.

6.4.2.3.1 Method 1

Fraction by time – 30 seconds
flow delay 0.0
peak threshold 5 %
Peak width 30 sec
Window 1 start 13.00 - end 15.00
2 start -18.00 end 23.00
3 start 1.30.00 end 1.40.00
Non peak window drain
Rack 12/13 mm test tubes
last tube 18
injection action overlay
6.4.2.3.2 Method 2

Fraction by drops - 30 seconds
Flow delay 30 seconds
Peak threshold none
Peak width 30 seconds
Window 1) 13.00-14.00 min
   2) 19.00-23.00 min
   3) 1:30:00-1:40:00 min
Non peak drain
50 test tubes
Injection action overlay

6.4.2.3.3 Method 3

Fraction by drops - 40 drops
Flow delay 0.05 seconds
Peak threshold none
Slope detection
Window 1) 13.00-14.00 min
   2) 19.00-23.00 min
   3) 1:30:00-1:40:00 minutes
Non peak drain
50 test tubes
Injection action overlay

6.4.2.3.4 Method 4

Fraction by time - 30 seconds
Flow delay 0.45
Percentage peak threshold 1 %
Peak width 15 seconds
Window 1) 15.00-23.00 min
   2) 1:30:00-1:40:00 minutes
58 test tubes
Injection action overlay

6.4.2.3.5 Method 5

Fraction by time - 30 seconds
Flow delay 0.45
Percentage peak threshold 1%
Peak width 15 seconds
Time windows 1) 14.00-15.00 min
              2) 16.30-17.00 min
              3) 19.20-21.00 min
              4) 21.00-21.40 min
              5) 1:30:00-1:40:00 minutes

58 test tubes
Injection action overlay

6.4.2.3.6 Method 6

Fraction by time - 30 seconds
Flow delay 0.45
Peak threshold none
Peak width no slope detection
Time window 1) 14.00-15.00 min
              2) 16.30-17.00 min
              3) 19.20-21.00 min
              4) 21.00-21.40 min
              50 1:30:00-1:40:00 MINUTES

6 bottles
Injection action overlay
6.4.2.3.7 Method 7

Fraction by drops – 40 drops
Flow delay 0.45
Peak threshold 5 %
Peak width, no slope detection
Time windows 1) 19.00-24.00 min
2) 1:30:00-1:40:00 minutes
Non peaks drain
9 flasks
injection action overlay

6.4.3 Results and discussion

Program 6.4.2.3.1. Following this time collection program, nine different fractions were collected and analysed. In each of the fractions collected, two or more compounds were observed. Each sample showed the presence of two or more peaks at 19.9, 20.5 and 22 minutes.

To improve separation, in order to facilitate the collection of individual compounds, Program 6.4.2.3.2 was run with a focus on collection of samples by drops. The complexity of the bran extract was again reduced, as observed by comparison of the sample prior to fractionation (Figure 6.19) with the collected fractions 1 and 2 (Figures 6.20 and 6.21 respectively). Fraction 1 showed one peak at 19.8 minutes but contained many minor peaks before 11 minutes. Fraction 2 showed a peak at 19.9, 20.4 and 21 minutes suggesting that the peaks were spliced with many peaks, again observed before 11 minutes and after 24 minutes.
Figure 6.19. Alkaline bran extract. Compounds were separated on a C18 column using a gradient elution system of formic acid – water and methanol at 0.7 mL.min$^{-1}$. Detection with UV-visible spectroscopy at 280 nm.
Figure 6.20 Alkaline bran extract fraction 1. Compounds were separated on a C18 column using a gradient elution system of formic acid –water and methanol at 0.7 mL.min$^{-1}$. Detection with UV-visible spectroscopy at 280 nm.
To limit the collection of compounds before 11 minutes, so as to capture the major compounds at 12 minutes and between 19 and 23 minutes, program 6.4.2.3.3. was employed. Seven different fractions were collected. All fractions displayed compounds at 2 and 4 minutes with collected fractions 1 showing a peak at 20.3 minutes. Fraction 2 showed peaks at 21, 24 and 28 minute, the target compounds at higher peak areas (Figure 6.22). The chromatogram of the third fraction was further complicated by numerous compound detected before 12 minutes (Figure 6.23). Whilst simplified chromatograms were observed, the objective was not achieved using this method.
Figure 6.22. Fraction collection by drops with compound detected and collected between 13-14 minutes and 19-23 minutes, flask 1. Compounds were separated on a C18 column using a gradient elution system of formic acid–water and methanol at 0.7 mL.min⁻¹. Detection with UV-visible spectroscopy at 280 nm.
Figure 6.23. Fraction collection by drops with compound detected and collected between 13-14 minutes and 19-23 minutes, flask 2. Compounds were separated on a C18 column using a gradient elution system of formic acid –water and methanol at 0.7 mL.min\(^{-1}\). Detection with UV-visible spectroscopy at 280 nm.

Further optimisation of the fraction collection methods was conducted in Program 6.4.2.3.4. Collection of compounds above a 1 % peak threshold was employed. Analysis of the samples collected showed peaks at 18.9, 19.2 and 19.8 minutes with a major peak being at 19.2 minutes. Compounds eluting before 12 minutes were successfully eliminated with focus on the peak detection window altered to 15 – 23 minutes.
The challenge remained to obtain a single compound in the collected fractions. The collection window times were modified as described in Program 6.4.2.3.5 to improve compound separation. Despite the increase in collection times, the capture of individual compounds was not achieved.

Removal of slope detection from the collection program (6.4.2.3.7) with continued implementation of many collection windows resulted in the successful separation of a single compound, at a retention time of 19.7 minutes. More than one compound was identified in the other fractions collected, with compounds identified at 19, 20 and 21 minutes. This method was repeated to confirm success. Unfortunately, a single compound at 19 minutes could not be achieved upon duplication.

6.4.4 Conclusion

Despite numerous attempts to develop a fraction collection program that could consistently separate and collect individual compounds, it was not achieved. This is a reflection again of the incredible similarity of the numerous flavonoid and phenolic compounds found in the wheat bran extracts.
CHAPTER 7. CHARACTERISATION AND QUANTIFICATION OF FLAVONOID AND PHENOLIC COMPOUNDS IN AUSTRALIAN WHEAT

7.0 Introduction

The following chapter focuses on the semi quantification and characterisation of wheat phenolic and flavonoid compounds. Firstly wheat flour and bran characteristics were examined to obtain an understanding of differences between wheat grain portions. The characteristics of the major pigments identified in Rosella wheat bran were examined in detail and compounds separated by RP-HPLC were tentatively identified by examination of their UV visible profiles using PAD and comparison with standard compound. A number of different wheat bran extracts were examined. Finally, the relative concentration of flavonoid pigments in 17 different wheat varieties are reported and compared and details of the major pigment identity provided and compared.

7.1. COMPARISON OF ROSELLA FLOUR AND BRAN

7.1.1. Introduction

Much of the focus in previous experiments has been on method development for flavonoid characterisation. To obtain the highest concentration of flavonoid compounds bran has been used in preference to flour. As the flour of wheat is used for commercial applications including noodle production, characterisation and quantification of wheat flour is more relevant. The extracts of wheat flour and bran were compared.

The following experiment was conducted to examine both the flavonoid compounds extracted using a small-scale alkaline water method and to examine the differences in flavonoid profiles of bran and flour.
7.1.2 Method and materials

7.1.2.1 Flavonoid extraction

Rosella flour and bran (Section 3.4.1) was extracted following the adapted methodology developed by (Feng and McDonald, 1989), (Section 3.6.3.1).

7.1.2.2 Sample purification

The extracts were purified using an Amberlite Xad-2 column, which was prepared by addition of enough Amberlite to fill a 2.8 X 7 cm column. The Amberlite was added dry to the column and conditioned by eluting with 300 mL dionised water (modification of method Feng et al, 1988). The sample was applied to the column then was washed with 300 mL water followed by 10 mL methanol. The sample was eluted with 50 mL methanol. After elution from the column, the solution was reduced under rotary evaporation to approximately 10 mL. Samples were either reduced by rotary evaporation to dryness or by evaporation on a heating mantle (60° C) depending on the amount of water in solution. Once reduced samples were redissolved in methanol for transferral to HPLC vials.

7.1.2.3 Sample analysis

A Varian system (Section 3.5.1.1) with a C18 column (Section 3.5.1.6) and guard column (Section 3.5.1.8) was used to separate the compounds. A gradient elution system of formic acid-water (5:95, v/v) adjusted to pH 3 with 10 M NaOH (solvent A) and methanol (solvent B), under the following linear gradient conditions was employed:
Table 7.1. Flavonoid separation gradient elution method

<table>
<thead>
<tr>
<th>Time (minute)</th>
<th>Solvent A %</th>
<th>Solvent B %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>93</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>93</td>
<td>7</td>
</tr>
<tr>
<td>8</td>
<td>85</td>
<td>15</td>
</tr>
<tr>
<td>25</td>
<td>25</td>
<td>85</td>
</tr>
<tr>
<td>27</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>40</td>
<td>20</td>
<td>80</td>
</tr>
</tbody>
</table>

The flow rate was set at 0.7 mL.min\(^{-1}\) with UV-visible detection at 280. Samples were injected via a 50 µL at 60 µL injection volumes.

7.1.3 Results and discussion

The solution obtained from the first bran extraction was a strong white wine yellow colour indicative of flavonoids. The flour extract was a cloudy white colour containing particles in suspension.

Comparable results for duplicates were not obtained indicative of the variability of the extraction and reduction method used (Figure 7.1 bran a and b and flour c and d). Higher concentrations of sample were obtained for the bran duplicate (Figure 7.1, b) and for flour duplicate (Figure 7.1, d). Semi quantification of compounds was not conducted due to the variability in results. For quantification the sample reduction method needs to be refined.
Figure 7.1. Rosella wheat bran A and B, wheat flour C and D. Compounds were separated on a C18 column using a gradient elution system of formic acid – water and methanol at 0.7 mL.min\(^{-1}\). Detection with UV-visible spectroscopy at 280 nm.
The lower concentration of sample obtained for the flour and bran extracts (Figure 7.1 a and c) enable more defined chromatographic separation of compounds and facilitated comparison between the flour and bran extracts.

In the bran extract, major peaks were observed at 9.9, 15.6, 16.8, 20.8, 21.63 and 29.51 minutes. For the flour extract compound profiles were slightly different to that of the bran samples. No peak was observed at 9.9 minutes. A major peak was observed at a similar retention time to the bran extract at 16 minutes. In the flour extract this was observed as a split peak. This may be indicative of the 2 forms of apigenin thought to be within wheat (Feng et al, 1988), which could not be separated from each other using this chromatographic system. Similar chromatographic profiles were observed for the flour and bran extract between 19 and 26 minutes. The flour sample displayed a greater abundance of compounds eluting from 26 minutes with poorly resolved compounds also detected at 35 and 38 minutes in the flour extract.

A summary of the similarities and differences is available in Table 7.2 comparing sample retention times.
Table 7.2 Peak retention time comparisons of bran and flour samples A and C.

<table>
<thead>
<tr>
<th>Retention time (minutes)</th>
<th>Flour</th>
<th>Bran</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>9.96</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>11.8</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>12.69</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>12.99</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>13.15</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>13.16</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>13.91</td>
<td></td>
</tr>
<tr>
<td>14.25</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>14.48</td>
<td>14.58</td>
<td></td>
</tr>
<tr>
<td>15.47</td>
<td>15.34</td>
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<tr>
<td>15.64</td>
<td>15.64</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>16.25</td>
<td></td>
</tr>
<tr>
<td>16.60</td>
<td>16.51</td>
<td></td>
</tr>
<tr>
<td>16.75</td>
<td>16.81</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>17.32</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>17.67</td>
<td></td>
</tr>
<tr>
<td>18.07</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>18.84</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>19.70</td>
<td>19.77</td>
<td></td>
</tr>
<tr>
<td>20.35</td>
<td>20.35</td>
<td></td>
</tr>
<tr>
<td>20.75</td>
<td>20.80</td>
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<table>
<thead>
<tr>
<th>Retention time (minutes)</th>
<th>Flour</th>
<th>Bran</th>
</tr>
</thead>
<tbody>
<tr>
<td>21.21</td>
<td>21.21</td>
<td></td>
</tr>
<tr>
<td>21.58</td>
<td>21.63</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>22.05</td>
<td></td>
</tr>
<tr>
<td>22.4</td>
<td>22.37</td>
<td></td>
</tr>
<tr>
<td>22.87</td>
<td>22.97</td>
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</tr>
<tr>
<td>23.32</td>
<td>23.34</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>23.65</td>
<td></td>
</tr>
<tr>
<td>23.92</td>
<td>23.95</td>
<td></td>
</tr>
<tr>
<td>24.30</td>
<td>24.36</td>
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<td>26.02</td>
<td>26.05</td>
<td></td>
</tr>
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<td>26.86</td>
<td>26.90</td>
<td></td>
</tr>
<tr>
<td>27.39</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>27.87</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>28.42</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>28.60</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>29.43</td>
<td>29.51</td>
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</tr>
<tr>
<td>30.03</td>
<td>-</td>
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<tr>
<td>30.65</td>
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<td>31.83</td>
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<td>35.78</td>
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</tr>
<tr>
<td>38.38</td>
<td>-</td>
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</tr>
</tbody>
</table>

7.1.4 Conclusion

The purpose of this experiment was to establish if flour extracts could be analysed the same way as those for wheat bran extracts. These results indicate that this is possible as flour samples were successfully characterized using the same extraction and separation methods for bran.
Chromatographic profiles of bran and wheat flour extracts showed similar major compounds. Differences were observed for the flour extract, which showed few early eluting compounds thought to be low molecular weight phenolic compounds and less polar compounds with longer retention times that were not evident within the profile of the bran extract.

7.2 TENTATIVE IDENTIFICATION OF FLAVONOID AND PHENOLIC COMPOUNDS IN WHEAT

7.2.1 Introduction

Tentative identification of compounds can be achieved using UV-Visible absorption. In this study, the UV visible profiles using PAD of the RP-HPLC separated compounds of wheat bran extracts were examined, to assist in the tentative identification of the aglycone structure of the flavonoid and phenolic compounds. The UV visible profiles of the compounds within the wheat bran were compared to available standards. Several different wheat bran extracts were examined as it was found that differences in compound separation occurred when different columns had been used as well as different extraction processors which enabled different other compounds within the wheat to be observed more readily.

7.2.2 Methods and materials

7.2.2.1 Flavonoid extraction and purification

Rosella bran (Section 3.4.1) was extracted following the methodology developed by (Feng and McDonald, 1989), (Section 3.6.3.1) and purified according to the method described in Section 7.1.2.2. A methanol extracted bran sample was extracted following the procedure outlined in Section 3.6.3.5.

7.2.2.2 Flavonoid analysis

A Varian system with photodiode array detection (Section 3.5.1.2), a C18 column (Section 3.5.1.6) and guard column (Section 3.5.1.8) were used to
separate the compounds. The gradient elution system described in Section 3.6.1.1 was employed to separate compounds.

7.2.2.3 Standard preparation

A number of different flavonoid and phenolic standards were used to develop an understanding of the spectral characteristics of these compounds. 50 µg.ml\(^{-1}\) solutions were prepared as described in Section 3.3.5.2.

7.2.3 Results and discussion

A number of compounds were observed and the base structure of the wheat bran compounds tentatively identified by comparison of the UV-visible profiles of with the standards. Figure 7.2 shows the UV-visible profiles of the phenolic and flavonoid standards used to assist with identification.
Figure 7.2. UV-visible profile of standard compounds obtained using RP-HPLC with PAD.

Rosella wheat bran contained thirteen different compounds with UV-visible absorption spectra (Figure 7.3).
Figure 7.3. Compounds identified in small scale alkaline extracted Rosella wheat bran with UV-visible activity. Compounds were separated on a C18 column using a gradient elution system of formic acid –water and methanol at 0.7 mL.min\(^{-1}\). Detection with UV-visible spectroscopy at 280 nm.

The UV-Visible profiles of these compounds are displayed in Figure 7.4. Peaks 1 and 11 displayed a similar UV profile to that of catechin. Peaks 6 and 7 a similar UV profile to that of apigenin aglycone. This is consistent with the findings of Feng and his colleagues (1988) who where able to successfully isolate and identify apigenin-6-C-arabinoside-8-hexoside and apigenin-6-C-hexoside-8-C-pentoside in the bran of hard red spring wheat. Peak 9 displayed a UV profile characteristic of chlorogenic acid. Chlorogenic acid has not been reported in wheat grain before, though it has been identified in the leaves of *triticum aestivum* (Harder and Christensen, 2000). Ferulic acid has been previously reported in wheat (Wang and Mares, 1995) and most recently has been quantified in three different varieties of milled wheat (Adom et al., 2005). In this experiment, the Rosella bran extract was not found to contain ferulic acid. This may be due to separation limitations rather than actual absence from the bran, as later experimentation detected ferulic acid within Rosella
flour and amongst 17 other varieties of wheat as discussed in Section 7.3. Peaks 2, 3, 4, 5, 10, 12 and 13 were not comparable to standards.
Figure 7.4. UV-visible profiles of wheat bran compounds separated by RP-HPLC with PAD.
Figure 7.4. UV-visible profiles of wheat bran compounds separated by RP-HPLC with PAD.
A methanol extracted Rosella wheat bran extract displayed a number of compound with chromophoric activity (Figure 7.5). The apigenin based compounds were again evident with the second apigenin compounds being at a much greater abundance than the first, (Figure 7.6 Peaks 2 and 3 respectively). An additional flavonoid type compound was observed eluting prior to the apigenin compounds. The absorption pattern of this compound is typical of flavonoids with two peaks traditionally designated band I (300-380 nm) and band II (240 to 280 nm) (Marby et al., 1970). Of interest is the decreasing slope of the first band from 275 , to 310 nm which appear to be typical of a hyroxyflavanone (Marby et al., 1970). Chlorogenic acid or ferulic acid were not evident within the profile of this sample.

Figure 7.5 Methanol extracted Rosella bran. Compounds were separated on a C18 column using a gradient elution system of formic acid –water and methanol at 0.7 mL.min⁻¹. Detection with UV-visible spectroscopy at 280 nm.
Figure 7.6. UV visible profiles of methanol extracted bran compounds.
An alkaline bran extract simplified by fraction collection, enabled the separation and tentative identification of ferulic acid along with apigenin and chlorogenic acid (Figure 7.7). Ferulic acid was observed eluting prior to the apigenin compounds (Figure 7.8).

Figure 7.7. Compound separation of alkaline extracted Rosella bran purified using fraction collection. Compounds were separated on a C18 column using a gradient elution system of formic acid –water and methanol at 0.7 mL.min⁻¹. Detection with UV-visible spectroscopy at 280 nm.
Figure 7.8. UV visible spectral profiles of compounds present in a partially purified Rosella wheat bran.

7.2.4 Conclusion

Ferulic acid, chlorogenic acid, apigenin and catechin derivatives have been tentatively identified within Rosella wheat bran. The presence of phenolic compounds with apigenin has not been previously reported though ferulic acid has previously been identified in wheat grain. Different extraction processors enabled the observation of these compounds, all identified within the one variety of Rosella wheat bran.
7.3 THE QUANTIFICATION OF FLAVONOID PIGMENTS IN DIFFERENT VARIETIES
OF WHEAT FLOUR

7.3.1 Introduction

Semi quantification of seventeen different wheat varieties was achieved using RP-HPLC. Catechin was used as an internal standard to enable quantification and comparison of the flavonoid and phenolic compounds identified within these wheat varieties.

7.3.2 Methodology and materials

7.3.2.1 Sample extraction

Wheat flour (9 g) was extracted in a round bottom flask (250 mL) by refluxing with methanol (90 mL) and internal standard to give a final sample concentration of 5 \( \mu \text{g mL}^{-1} \). The suspension was boiled under reflux for one hour with continuous stirring with a magnetic stirring bar. On cooling, the suspension was centrifuged at 5000 rpm for 15 minutes. The resultant supernatant was decanted into a 100 mL volumetric flask and made up to the mark with methanol. A 5 mL aliquot was taken and reduced under a stream of nitrogen. The sample was redissolved in 500 \( \mu \text{L} \) of methanol for semi quantification for the flavonoid and phenolic compounds extracted using RP-HPLC.

7.3.2.2 HPLC conditions and equipment

Compound separation was achieved using a C18 column (Section 3.5.1.9) and guard column C18 (Section 3.5.1.8) Waters Alliance system (Section 3.5.1.4). Column temperature was maintained at 25°C. A 20 \( \mu \text{L} \) sample volume was injected onto the column. A gradient elution system of methanol and 5 % formic acid in water (Section 3.6.1.1) with continuous degassing was employed to assist good separation.
7.2.2.3 Standard preparation

A number of different flavonoid and phenolic standards were used to develop an understanding of the spectral characteristics of these compounds. From stock solutions, 5 µg.ml⁻¹ concentrations were prepared as described in Section 3.3.5.2.

7.3.2.3 Tentative identification and semi quantification of wheat flour varieties

A variety of available standard flavonoid and phenolic compounds were used to assist in the detection of these compounds in the wheat extracts by comparing standard retention times and their UV visible adsorption profiles with those compounds present within the wheat extracts.

Sometimes over 50 different compounds were separated using these chromatographic conditions. If the unknown peaks did not match with a standard but had an absorption profile and consistent retention time within the different wheat samples, the concentrations of these unknown peaks were also determined.

For compounds with retention times but no spectral absorption with peak areas detected, the compounds individual concentration was not determined, however, the peak area count was added to the total flavonoid area count to enable overall pigment concentration determination of each different wheat variety.

A methanol blank was run to identify compounds that elute from the column under these conditions that may contribute to background noise. A total of thirteen different compounds were separated and detected by the system: the concentration of these compounds was subtracted from the total concentration of flavonoids identified in each wheat variety.

The reproducibility of peak retention time and injection volume was assessed to validate the accuracy of the chromatographic method. This was achieved by repeat
injections (7) of the IS and calculation of the relative standard deviation between samples. Less than 20 % variation between samples was deemed acceptable.

7.3.2.5 Concentration calculations

The relative concentration of phenolic and flavonoid compounds was determined as described in Section 3.6.1.3.1. Nine g of flour was extracted with the addition of 5 µg.mL⁻¹ of the IS was added. A 20 µL volume was injected with a dilution factor of 20. The data is calculated from individual analysis of each wheat variety.

7.3.3 Results and discussion

Chromatographic repeatability was examined by comparing the results of seven injections of the Batavia wheat extract with a focus on the activity of the catechin standard (Table 7.3). Injection and analysis repeatability was excellent with 0.1 % relative standard deviation between the elution times of the seven samples and a 10.72 % RSD for peak area.
Table 7.3. Injection reproducibility

<table>
<thead>
<tr>
<th>Trial #</th>
<th>Retention time (min)</th>
<th>Peak area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16.36</td>
<td>231587</td>
</tr>
<tr>
<td>2</td>
<td>16.34</td>
<td>196834</td>
</tr>
<tr>
<td>3</td>
<td>16.32</td>
<td>248890</td>
</tr>
<tr>
<td>4</td>
<td>16.31</td>
<td>225462</td>
</tr>
<tr>
<td>5</td>
<td>16.337</td>
<td>222025</td>
</tr>
<tr>
<td>6</td>
<td>16.33</td>
<td>186141</td>
</tr>
<tr>
<td>7</td>
<td>16.32</td>
<td>248011</td>
</tr>
<tr>
<td>STD</td>
<td>0.016542874</td>
<td>23872.0057</td>
</tr>
<tr>
<td>AV</td>
<td>16.331</td>
<td>222707.143</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>0.101297371</td>
<td>10.7190122</td>
</tr>
</tbody>
</table>

In the wheat flour extracts, a number of compounds were tentatively identified by comparison of their UV visible absorbance spectra with the standard absorbance, as catechin, chlorogenic acid, apigenin and ferulic acid. A representation of the wheat profiles and complexity is available in Figure 7.9.

In the chromatograms of the Batavia wheat samples seven major compounds were consistently observed. Their retention times with the absorbency of these compounds and of the compounds that had spectra characteristic of standard compounds are listed in Table 7.4. These major compounds were compared across the different varieties of wheat. More information on the UV visible spectral characteristics of standard flavonoid and phenolic compounds is available in Appendix 1.
Figure 7.9 Representation of the complexity of the flour separated by RP-HPLC. Compounds were separated on a C18 column using a gradient elution system of formic acid–water and methanol at 0.7 mL.min\(^{-1}\). Detection with UV-visible spectroscopy at 280 nm.
Table 7.4 Summary of major compounds in Batavia wheat flour and compounds with the spectral characteristics of standards. These compounds were compared across wheat varieties.

<table>
<thead>
<tr>
<th>IS</th>
<th>uv-visible spectra</th>
<th>Rt (min)</th>
<th>Peak area</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS</td>
<td>Catechin 273.3</td>
<td>16.36</td>
<td>231587</td>
</tr>
<tr>
<td>1</td>
<td>Ferulic acid 322.1</td>
<td>19.29</td>
<td>13519</td>
</tr>
<tr>
<td>2</td>
<td>Unknown 279.2</td>
<td>20.31</td>
<td>654495</td>
</tr>
<tr>
<td>3</td>
<td>Apigenin 272.1, 340.9</td>
<td>21.37</td>
<td>44134</td>
</tr>
<tr>
<td>4</td>
<td>Chlorogenic acid</td>
<td>22.57</td>
<td>28688</td>
</tr>
<tr>
<td>5</td>
<td>Unknown 276.8, 327.8</td>
<td>28.59</td>
<td>325978</td>
</tr>
<tr>
<td>6</td>
<td>Unknown 283.9</td>
<td>34.61</td>
<td>298320</td>
</tr>
<tr>
<td>7</td>
<td>Unknown 280.4</td>
<td>41.82</td>
<td>140493</td>
</tr>
<tr>
<td>8</td>
<td>Unknown 279.2</td>
<td>42.93</td>
<td>137110</td>
</tr>
</tbody>
</table>

For the quantification of flavonoid and phenolic compounds the above compounds were compared across all the wheat varieties analysed.

In the wheat flour extracts, a number of compounds were tentatively identified by comparison of their UV-visible absorbance spectra with the standard absorbance, as catechin, chlorogenic acid, apigenin and ferulic acid.

Strong absorbency readings for unidentified compounds at 12.3 minutes were observed with a similar UV profile to catechin having an absorbance maximum at 273.3 nm.

Observation of a high peak area was not reflective of absorbance detection. Many compounds with small peak areas showed compounds with UV visible absorbance.

A range of different total flavonoid and phenolic compound concentrations were observed (Table 7.5). The Rosella, Tammin, and Eradu varieties displayed the
lowest total relative concentration of flavonoid and phenolic compounds. The highest concentrations were observed for Yanac and Goldmark varieties.

Table 7.5 Semi quantification of the major compounds identified in seventeen different wheat flour varieties.

<table>
<thead>
<tr>
<th>Variety</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rosella 9</td>
<td>0.02</td>
<td>2</td>
<td>0.2</td>
<td>0.18</td>
<td>0.25</td>
<td>0.14</td>
<td>0.38</td>
<td>5.52</td>
<td></td>
</tr>
<tr>
<td>Wagga Rosella</td>
<td>0.01</td>
<td>1.33</td>
<td>0.21</td>
<td>0.2</td>
<td>0.2</td>
<td>0.42</td>
<td>4.94</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tammin 13</td>
<td>0.01</td>
<td>1.13</td>
<td>0.19</td>
<td>0.26</td>
<td>0.19</td>
<td>0.27</td>
<td>3.94</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hartog 15</td>
<td>3.12</td>
<td>0.23</td>
<td>0.05</td>
<td>0.27</td>
<td>0.44</td>
<td>7.18</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eradu 17</td>
<td>0.27</td>
<td>0.16</td>
<td>0.28</td>
<td>4.46</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trident 19</td>
<td>0.02</td>
<td>2.26</td>
<td>0.15</td>
<td>0.11</td>
<td>0.29</td>
<td>0.19</td>
<td>0.47</td>
<td>0.6</td>
<td>6.28</td>
</tr>
<tr>
<td>Cadoux 23</td>
<td>0.04</td>
<td>0.7</td>
<td>0.48</td>
<td>0.15</td>
<td>0.49</td>
<td>0.35</td>
<td>5.19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Machete 21</td>
<td>3.56</td>
<td>0.51</td>
<td>1.33</td>
<td>1.29</td>
<td>15.15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Goldmark 25</td>
<td>0.05</td>
<td>7.37</td>
<td>0.51</td>
<td>0.23</td>
<td>1.69</td>
<td>0.74</td>
<td>0.92</td>
<td>17.35</td>
<td></td>
</tr>
<tr>
<td>Yanac 27</td>
<td>0.14</td>
<td>7.09</td>
<td>0.42</td>
<td>1.64</td>
<td>0.72</td>
<td>0.76</td>
<td>17.96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vectis 29</td>
<td>0.6</td>
<td>0.26</td>
<td>1.04</td>
<td>0.38</td>
<td>0.43</td>
<td>9.15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Merring 39</td>
<td>0.11</td>
<td>0.08</td>
<td>0.57</td>
<td>0.53</td>
<td>0.73</td>
<td>0.6</td>
<td>14.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Batavia 31</td>
<td>0.08</td>
<td>6.47</td>
<td>0.35</td>
<td>0.5</td>
<td>0.56</td>
<td>15.77</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Suneca 33</td>
<td>0.05</td>
<td>5.44</td>
<td>0.62</td>
<td>0.31</td>
<td>0.4</td>
<td>0.47</td>
<td>0.6</td>
<td>12.88</td>
<td></td>
</tr>
<tr>
<td>Janz 35</td>
<td>0.13</td>
<td>7.04</td>
<td>0.54</td>
<td>0.33</td>
<td>0.5</td>
<td>0.65</td>
<td>0.81</td>
<td>15.05</td>
<td></td>
</tr>
<tr>
<td>Katunga 37</td>
<td>1.57</td>
<td>0.51</td>
<td>0.99</td>
<td>1.01</td>
<td>14.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Different relative concentrations of each of the major compounds were observed across the varieties of wheat flour. Not all of the eight major pigments focussed on, were present in all of the wheat varieties. A number of varieties lacked the presence of the compound 4 tentatively identified as chlorogenic acid. In fact, this compound was the least frequently observed. Apigenin (compound 3) was observed in all wheat varieties. This form of apigenin was observed at a retention time of 21.3
minutes with a spectral absorption of 272.1, 337.3 nm. Ferulic acid (compound 1) was observed in all wheat flours except for Katunga. The highest concentration of ferulic acid was observed in the Yanac, Merring and Janz wheat flours. Wheat varieties that displayed both ferulic acid and chlorogenic acid (compound 4) included Trident, Cadoux, Goldmark, Vectis, Suneca, and Janz. Apigenin (compound 3) was observed in all wheat varieties, with the highest concentrations observed in Katunga, Suneca and Merring wheats. A number of different wheats contained two forms of apigenin (Table 7.6). Euradu flour contained three forms of apigenin. The distinguishing factor was the band II absorption at 272.9 nm detected in each form. Interestingly Rosella flour did not appear to contain the two forms of apigenin previously identified in the bran of this variety (Section 7.2.3).

Table 7.6 Forms of apigenin identified in several wheat flour varieties.

<table>
<thead>
<tr>
<th>Variety</th>
<th>Rt (min)</th>
<th>Absorption maxima (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Euradu</td>
<td>20.66</td>
<td>272.1, 332.6</td>
</tr>
<tr>
<td></td>
<td>21.36</td>
<td>272.1, 334.9</td>
</tr>
<tr>
<td></td>
<td>21.86</td>
<td>272.1, 324.2</td>
</tr>
<tr>
<td>Cadoux</td>
<td>20.61</td>
<td>272.1, 330.2</td>
</tr>
<tr>
<td></td>
<td>21.34</td>
<td>272.1,336.1</td>
</tr>
<tr>
<td>Machete</td>
<td>20.63</td>
<td>272.1, 325.4</td>
</tr>
<tr>
<td></td>
<td>21.37</td>
<td>272.1, 334.9</td>
</tr>
<tr>
<td>Vectis</td>
<td>20.64</td>
<td>272.1, 333.8</td>
</tr>
<tr>
<td></td>
<td>21.34</td>
<td>272.1, 336.1</td>
</tr>
<tr>
<td>Katunga</td>
<td>20.62</td>
<td>272.1, 333.8</td>
</tr>
<tr>
<td></td>
<td>21.34</td>
<td>272.1, 337.8</td>
</tr>
</tbody>
</table>

7.3.4 Conclusion

It has been recognized that wheat flour contains many different flavonoid and phenolic compounds and for the first time the complexity of these compounds has been reported. Tentative identification with standard UV visible spectral comparison found at least one form of apigenin in the flours with several varieties displaying two
or three forms of apigenin. All flours contained ferulic or chlorogenic acid. Differences in the relative concentrations of pigments were observed within seventeen different wheat flours. Differences in total pigment content between wheat varieties were also observed. Further research is required to isolate or separate these compounds to facilitate their definitive identification in order to increase the understanding of the impact on flour and noodle colour that these compounds may have individually or in combination.
CHAPTER 8: THE CHARACTERISATION AND QUANTIFICATION OF CAROTENOID COMPOUNDS IN AUSTRALIAN WHEAT

8.0 Introduction

Product colour is an important quality parameter that influences consumer perception and the subsequent choice of products. The inherent colour of flour can influence the wheat variety selected for a particular product and the manufacturing processes to produce consumer acceptable food. The focus of the following chapter is on the characterisation and quantification of carotenoid pigments in a number of noodle wheat varieties. The information from this chapter will assist in confirming the success of methodologies developed for the characterisation and quantification of wheat flour. The many different compounds in wheat flour varieties will be characterized and the relative concentrations of these compounds compared.

The first section of this chapter focuses on characterisation of Rosella wheat flour using RP-HPLC coupled with PAD to examine the UV visible absorption spectra of separated compounds. Secondly, the mass spectral profiles of this wheat were analysed and discussed. Lastly, the findings from the semi quantification and characterisation of thirteen different wheat varieties are discussed.

8.1 SPECTRAL ANALYSIS OF CAROTENOID PIGMENTS IN WHEAT

8.1 1. Introduction

A method for the rapid but simple analysis of the carotenoid compounds will help in the identification of high or low pigmented wheat and the development and selection of wheat cultivars for specific niche markets. Existing methods for wheat carotenoid analysis do not facilitate the comprehensive characterisation of carotenoid compounds.
RP-HPLC coupled with photodiode array detection offers a powerful tool for the tentative identification of compounds. Samples may be characterised using UV visible spectroscopy and quantified at the same time.

The following experiment was conducted to establish a chromatographic characterisation method, to enable the quantification and tentative identification of carotenoid pigments in wheat.

8.1.2 Methods and materials

8.1.2.1 Pigment extraction

The wheat varieties Janz and Rosella were extracted (Section 3.4.3) for carotenoids following a modification of the procedure described by Wootton, 1996. In a 250 mL round bottom flask, 20 g of Rosella flour was extracted with 40 mL acetone, 60 mL petroleum ether together with the antioxidants magnesium carbonate (0.1 g) and ascorbic acid (1.0 g). Prior to extraction, β-apo-8'-carotenal (50 µg.mL⁻¹) standard was added for compound identification purposes. The sample was refluxed for 30 minutes at setting 4 on the electromantle. To reduce solvent loss, two condensers in line were used. The solution was filtered through No. 1 Whatman filter paper under vacuum. The flour sample was washed then transferred to a funnel and dried under vacuum.

The carotenoid solution was reduced to dryness by rotary evaporation then redissolved in 100 mL diethyl ether, for saponification. Saponification was achieved by addition of 100 mL 20 % potassium hydroxide to the solution. The sample was covered with foil and left at room temperature overnight. The alkaline solution was removed by washing 4-5 times with 100 mL deionised water, in a separating funnel, or until the eluate was at neutral pH. The sample was reduced under rotary evaporation until dry then transferred to HPLC vials by redissolving in acetone. The samples were centrifuged in eppendorf tubes and the clear solution transferred to HPLC vials. The samples were reduced to dryness under nitrogen, at room
temperature, then redissolved in 500 µl acetone prior to HPLC analysis. It should be noted that at all stages of the process nitrogen was used to flush air from the flasks, vials and tubes to reduce oxidation.

8.1.2.2 Flour colour analysis.

Before and after the extraction of carotenoids the flour samples were analysed using a Minolta chromometer to measure the lightness, (L), brightness (b*) and hue or redness (a*) of the flours (Section 3.6.2). The sample was transferred to a small Petrie dish and lightly packed for analysis, five readings were taken then the mean values calculated.

8.1.2.3 Equipment.

A Hewlett Packard 1040 A HPLC detection system with PAD and software with a Biorad HPLC (model 1330) were employed (Section 3.5.1.3).

8.1.2.4 HPLC method development.

To enable the analysis of the carotenoids using the Hewlett Packard system an isocratic elution system needed to be developed. The gradient elution system described in Section 3.6.1.2 using acetonitrile -water and ethyl acetate was modified to allow for the isocratic system. It was established that at 85% ethyl acetate and 15 % acetonitrile -water that separation of the most of the carotenoid compounds in wheat occurred. An isocratic elution system of 85 % ethyl acetate and 15 % acetonitrile water, for a run time of 15 minutes at a flow rate of 0.5 mL.min-1 was employed. Three standards, lutein, β-apo-8-carotenal and β-carotene were examined using this method. Samples were detected between an absorbance of 300- 600 nm and separation was achieved using a C18 column (Section 3.5.1.7) with a C18 guard column (Section 3.5.1.8). The peak purity of the separated compounds was determined using the software provided, within 99% accuracy.
8.1.3 Results and discussion

Using the isocratic elution system the three standard compounds displayed good baseline-to-baseline separation Figure 8.1.

Figure 8.1. Isocratic elution using an isocratic elution system of 85 % ethyl acetate and 15 % acetonitrile water for a run time of 15 minutes at a flow rate of 0.5 mL.min⁻¹ was employed. Compounds are lutein (RT 4.73 min), b-apo-8-Carotenal (RT 5.41 min) and b-carotene (RT 7.3 min.)

The internal standard b-apo-8'-Carotenal did not display characteristic spectra (Figure 8.2), with no clear absorption maxima.
Lutein and β-carotene displayed the expected spectral pattern with maximum absorbance at 418, 446 and 474 nm and 426, 457 and 476 nm for lutein and β-carotene respectively.

The chromatographic profile of a Rosella wheat extract displayed seven peaks with baseline-to-baseline separation (Figure 8.3). A split peak with a retention time of 4.98 minutes was also observed. This suggests that the two compounds may be co-eluting under these conditions. Zeaxanthin is the steroisomer of lutein. They differ from each other in the position of the OH functional group on the molecule. It is, therefore, possible that at this retention time, zeaxanthin and lutein are co-eluting.
Figure 8.3. Rosella flour extract exhibiting 7 peaks at 4.98, 5.69, 677, 7.98, 8.33, 9.31, 10.36 and 11.15 minutes. Compounds were separated using an isocratic elution system of 85% ethyl acetate and 15% acetonitrile water for a run time of 15 minutes at a flow rate of 0.5 mL.min⁻¹ was employed.

The Janz flour extract showed a similar chromatographic profile (Figure 8.4).

Figure 8.4. Janz flour extract exhibiting 7 major peaks at 4.95, 5.70, 6.73, 7.84, 8.99, 10.08 and 10.87 minutes. Isocratic elution using 85% ethyl acetate and 15% acetonitrile water for a run time of 15 minutes at a flow rate of 0.5 mL.min⁻¹ was employed.

Compounds detected in the two different flour extracts showed similar retention times but there were some observable differences in spectral absorbance maxima. Compounds detected in the Rosella wheat flour at 4.8 and in Janz flour at 4.93
minutes were thought to be lutein. The spectral absorption maxima of these compounds did not, however, displayed the exact absorption maxima of the standard (Table 8.1). The variability in the retention time was thought be due to manual sample injection. Variability of the wheat flour spectral absorption when compared to standards may be due to the co-elution of compounds at these retention times within the wheat extracts.

Table 8.1. Peak retention times. Purity and absorption maxima’s for carotenoid standard and a Rosella wheat flour extract.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Peak retention time (min)</th>
<th>Absorption maxima</th>
<th>Peak purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lutein</td>
<td>4.46</td>
<td>418, 446, 474</td>
<td>999</td>
</tr>
<tr>
<td>β-apo-8’-carotenal</td>
<td>5.22</td>
<td>Not determinable</td>
<td>999</td>
</tr>
<tr>
<td>β-carotene</td>
<td>7.18</td>
<td>426, 457, 476</td>
<td>100</td>
</tr>
<tr>
<td>Rosella flour</td>
<td>1</td>
<td>416, 442, 476</td>
<td>988</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>422sh, 448, 470</td>
<td>Impure</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>422sh, 447, 470</td>
<td>991</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>420sh, 446, 470, 474</td>
<td>997</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>422sh, 443, 470, 474</td>
<td>996</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>420sh, 440, 470</td>
<td>1 spec found only</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>416sh, 440, 470</td>
<td>Impure</td>
</tr>
<tr>
<td>Janz flour</td>
<td>1</td>
<td>420sh, 444, 472</td>
<td>999</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Internal standard</td>
<td>989 impure</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>420, 442, 472</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>420sh, 446, 473</td>
<td>965 impure</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>420sh, 444, 473</td>
<td>988 impure</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>420sh, 441, 469</td>
<td>980 impure</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>425sh, 444, 473</td>
<td>998 pure</td>
</tr>
</tbody>
</table>

Compounds detected between 4 and 8 minutes within the two varieties of wheat comprised similar retention times. Due to the differences in the spectral absorption patterns of these compounds they could not be considered to be the same.
8.1.4 Conclusions

Using this system of separation and detection co-eluting peaks at 4.95 minutes were observed, suggesting that lutein and zeaxanthin may not be effectively separating from each other. Compounds with similar retention times were identified between the two varieties of wheat. Variance in the maximum absorption of standards has suggested spectral detection problems. Wheat flour carotenoids could not be confidently characterised using this method.

8.2 EXAMINATION OF CAROTENOID PIGMENTS IN AUSTRALIAN WHEAT

8.2.1 Introduction

The following is an account of preliminary investigations into the structural elucidation of carotenoid standards and compounds extracted from wheat flour using electrospray mass spectroscopy. Rosella wheat flour and a TLC separated component of this extract were chromatographically characterised using RP-HPLC together with standard compounds.

8.2.2 Methods and materials

8.2.2.1 Pigment extraction.

Carotenoids were extracted from Rosella wheat (Section 3.4.3) following the procedure described in Section 8.1.2.1.

8.2.2.2 Pigment analysis

The extract was examined using the ethyl acetate and 90 % acetonitrile RP-HPLC method as described in Section 3.6.1.2, with separation on a C18 column (Section 3.5.1.7) with a C18 guard column (Section 3.5.1.8). Peaks were detected at 450 nm, separation was achieved with a flow rate of 0.7 ml.min⁻¹.
8.2.2.3 Compound purification for structural identification

Using the pigment extraction method described in Section 8.1.2.1, a carotenoid compound, unknown 1, was separated by Thin Layer Chromatography (TLC). TLC separation was achieved using silica gel plates developed with hexane-isopropyl alcohol-diethyl ether-acetone-acetic acid (85:12:1:4:1, v/v) (Lepage and Sims 1968), the compound displayed an Rf value of 0.47 similar to lutein, Rf 0.48.

8.2.2.4 Compound identification.

8.2.2.4.1 UV-visible spectroscopy

Unknown 1 was examined by UV-visible spectrophotometry (Section 3.5.2) between 250 - 550 nm and by RP-HPLC. Information was compared to β-carotene and lutein standards.

8.2.2.4.2 Mass spectral analysis

All samples were analysed in the positive ion mode using EMS (Section 3.6.6.1) with an acetonitrile-isooamyl alcohol solvent system. To verify methodology prior to analysis of the wheat flour extract, mass spectra of β-carotene (300 µM) and lutein (292 µM) were examined. To enhance ionisation of these neutral compounds 0.1 % chloroform was added to the lutein and unknown 1 samples solvent system as an oxidant.

8.2.3 Results and discussion

RP-HPLC analysis enabled the detection of several major compounds (Figure 8.5) within the wheat extract. Baseline separation of the less polar compounds eluting after 38 minutes was not achieved so it is recommended that the runtime be extended for future analysis. A compound within the flour extract, with a retention
time of 38.3 minutes was observed, a retention time consistent with that of the β-carotene standard.

It was established that the unknown 1 compound, purified by TLC contained a number of different compounds. A compound with the same retention time as the lutein standard was detected at 15.0 minutes.

Figure 8.5. RP-HPLC profile of an unpurified flour carotenoid extract (A) and unknown 1 (B) purified from the carotenoid extract by TLC. The extract was examined using ethyl acetate and 90% acetonitrile isocratic elution over a run time of 50 minutes at a flow rate of 0.7 mL.min⁻¹.

The unknown 1 sample mixture displayed absorption spectra characteristic of carotenoids with absorption maxima at 438, 455 and 484 nm, an absorption spectral pattern characteristic of other carotenoids such as β-carotene (437, 467 and 491 nm) and Lutein (435, 457 and 487 nm).

The mass spectrum of β-carotene showed a low abundance of deprotonated ions, which made it difficult to distinguish molecular ion peaks and fragment ions from background noise. A base peak was observed at m/z 536.3 [M]+. Removal of CH₃
groups and cleavage of the cyclic end groups by retro-Diels-Alder (RDA) fragmentation may have resulted in the fragment ion and base peak at 481.2 [M-56]+ (Caccamese and Garozzo, 1990) (Figure 8.5). The lutein standard exhibited a molecular ion peak at m/z 568 [M]+ (Figure 8.6). Removal of CH₃ groups at the conjugated double bond site, on the cyclic end-groups, may account for the fragment ion at m/z 538.4 [M-30]+. Further fragments of the cyclic end groups following RDA fragmentation probably resulted in the formation of the base peak at m/z 547.2 [M-112]+.

A high molecular weight peak was observed at m/z 713, in the spectrum of unknown 1 (Figure 8.7). A peak at m/z 457.2 detected in the lutein spectrum was also present in the spectrum of unknown 1. This suggests compound similarity to lutein but as previously mentioned, no further structural elucidation was relevant as the sample was impure.
Figure 8.6. Electrospray mass spectra of β-carotene.

Figure 8.7 Electrospray mass spectra of lutein.
8.2.4 Conclusions

RP-HPLC of a carotenoid extract of flour confirmed the existence of at least seven major carotenoids among which β-carotene was observed.

A TLC collected fraction of the unpurified extract, showed several different compounds. A compound within the fraction exhibited a RP-HPLC retention time similar to that of lutein. More effective purification methods are required to facilitate conclusive structural assessment.

The molecular structure of lutein was observed using this mass spectral method application. Low ion abundance prevented structural ions from being distinguished from background noise, in the case of β-carotene.
RP-HPLC requires improvement, to enhance resolution of the less polar carotenoids, which may possibly be improved by increasing gradient increment times and decreasing flow rate.

A suitable carotenoid standard needs to be found so that it can be used as an internal standard, for quantification of unknowns and to ensure that degradation of the carotenoids during extraction, purification and EMS analysis is not occurring.

8.3 THE QUANTIFICATION OF CAROTENOID COMPOUNDS IN AUSTRALIAN WHEATS

8.3.1 Introduction

It has been established that RP-HPLC analysis can facilitate the separation, characterisation and semi quantification of a Rosella flour extract. This section focuses on characterising and comparing the relative concentrations of thirteen different varieties of wheat flour, to gain greater insight into the nature of carotenoid compounds in wheat.

8.3.2 Method and materials

8.3.2.1 Sample extraction

Wheat flour (Section 3.4.3) was extracted following a method for total carotenoid content (AACC, 1983), Section 3.6.5.4. Duplicate samples of each variety were extracted routinely.

8.3.2.2. Sample preparation for RP-HPLC

To obtain an analysable concentration of carotenoid extract, 2 ml of the 40 mL n-butanol extract was reduced to dryness under nitrogen at 40°C then redissolved in 100 µl water saturated n-butanol. For quantification, 2.5 µg of β-apo-8'-carotenal was
added to the solution after extraction, prior to analysis. The sample was contained in a vial insert within the HPLC vial to ensure correct application of sample volume.

8.3.2.3. Chromatographic conditions

Separation was achieved on C18 (Section 3.5.1.7), C18 guard column (Section 3.5.1.8), using the Varian chromatographic system, Section 3.5.1.1. Samples (94 µl) were injected via a 50 µl loop giving a final injection volume of 50 µl. A gradient elution system using acetonitrile-water and ethyl acetate as described in Section 3.6.1.2 enabled separation. Solvents were applied at a flow rate of 0.7 mL and compounds were detected at 436 nm in accordance with the transmissive index of β-carotene of 436 nm and with the spectroscopic method (Section 3.6.5.4).

8.3.2.4 Quantification by the external standard method.

Quantification involved individual compound concentration calculation against β-apo-8'-carotenal (2.5 µg.mL⁻¹) with this standard added after extraction to facilitate comparison between the AACC method and HPLC methodology in Section 3.6.1.3.2.

8.3.3 Results and discussion

Similar chromatographic profiles were observed for each of the samples. Every variety examined displayed 6 major peaks as represented in the profile of Janz variety of wheat (Figure 8.5). A broad compound eluting at around 9 minutes matched the retention time of the lutein standard (Figure 8.6). The standard, β-apo-8'-carotenal was observed at 13 minutes. β-carotene was observed as a minor peak within the samples at around 22 minutes.

The relative concentrations of the six major compounds within each of the varieties were calculated and compared to construct a profile of the differences in yellow pigment content between varieties (Table 8.2).
Figure 8.9. Chromatographic profile of Janz flour with the six major peaks identified. Separation was achieved using a C-18 column with a gradient elution system of acetonitrile–water (90:10 v/v) and ethyl acetate at a flow rate of 0.7 mL.min⁻¹ with UV-visible detection at 436 nm.

Figure 8.10. Standard chromatographic profile. Separation was achieved using a C-18 column with a gradient elution system of acetonitrile–water (90:10 v/v) and ethyl acetate at a flow rate of 0.7 mL.min⁻¹ with UV-visible detection at 436 nm.
Table 8.2 Comparison of major pigments identified in 13 different varieties of wheat flours.

<table>
<thead>
<tr>
<th>Wheat variety</th>
<th>Concentration of compound µg.g⁻¹</th>
<th>Major peak comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Meering</td>
<td>0.4</td>
<td>0.38</td>
</tr>
<tr>
<td>Machete</td>
<td>0.24</td>
<td>0.3</td>
</tr>
<tr>
<td>Vectis</td>
<td>0.81</td>
<td>0.7</td>
</tr>
<tr>
<td>Janz</td>
<td>0.39</td>
<td>0.37</td>
</tr>
<tr>
<td>Eradu</td>
<td>0.25</td>
<td>0.32</td>
</tr>
<tr>
<td>Hartog</td>
<td>0.18</td>
<td>0.37</td>
</tr>
<tr>
<td>Yanac</td>
<td>0.21</td>
<td>0.25</td>
</tr>
<tr>
<td>Suneca</td>
<td>0.28</td>
<td>0.48</td>
</tr>
<tr>
<td>Goldmark</td>
<td>0.35</td>
<td>0.52</td>
</tr>
<tr>
<td>Rosella</td>
<td>0.39</td>
<td>0.4</td>
</tr>
<tr>
<td>Batavia</td>
<td>0.44</td>
<td>0.46</td>
</tr>
<tr>
<td>Trident</td>
<td>0.58</td>
<td>0.49</td>
</tr>
<tr>
<td>Cadoux</td>
<td>0.59</td>
<td>0.64</td>
</tr>
</tbody>
</table>

High concentrations of greater than 0.59 µg.g⁻¹ to 0.81 µg.g⁻¹ of lutein (peak 1) were observed within Cadoux, Trident and Vectis wheat flours. Vectis contained the highest overall yellow pigment content (4.21 µg.g⁻¹) followed by Cadoux (3.91 µg.g⁻¹) then Trident and Goldmark (2.84 µg.g⁻¹). The lowest concentration of yellow coloured compounds where observed in the Yanac (1.62 µg.g⁻¹) and Eradu (1.97 µg.g⁻¹) flours.

Lutein is not necessarily the predominant pigment in wheat as has been observed in this investigation with the detection of other compounds with higher concentrations. The predominant compound in the Goldmark variety was the compound designated 231.
peak 3, with a concentration of 0.49 µg.g\(^{-1}\) with lutein (peak 1) detected at a concentration of 0.35 µg.g\(^{-1}\). Further work is required to isolate and identify these major compounds within wheat.

The impact that the individual compounds have on wheat flour colour is not understood and requires further investigation. Total pigment content did appear to relate to the Minolta b* value of the wheat flour (Table 8.3). The Vectis variety was found to have the highest total pigment content, but did not have the highest b* value, and display the highest a* value (-1.14) and L value (99.43) readings. L measures white to black (perfect white, 100), a* measures hue, (a* positive the degree of redness, a* negative colour towards green), b* the yellowness of flour. From this, it may be inferred that high total pigment content has a greater influence on the hue and lightness of wheat flour than on the yellow colour or the flour. The Trident variety of flour was found to have the highest b* value of 10.36, the lowest value recorded was for the Wagga Rosella flour (b* 7.78). Further investigations are required to statistically confirm this observation.
Table 8.3. Comparison of duplicate extract wheat flour variety total pigment content. Concentrations are also compared to the reading of Minolta Chroma Meter the colour space parameters that measure L* (lightness), b* (yellowness) and a* (redness).

<table>
<thead>
<tr>
<th>Flour variety</th>
<th>Total pigment (ug/g)</th>
<th>b* value</th>
<th>L value</th>
<th>a* value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meering</td>
<td>2.31</td>
<td>8.98</td>
<td>95.89</td>
<td>-0.77</td>
</tr>
<tr>
<td>Machete</td>
<td>2.12</td>
<td>8.83</td>
<td>97.78</td>
<td>-0.62</td>
</tr>
<tr>
<td>Vectis</td>
<td>4.21</td>
<td>9.19</td>
<td>99.43</td>
<td>-1.14</td>
</tr>
<tr>
<td>Janz</td>
<td>2.45</td>
<td>8.29</td>
<td>96.3</td>
<td>-0.55</td>
</tr>
<tr>
<td>Eradu</td>
<td>1.97</td>
<td>8.38</td>
<td>94.52</td>
<td>-0.52</td>
</tr>
<tr>
<td>Hartog</td>
<td>2.07</td>
<td>9.63</td>
<td>93.93</td>
<td>-0.72</td>
</tr>
<tr>
<td>Yanac</td>
<td>1.62</td>
<td>8.39</td>
<td>96.76</td>
<td>-0.44</td>
</tr>
<tr>
<td>Suneca</td>
<td>2.68</td>
<td>9.18</td>
<td>97.77</td>
<td>-0.65</td>
</tr>
<tr>
<td>Goldmark</td>
<td>2.84</td>
<td>9.34</td>
<td>98.35</td>
<td>-0.84</td>
</tr>
<tr>
<td>Wagga</td>
<td>2.29</td>
<td>7.78</td>
<td>98.08</td>
<td>-0.76</td>
</tr>
<tr>
<td>Rosella</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Batavia</td>
<td>2.67</td>
<td>9.96</td>
<td>97.42</td>
<td>-1.05</td>
</tr>
<tr>
<td>Trident</td>
<td>2.84</td>
<td>10.36</td>
<td>95.59</td>
<td>-1.12</td>
</tr>
<tr>
<td>Cadoux</td>
<td>3.91</td>
<td>8.37</td>
<td>94.89</td>
<td>-0.53</td>
</tr>
</tbody>
</table>

8.3.4 Conclusion

All of the wheat flours examined contained a lutein type pigment, with β-carotene tentatively identified. For the first time, the presence of 5 other major compounds within wheats has been observed in all varieties investigated during this study. The lutein compounds within wheat varieties does not necessarily represent the predominant pigment responsible for the yellow colouration of wheat flours, as it was observed that wheat flour contain five other major compounds, some of which were observed at higher concentrations than lutein. Further research is required to isolate
and identify these compounds and to increase the understanding of their individual contribution to the yellow colouration of flours. Each wheat variety displayed a unique pigment profile observed by differences in the concentration of these major compounds. High total pigment concentrations did not necessarily reflect a high yellow colour value (b* value) reading for the flours. Wheats with high total pigment contents tended to have a high a* value (hue reading) and L (lightness) reading. Further work is required to statistically validate these observations.

CHAPTER 8.4. COMPARISON OF THE AACC AND RP-HPLC TOTAL CAROTENOID QUANTIFICATION METHODS.

8.4.1 Introduction

Laboratories throughout Australia use the AACC method for the evaluation of total carotenoid content in wheat flour and products. This method enables comparison of the relative levels of natural yellow pigment against the transmissive index of β-carotene. Pigment values are regarded as absolute as it thought that the majority of carotenoid pigments are xanthophylls and not β-carotene. The methods most advantageous attributes are ease of analysis and extraction with low cost analysis requiring equipment no more complicated that a spectrophotometer. Its major limitation is that individual pigment compounds cannot be identified or quantified.

A highly specific method of analysis is available with the use of RP-HPLC. The HPLC method is more expensive to establish than the AACC method and requires technical expertise, however it has the advantage of providing information on individual carotenoid compounds and their quantities, relative to a standard, also it has been used successfully for the quantification and characterisation of fruit and vegetable carotenoids. RP-HPLC quantification can employ the use of an internal standard or external standard method for the quantification of individual pigments.
In this experiment to facilitate comparison between the AACC method and RP-HPLC method the external standard method using β-apo-8'-carotenal was employed. Whilst the ES method is considered to be less accurate due to the variability between manual sample injection the employment of autosamplers in this instance has resulted in excellent sample injection reproducibility. The reproducibility and repeatability of injection volume and in detection sensitivity during the run will not influence the accuracy and precision of calculated concentrations.

This work tests the most commonly used method of carotenoid content analysis against that of the more sophisticated RP-HPLC method of pigment quantification, for accuracy and reproducibility of pigment concentration in wheat flours.

8.4.2 Methods and materials

8.4.2.1 Sample materials

Australian Standard white flour was kindly supplied by Agrifood. For more information on the characteristics of this wheat refer to Section 3.4.3. Duplicate analysis of carotenoid content was carried out on nine wheat flour samples. For all evaluations the mean of duplicated was reported.

8.4.2.2 Pigment extraction

Wheat flour was extracted following a method for total carotenoid content (AACC, 1983) Section 3.6.5.4. Duplicate samples of each variety were routinely extracted.

8.4.2.3 Standard preparation.

A 1000 μg.mL⁻¹ solution of β-apo-8'-Carotenal standard was prepared in a McCartney bottle by weighing 0.01 g using an analytical balance and dissolving the standard in 10 mL analytical grade n-butanol. The following formula was used to obtain a 2.5 μg.mL⁻¹ sample for addition into wheat extracts of 500 μl:

235
\[ C_1 V_1 = C_2 V_2 \]

To find the second volume required convert to

\[ V_1 = \frac{C_2}{C_1} \times V_2 / C_1 \]

Example

\[ V_1 = 2.5 \times 500 \]
\[ \frac{1000}{1000} \]
\[ = 1.23 \mu l \]

8.4.2.4 RP-HPLC analysis

8.4.2.4.1 Sample preparation

To obtain an analysable concentration of carotenoid extract, 2 ml of the 40 mL n-butanol extract was reduced to dryness under nitrogen at 40°C and redissolved in 100 µl water saturated n-butanol. For quantification, 6.5 µg of β-apo-8'-carotenal was added to the solution prior to analysis. The sample was contained in a vial insert within the HPLC vial to ensure correct application of sample volume onto the column.

8.4.2.4.2 Chromatographic conditions

A Varian chromatography system Section 3.5.1.1 was used for analysis. Separation was achieved on C18 column (Section 3.5.1.7) with a C18 guard column (Section 3.5.1.8). Samples (94 µL) were injected via a 50 µL loop giving a final injection volume of 50 µL. A gradient elution system using acetonitrile-water and ethyl acetate as described in Section 3.6.1.2, enabled separation. Solvents were applied at a flow rate of 0.7 ml and compounds were detected at 436 nm, in accordance with the transmissive index of β-carotene of 435.8 nm and with the AACC spectroscopic method.
8.4.2.4.3 Quantification of total carotenoid content.

8.4.2.4.3.1 Quantification by external standard method.

Quantification involved total carotenoid content calculation against \( \beta \)-apo-8'-carotenal (2.5 \( \mu \)g.mL\(^{-1} \)) standard following the method described in Section 3.6.1.3.2. This involved multiplication of the total area count of the sample by the concentration of standard selected, divided by that standard’s peak area count. Calculations were adjusted to consider the injection volume, amount of initial sample used and the amount of wheat extracted.

Sample reproducibility was established by comparing the response of duplicate samples extracted and working out the RSD (Section 3.6.1.3.3.). For result reliability samples with less than 10 % variability were reported.

8.4.2.4.3.2 Quantification by AACC method

Wheat extracts were quantified as described in Section 3.6.5.4 to establish total pigment content by spectroscopy.

8.4.3 Results and discussion

Variations in concentration values were obtained using the different quantification methods (Table 8.4). The HPLC method resulted in higher total carotenoid concentrations than the AACC method. Concentrations values, obtained using the RP-HPLC method ranged from 1.8- 4.1 \( \mu \)g.mL\(^{-1} \) depending on the wheat variety. Concentrations determined using the AACC method showed a range from 1.4 \( \mu \)g.mL\(^{-1} \) to 2.9 \( \mu \)g.mL\(^{-1} \). The wheat with the lowest concentration of carotenoids, established using the AACC method (Janz) was not the same variety as the wheat found to have the lowest concentration using the RP-HPLC method (Eradu).
Table 8.4. Comparison of concentration values obtained from duplicated samples, comparing the AACC method with the RP-HPLC method.

<table>
<thead>
<tr>
<th>Sample</th>
<th>AACC method µg.mL⁻¹</th>
<th>RP-HPLC ES method µg.mL⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vectis</td>
<td>2.9</td>
<td>4.1</td>
</tr>
<tr>
<td>Suneca</td>
<td>1.7</td>
<td>2.9</td>
</tr>
<tr>
<td>Eradu</td>
<td>1.5</td>
<td>1.8</td>
</tr>
<tr>
<td>Janz</td>
<td>1.4</td>
<td>2.4</td>
</tr>
<tr>
<td>Goldmark</td>
<td>2.0</td>
<td>2.7</td>
</tr>
<tr>
<td>Trident</td>
<td>2.0</td>
<td>2.7</td>
</tr>
<tr>
<td>Cadoux</td>
<td>2.6</td>
<td>3.4</td>
</tr>
<tr>
<td>Batavia</td>
<td>2.1</td>
<td>2.7</td>
</tr>
<tr>
<td>Machete</td>
<td>1.6</td>
<td>2.1</td>
</tr>
</tbody>
</table>

It is not surprising that the RP-HPLC method of total carotenoid quantification offers more sensitive analysis and, therefore, higher concentration values are expected. These higher concentration values reflect greater accuracy and sensitivity of compound detection and analysis whereby the concentration of each individual pigment separated are calculated.

A range of compounds with different polarities shown by the different compound retention times were present in the wheat samples, as represented in Vectis wheat (Figure 8.11) and for comparison all wheat varieties analysed (Figure 8.12). Compounds eluting before the ES at 14 minutes are oxygenated carotenoids and include lutein at 0.81 µg.g⁻¹ (peak 1). Those eluting after the IS are hydrocarbon carotenoids and include β-carotene at 0.04 µg.g⁻¹ (peak 5) present in very low quantities. Vectis displayed 6 major compounds.
Figure 8.11 RP-HPLC chromatograph of Vectis wheat flour. Separation was achieved using a C-18 column with a gradient elution system of acetonitrile – water (90:10 v/v) and ethyl acetate at a flow rate of 0.7 mL.min⁻¹ with UV-visible detection at 436 nm.
Figure 8.12. RP-HPLC at 436 nm of different varieties of wheat flour. All samples are at an attenuation of 50 to enable direct visual comparison. Separation was achieved using a C-18 column with a gradient elution system of acetonitrile–water (90:10 v/v) and ethyl acetate at a flow rate of 0.7 mL·min⁻¹ with UV-visible detection at 436 nm.
Pigment profiles were similar for all the different varieties, each showing 5 major peaks and a similar cluster of co-eluting hydrocarbon carotenoid eluting between 23 and 27 minutes. Minor compounds were also observed eluting after the internal standard from 14 to 18.7 minutes. Different wheat varieties displayed different total carotenoid concentrations and ranged from 1.8 to 4.1 µg.g\(^{-1}\) (Figure 8.13). The largest peak areas where observed for Vectis and Cadoux which also contained the highest concentration of total carotenoid 4.1 and 3.4 µg.g\(^{-1}\) respectively. Cadoux contained two additional pigments eluting closely after lutein at 11.6 and 12.6 minutes, which were not observed in the other wheat varieties.

![Figure 8.13. Concentrations of total carotenoid concentrations in different wheat varieties determined using the AACC and HPLC methods.](image)

Examination of the relationship between the AACC and RP-HPLC showed that a low correlation between the two concentration determination methods existed (Figure 8.14).
8.4.4 Conclusion

Different total carotenoid concentrations were observed using the AACC method and RP-HPLC analysis with an external standard for semi-quantification. Higher concentrations were observed using the RP-HPLC methodology as expected with a higher degree of precision and accuracy from the improved detection and sensitivity of the analytical instrumentation used. For comprehensive information on pigment types and quantities the ES method may be used for wheat carotenoid characterisation and for the quantification of individual pigment types and detection of major compound that may influence colour. A weak relationship exists between the HPLC method and the AACC total carotenoid content method.
CHAPTER 9. PIGMENT IMPACT ON FLOUR AND NOODLE COLOUR.

9.0 Introduction

The following chapter focuses on gaining an understanding of the impact the flour carotenoid and flavonoid pigments have on noodle and noodle sheet colour.

The information is presented in two stages. The first stage focuses on assessment of colour impact using colour space analysis. The second stage enabled a more detailed assessment of the type and quantity of wheat pigments using RP-HPLC semi-quantification.

The colour of wheat flour and noodles has long been established as an important wheat quality parameter. It influences end product appearance and hence consumer preference, as it is indicative of quality and perceived taste of a product. In the instance of Chinese noodles, the quality of the product is dependent on the inherent wheat colour properties, texture and protein. Both the carotenoids together with the flavonoids are responsible for the yellow colouration of noodles. In the instance of alkaline noodles, the flavonoids respond to the addition of Kansui (alkaline salt) by producing a strong yellow colour. They are sensitive to the alteration in pH due to cation and anion exchange on the flavanyl structure. Under acid conditions the flour flavonoids become clear, under alkaline conditions bright yellow to orange depending on concentration.

As noodles are made from very simple ingredients, flour, water and salts, they are dependent on the inherent colour attributes of wheat. The wheat flour colour dictates its suitability for noodle production as well as grain hardness and protein, which influence the firmness and texture of the noodles.

A simple way to measure the colour attributes in terms of lightness, yellowness and hue is achieved using tristimulus colourimetry, CIELAB space parameters. Using colourimetry, a high correlation between carotenoid content and Minolta b* values
has been observed by several researchers (Hook et al, 1985; Oliver et al, 1992). Less information is available on the direct influence of the flavonoid compounds although it is understood that in the instance of alkaline noodles, the flavonoids respond by producing a strong yellow colour, due to the sensitivity of flavone structure to pH change (Oh et al., 1985). To obtain a greater understanding of the role both pigments have on the lightness, hue and yellowness of flour colour, pigments were removed from the flours that were compared with unextracted flours. To define further the role each pigment group plays with colour, both white salted and alkaline noodles were prepared from flours. Comparison was achieved by measurement of colour at different stages of extraction and noodle preparation.

9.1 Methods and materials

9.1.1 Colour analysis

Colour measurements of flour, noodle sheets and noodles, both raw and cooked, prepared from unextracted and pigment extracted flours with a Minolta meter (CR-300) following the Judd-Hunter L* a*, b* system described in Section 3.6.2.

9.1.2 Pigment extraction

These experiments were designed to specifically isolate the influence of each of the pigment groups on the noodle products. Pigments were extracted from 100 g of Batavia flour (Section 3.4.3) using three different techniques. Carotenoids were removed using hexane-diethyl ether as described in Section 3.6.5.1.2. For HPLC quantification of the carotenoids β-apo-8'-carotenal was added (50 µg.ml⁻¹) prior to analysis. Flavonoids were extracted with methanol as described in Section 3.6.3.2. Prior to extraction, kaempferol (50 µg.ml⁻¹) was added to facilitate flavonoid semi-quantification. All pigment was removed from the flour by extracting, firstly with hexane and diethyl ether as described in Section 3.6.5.1.2, then extracting the flour after drying at room temperature overnight, following the procedure described in Section 3.6.3.2.
9.1.3 Dough sheet and noodle preparation

Chinese alkaline and salted noodles were prepared following standard procedure Section 3.4.9.1 for alkaline noodles and Section 3.4.9.2 for white salted noodles. Dough sheets were prepared as described in Section 3.4.9.1, step 3. Noodles were prepared from carotenoid extracted flour, flavonoid extracted flour and total pigment extracted flour.

9.1.4 Noodle characteristics

To observe physical changes that may have occurred to the flour structure as a result of the extraction process, the optimum cooking time (Section 3.4.9.4) and water absorptivity of the noodles was assessed (Section 3.4.9.5). Noodle pH was also checked as described in Section 3.4.9.3.

9.1.5 HPLC of wheat flour pigments

A Varian chromatographic system as described in Section 3.5.1.1 was employed. To facilitate the separation and quantification of pigments a C18 column (Section 3.5.1.7) and C18 guard column (Section 3.5.1.8) were employed. For carotenoid analysis 70 µl of sample was injected. A gradient elution system using acetonitrile-water and ethyl acetate as described in section 3.6.1.2 enabled separation.

For flavonoid analysis a gradient elution system with methanol and formic acid-water was employed as described in Section 3.6.1.1 for separation with 70 µl of sample injected.

Result integrity was examined by determining injection reproducibility and the RSD (Section 3.6.1.3.3) of five injections of lutein, β-carotene and β-apo-8’carotenal as well as by assessing the extraction efficiency as described in Section 3.6.1.3.4. The extraction efficiency of the flavonoid was also assessed using this approach with the inclusion of a kaemferol to the sample prior to extraction.
9.1.6 Semi-quantification of pigments

The semi-quantification of the flavonoid and carotenoid pigments was undertaken as described in Section 3.6.1.3.2. The internal standard Calculations were adjusted to consider the injection volume, amount of initial sample used and the amount of wheat extracted. Only the fractions with either carotenoids removed or flavonoids removed were examined. An extract with both pigment groups removed was not examined.

9.2 Results and discussion

Removal of carotenoid and total pigments from the flour caused a marked increase in flour lightness (Figure 9.1) an increase in flour redness and a decrease in flour yellowness. The yellowness (b* value) of the flour reduced marginally with removal of flavonoids. The removal of both pigment groups (Method 3) resulted in total yellow colour loss. This clearly shows that both carotenoids and flavonoids influence flour colour. Comparison of the unextracted flour with flavonoid extracted flour showed only minor colour change. The influence of flavonoids was only observed when both pigment groups were removed.

With removal of the pigments from the wheat the flour characteristics altered. Flour extracted for carotenoids became a fine red tinged powder. Flour with flavonoids extracted became dough like and sticky and dried into large particles. To ensure that all flour was of the same particle size, flours were passed through a 30-mesh sieve prior to noodle making. The flavonoid extracted flour required grinding with a mortar and pestle to obtain a fine flour particle size. Solvent extraction resulted in flour moisture removal. To achieve dough that held its form, additional salt and alkaline solution (2 mL) was added to these flours.
The alkaline and salt noodle colour attributes were examined colourimetrically, (Figure 9.2). The raw noodle sheets were used for colour comparison. The addition of salt or alkali to the same sample caused a different colour response, which was observed for all flour treatments. Salted noodles displayed higher a* value readings and lower b* value readings than alkaline noodles although difference between each are only slight. Noodle brightness (L* value) for each type of noodle was similar.

When the alkaline and salted noodle colours were compared with the results obtained for the flour (Figure 9.2, unextracted) a large difference in the lightness, hue and yellowness was observed for all treatments. With the addition of kan sui noodles became redder and more yellow. Both the carotenoid and flavonoid pigments showed higher b* values.
Differences between the colour of alkaline noodle sheets, raw and cooked noodles were observed (Figure 9.3 and photographically represented Figure 9.4). This is principally due to different surface reflectance. Dough sheets are flat whilst noodle stacks are uneven. The unevenness of the noodle stack resulted in a higher incidence of light scattering and subsequently a reduction in L* value. This was observed in all flour treatments but was most noticeable by comparison of the unextracted flour dough with the raw noodle.

With the cooking of the noodles, a reduction in the lightness, hue and yellowness was observed in all treatments. This was thought to have occurred due to heat and moisture induced changes to the light reflection and refraction properties of the flour and, perhaps, changes in pigments. Changes in the physical structure of the flour were observed. Unextracted flours exhibited a longer optimum cooking time and
displayed a higher water absorptivity percentage than flours (22 minutes versus 13 minutes and 278 % versus 199 % respectively). These differences indicated that the extraction process itself caused changes to flour starch and protein components enabling faster water uptake (Personal communications, 1998).

The removal of carotenoids caused a reduction in lightness and yellow colouration and an increase in hue observable at all stages of noodle processing. The removal of flavonoids resulted in a slight reduction in the lightness of dough sheets and a significant reduction in the lightness of raw and cooked noodles. A marked reduction in hue with colour measurement moving towards green and an increase in b* values was observed for all stages. This increase is assumed to have occurred due to the addition of alkaline solution to the noodle, as an increase in brightness was not observed in flavonoid extracted flours. It also would appear that with the preparation of flavonoid extracted flour not all of the flavonoids were removed during the methanol extraction process. With the removal of both pigment groups a reduction in yellow colouration was again observed. This suggests that there were not any flavonoid pigments remaining to react with the alkali solution.
Figure 9.3. Colour comparison of dough sheets, raw and cooked noodles.
Alkaline treatment results in a darker colour noodle most readily observed for noodles prepared from un-extracted flour (Figure 9.5). With the removal of flavonoids noodles appear quite yellow, attributed to the yellow colour contribution of carotenoids. With the removal of carotenoid both alkaline and salted noodles appear dull. Flavonoid and carotenoid extracted flour offers a noodle colour that is much lighter signifying the impact that pigment groups have on flour colour.
Colour development is very evident with the alkaline treatment attributed to the flavonoid pigments, which respond to pH changes. In this trial the presence of flavonoids and carotenoids in the flour was found to influence the lightness, hue and yellowness of the flour, dough, raw and cooked noodles. The removal of both pigment groups resulted in whiter, redder flour with less yellow colouration. Carotenoids appeared to contribute more to yellow colouration than flavonoids. Before this can be confirmed, however, it is imperative that total flavonoid extraction with methanol be proven to have occurred. The pigment influences were best observed by comparison of flour treatment measurements, as both pigment types were influenced by the addition of salt and alkaline solutions in noodle preparation. In this trial, many different factors influenced flour and noodle pigment analysis. Flours were structurally altered, both pigment groups were found to respond to salt and alkali solutions, and colour values were found to differ with measurement of the different stages of noodle processing. Cooked noodle measurements showed the lowest colour values for all flour treatments.
Further examination or elimination of these variables is required to definitively establish the influence of each pigment group, however, this method has clearly provided more evidence on the role both flavonoids and carotenoids play on wheat colour.

Extraction efficiency was determined for the carotenoid compounds by calculating the amount of IS identified in the sample and was found to be 54 % for the extraction of carotenoids. Injection reproducibility was determined by injecting a standard solution 3 times (Table 9.1) and by calculating the reproducibility of standard added to sample (Table 9.2) this also enabled determination of the extraction efficiency for removal of carotenoids.

Standard injection reproducibility was found to be very good with 1.4 % relative standard deviation for lutein and 3 % for β-carotene. IS reproducibility in sample was found to be 0.10 % coefficient of variation, for retention time and 8.20 % for peak area counts. For flavonoids, the injection reproducibility was assumed to be similar to that of the carotenoids.
Table 9.1. RP-HPLC injection reproducibility

<table>
<thead>
<tr>
<th>Run No.</th>
<th>PEAK AREA</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lutein (5 µg.g⁻¹)</td>
<td>β-apo-</td>
<td>β-carotene (10 µg.g⁻¹)</td>
</tr>
<tr>
<td></td>
<td>β-apo- 8′carotenal (50 µg.g⁻¹)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>611204</td>
<td>1215743</td>
<td>44656</td>
</tr>
<tr>
<td>35</td>
<td>614054</td>
<td>1216580</td>
<td>43058</td>
</tr>
<tr>
<td>36</td>
<td>607714</td>
<td>1226473</td>
<td>41550</td>
</tr>
<tr>
<td>37</td>
<td>595085</td>
<td>1223697</td>
<td>42801</td>
</tr>
<tr>
<td>AV</td>
<td>607014.3</td>
<td>1220623</td>
<td>43016.25</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>8364.782</td>
<td>5286.216</td>
<td>1276.29</td>
</tr>
<tr>
<td>RSD</td>
<td>1.40%</td>
<td>0.40%</td>
<td>3%</td>
</tr>
</tbody>
</table>

Table 9.2. Characteristics of standards added to Batavia wheat flour and extracted with this flour.

<table>
<thead>
<tr>
<th>Run No.</th>
<th>Sample</th>
<th>Retention time of β-apo-8′carotenal</th>
<th>Peak area of β-apo-8′carotenal</th>
<th>Extraction efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>46</td>
<td></td>
<td>12.92</td>
<td>118276</td>
<td>Av IS1092271</td>
</tr>
<tr>
<td>47</td>
<td></td>
<td>12.95</td>
<td>108807</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td></td>
<td>12.94</td>
<td>128313</td>
<td></td>
</tr>
<tr>
<td>Av</td>
<td></td>
<td>12.93667</td>
<td>118465.3</td>
<td>54 %</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.015275</td>
<td>9754.378</td>
<td>54 %</td>
<td></td>
</tr>
<tr>
<td>RSD</td>
<td>0.10%</td>
<td>8.20%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A number of major peaks were detected in the Batavia flour (Figure 9.6). Consistent with previous findings, a peak tentatively identified as lutein eluted early at around 10 minutes with the hydrocarbon carotenoids detected after the IS from 20 minutes onward.
Figure 9.6. Batavia wheat extracted for carotenoids and analysed at a wavelength of 420 nm using a gradient elution system of acetonitrile water (90:10 v/v) at 40 % and ethyl acetate at 60 % to 100 % in 30 minutes and held for 10 minutes.

The five major peaks were quantified and found to represent approximately 38.1, 23.7, 3.5, 7.0 and 17.6 µg.g⁻¹ of carotenoids respectively (Table 9.3). The carotenoid pigments were found to contribute 148.7 µg.g⁻¹ of colour to the noodle sheets and noodles.
Table 9.3. Major carotenoid and total carotenoid pigment contribution to flour, noodle sheet and noodle colour.

<table>
<thead>
<tr>
<th>Peak</th>
<th>RT</th>
<th>Peak area</th>
<th>Concentration ug.g⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10.34</td>
<td>2818696</td>
<td>38.09</td>
</tr>
<tr>
<td>IS</td>
<td>15.67</td>
<td>2590187</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>26.05</td>
<td>1755751</td>
<td>23.72</td>
</tr>
<tr>
<td>3</td>
<td>30.79</td>
<td>259209</td>
<td>3.50</td>
</tr>
<tr>
<td>4</td>
<td>32.55</td>
<td>519633</td>
<td>7.02</td>
</tr>
<tr>
<td>5</td>
<td>23.99</td>
<td>1305534</td>
<td>17.64</td>
</tr>
<tr>
<td>Total carotenoids</td>
<td></td>
<td>11005596</td>
<td>148.71</td>
</tr>
</tbody>
</table>

A relatively low extraction efficiency of 53 % was obtained for flavonoids extracted from the flour. Despite this, however, a number of flavonoid pigments were identified in the flavonoid extracted flour with three major peaks identified at 6 minutes 9.8 and at 18 minutes. The IS eluted at 26 minutes (Figure 9.7). These compounds were found to contribute 48.1, 24.1 and 72.3 µg.g⁻¹ (Table 9.4) for a total flavonoid pigment concentration, and hence the influence on flour and noodle colour was 209 µg.g⁻¹.
Figure 9.7. Batavia wheat flour extracted for flavonoid pigments and examined at 280 nm using a methanol formic acid water gradient election system.
Table 9.4. Major flavonoid and total flavonoid pigment contribution to flour, noodle sheet and noodle colour.

<table>
<thead>
<tr>
<th>Peak</th>
<th>RT</th>
<th>Peak area</th>
<th>Concentration µg.g⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.06</td>
<td>1211312</td>
<td>48.07</td>
</tr>
<tr>
<td>2</td>
<td>9.81</td>
<td>606505</td>
<td>24.07</td>
</tr>
<tr>
<td>3</td>
<td>18.56</td>
<td>1821000</td>
<td>72.26</td>
</tr>
<tr>
<td>IS</td>
<td>26.68</td>
<td>441015</td>
<td></td>
</tr>
<tr>
<td>Total flavonoids</td>
<td></td>
<td>5272028</td>
<td>209.20</td>
</tr>
</tbody>
</table>

9.3 Conclusion.

Both pigment groups contribute to the colouration of flours and to the colour of alkaline and salted noodles.

Carotenoid compounds appear to contribute more to the yellowness of the flour consistent with the findings of others and the flavonoids to the colouration of alkaline noodles even more so to the hue of noodle sheets and noodle colour.

Flavonoids were found to contribute an approximate concentration of 209 µg.mL⁻¹ whilst carotenoids were found to contribute a slightly lower concentration contribution of approximately 148 µg.mL⁻¹.
CHAPTER 10: METHODOLOGY INVESTIGATIONS FOR THE COMPREHENSIVE IDENTIFICATION OF FLAVONOID PIGMENTS

10.0 Introduction

The principal aim of the following experiments was to identify methods that would facilitate the comprehensive identification of the major flavonoid pigments in wheat. RP-HPLC with PAD has enabled the characterisation of several different varieties of wheat flour and bran flavonoid as well as phenolic compounds. For conclusive identification several different structural identification techniques that provide supporting information are required to confirm compound identity. Supporting structural information may be gained from mass spectral data, which provides information on the molecular ion and the position of OH, methyl and sugar functional groups of flavonoids. Current methods for flavonoid mass spectral analysis include Electrospray Mass Spectroscopy (EMS) and Fast Atom Bombardment Mass Spectroscopy (FAB). Traditional methods have involved GCMS with derivatisation of the flavonoid to facilitate its ionisation and subsequent identification.

Three different mass spectral techniques were employed to established the most appropriate method EMS, FAB and Gas Chromatography Mass Spectroscopy (GC-MS). For analysis by EMS and FAB the complex flavonoid extracts required purification. For GCMS analysis flavonoid extracts required derivatisation to make the non volatile flavonoids volatile. Nuclear magnetic resonance (NMR) spectroscopy was also employed to assist in confirmation or otherwise of compound derivatisation and to provide structural information. The function of the different trials conducted was to determine a superior method for the structural determination of flavonoids, by analysis of several flavonoid standards and a wheat flavonoid extract. A method that produced strong molecular ion and fragment ion ionisation that would readily support compound identification was considered superior.
10.1 FAST ATOM BOMBARDMENT MASS SPECTROSCOPY

10.1.1 Introduction

Fast Atom bombardment mass spectroscopy is a soft ionisation method that may be used for the analysis of non-volatile organic compounds such as flavonoids. The technique involves dissolving the sample in a polar matrix (i.e. thioglycerol) and application of the sample onto the end of a probe, which is inserted into the mass spectrometer. The sample is bombarded with a fast stream of either xenon atoms or caesium ions. Both positive and negative ions are produced and are analysed by the mass spectrometer.

In a paper published by de Koster and his colleague (1985) the FAB-MS of flavonoids was described. The positive ion spectra showed an abundance of [M+H]+ ions. The aglycone structure and monosaccharide units for glycosides were readily distinguishable. The type of linkage to the aglycone and the position on of monosaccharide units was not determinable.

Fast atom bombardment MS was applied to the analysis of purified flavonoids extracted from Rosella wheat bran. These samples were purified using open column chromatographic techniques employing size exclusion principles and by RP-HPLC. The flavonoid standards rutin and quercetin were also analysed.

10.1.2 Method and Materials

10.1.2.1 Extraction and open column purification procedures.

Bran from the wheat variety Rosella (Section 3.4.1) was extracted with alkaline water as described in Section 3.6.3.1. The extract was purified on open column chromatography as described in Section 3.6.4.1. The alkaline extract was examined by RP-HPLC following the procedure described Section 3.5.3.1 using a C18 column (Section 3.5.1.6) and guard column (Section 3.5.1.8).
A methanol extract from Rosella bran (Section 3.6.3.1) was collected from open column purification on Sepadex G-15, between 40 mL to 200 mL of the methanol eluate (Section 3.6.4.1). Further purification of the extract was conducted using fraction collection post RP-HPLC column separation, as described in Section 6.4.2.3.2. The methanol extract was examined by RP-HPLC following the procedure described Section 3.6.1.1 using a C18 column (Section 3.5.1.6) and guard column (Section 3.5.1.8).

### 10.1.2.2 Fast atom bombardment

Fast Atom bombardment of flavonoids was achieved using a A JEOL DX303 mass spectrometer as described in Section 3.5.3.2. Samples were prepared in thioglycerol matrix prior to analysis by mixing. The standards, rutin, quetcentin and the semi purified wheat flour extract were prepared in this manner.

### 10.1.3 Results and discussion

Before FAB analysis, the bran extracts were examined by RP-HPLC to assist in understanding their characteristics, the complexity and number of compounds within the extracts. The alkaline bran extract contained a number of different compounds (fifty five in all) of which four major pigment eluting from 18 to 20.4 minutes were evident (Figure 10.1). The methanol extract that was further purified by fraction collection contained less compounds (ten in all) at a lower relative intensity when compared to the alkaline extract, with major pigments evident at 19 to 22.4 minutes (Figure 10.2).
Figure 10.1. Chromatographic representation of alkaline extracted Rosella bran using a methanol and formic acid-water gradient elution system and a C-18 column and guard column with compound detection at 280 nm.
Figure 10.2 Chromatographic representation of methanol extracted Rosella bran purified by RP-HPLC post column fraction collection. Compounds were separated with a methanol and formic acid-water gradient elution system and a C-18 column and guard column, with compound detection at 280 nm.

The FAB mass spectra of rutin (Figure 10.3) and quercetin (Figure 10.4) display recognisable protonated molecular ions at m/z 303, as the base peaks. Unfortunately, this method did not enable the ionisation and detection of the fragment ions characteristic of these compounds at m/z 153, 121 and 165 (de Koster et al, 1985). The rutin spectra showed a peak for the glycosylated molecule at m/z 611 and that of the aglycone moiety (m/z 465) indicating the elimination of C₆H₁₀O₄. The quercetin standard showed high molecular weight peaks (m/z 315-514). The fragment ions at m/z 131 and 147, may be those formed by cleavage of the C ring in the flavonoid moiety originating from Retro-diels-Alder reactions (Figure 10.5).
Figure 10.3. Positive ion FAB mass spectra of the flavonoid standard rutin.

Figure 10.4. Positive ion FAB mass spectra of the flavonoid standard quercetin.

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The alkaline extracted sample (Figure 10.5) and the methanol extracted sample extract (Figure 10.6) show no clearly recognisable molecular ion characteristic of flavonoids at m/z 303. The methanol extract sample displayed an odd-electron ion ($M^+$) at m/z 301 which may represent the aglycone.

Figure 10.5 Positive ion FAB of the alkaline extract of bran.
A base peak was evident at m/z 153 in both mass spectra. This peak at m/z 153 may represent an ion fragment produced by the cleavage of the C ring from the flavonoid structure (Figure 10.7). The m/z ion 131 is present in both mass spectra of the flavonoid standards and in the alkaline extracts. The spectra of the extracts are therefore indicative of flavonoids although ultimate identification was not achieved.
10.1.4 Conclusions and Recommendations

FAB-MS of wheat bran flavonoid extracts did not facilitate conclusive identification of major pigments previously identified in wheat bran using RP-HPLC with PAD. It is thought that this was because sample purity was not sufficient to facilitate identification. The FAB mass spectra of quercetin and rutin taken in this present study differed from those published for these compounds, possibly a function of different instrumental analysis.

Similar fragmentation ions to standard compounds were detected for wheat flavonoid extracts supporting previous information from RP-HPLC analysis that the compounds isolated from wheat bran are flavonoids.

10.2 ELECTROSPRAY MASS SPECTROSCOPIC ANALYSIS OF FLAVONOIDS ISOLATED FROM ROSELLA WHEAT.

10.2.1 Introduction

Electrospray mass spectroscopy involves transference of the ions in a solution phase. The solution is ionized resulting in protonation or deprotonation of the molecule depending on the nature of the compound; for example amino acids are protonated and alcohol and acids deprotonated. As electrospray mass spectroscopy uses solutions, it can be coupled to liquid chromatography systems such as RP-HPLC for the separation of complex mixtures in order to identify individual pigments.

Initial experiments involved the analysis of flavonoid standards rutin and apigenin, the phenolic compound ferulic acid and two partially purified flavonoid mixtures extracted from Rosella wheat bran.
10.2.2 Method and materials

10.2.2.1 Sample preparation.

Samples of standards rutin, apigenin and ferulic acid (300 μM) were prepared by dissolving the compounds in methanol:water (50:50 %,v/v) and adding 1 % acetic acid. Rutin (300 mM) was prepared by dissolving 1.83 g in 1 L methanol: water (50/50 v/v) and heating with stirring until no precipitate was evident. An aliquot of this solution was diluted 1 mL in 9 mL solvent to obtain a 300 μM solution. Apigenin was prepared by dissolving 0.0081 g in 10 mL solvent to produce a 3 mM solution and then diluting 1/10 to get 300 μM. Ferulic acid was prepared by preparing 0.0058 g in 10 mL solvent and diluting this 1/10.

An alkaline bran extract and a methanol extracted bran as described in Section 10.1.2.1 were used in this investigation. The alkaline extract was prepared by diluting 20 μL of reduced solution in 9 mL solvent. The methanol extract by diluting 1 mL sample in 9 mL diluent.

10.2.2.2 Instrumentation

Mass spectra was obtained with a VG Bio Q triple-quadropole MS as described in Section 3.5.3.3. Spectra were recorded in the positive and negative mode for the standard rutin. All other samples were recorded in the negative mode only at varying voltages, in an attempt to find the optimum (Section 3.6.6.3.1).

10.2.3 Results and discussion

The flavonoid standard rutin was examined in the negative ion mode at an initial B<sub>i</sub> voltage of 85 v (Figure 10.8). The deprotonated molecular ion peak was clearly evident at 609.1 m/z (M-H). No evidence of fragmentation was present. To visualise the aglycone fragment and removal of the sugar O-glycosidic linkage, the voltage was increased to B<sub>i</sub> = 150 v (Figure 10.9). Detected was a daughter ion at m/z 268
609.1, along with a high intensity deprotonated aglycone ion at m/z 300. A signal at m/z 465 representing removal of a rhamnosyl moiety with fragmentation of the glycosidic linkage of the sugar was not evident. To increase fragmentation signals, the second quadropole was turned on and argon was used for solvent nebulising and drying. The first quadropole acted as a filter and the second as a multiplier, however, resolution is reduced. No signals were observed due to an insufficient number of ions. The voltage was reduced to \( B_1 = 115 \) v (Figure 10.10), to ensure that excessive energy was not applied. The molecular ion peak was observed at a greater intensity than the aglycone peak at 300 m/z caused by a reduction in energy.

![Figure 10.8. Electrospray mass spectra of rutin in the positive ion mode at \( B_1 = 75 \) v.](image)

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Figure 10.9. Electrospray mass spectra of rutin in the positive ion mode at $B_1 = 150 \text{ v.}$

Figure 10.10. Electrospray mass spectra of rutin in the positive ion mode at $B_1 = 115 \text{ v.}$
The positive ion spectra of rutin (Figure 10.11) collected at $B_1 = 75$, did not display a molecular ion peak clearly distinguishable from the background noise. The protonated aglycone signal was observed at m/z 302.9. Other real peaks at m/z 272.9, 288.8 and 537.8 were present. These ions are not characteristic of fragment ions. Their presence in other positive ion spectra was not observed. In addition, their presence cannot be accounted for as these ions were not present in the background spectra.

![Data File: SU4. Acquired on 25-Jan-96 at 18:15 La Trobe University Sample: ES+ Rutin MeOH/H2O/HCl acid B1=75](image)

**Figure 10.11. Positive ion mode of rutin at a voltage of $B_1 = 75$ v.**

When the voltage was increased to $B_1 = 115$ the protonated aglycone signal at m/z 303 was observed although the protonated molecular ion peak at m/z 611 was still not evident (Figure 10.12). A high intensity signal at m/z 419.9 has not been accounted for.
Figure 10.12. Positive ion mode of rutin at a voltage of \( B_1 = 115 \) v.

Reduction of the ionisation energy to \( B_1 = 33 \) prevented fragmentation of the molecular ion and therefore its visualization, as a signal at m/z 611.1 (Figure 10.13). This confirmed that fragmentation of the ion had occurred at the higher ionisation energies applied.
A second high intensity peak present in the spectra of rutin at m/z 279.1 was found to be a solvent peak when a background scan was run (Figure 10.14).

Figure 10.14. Positive ion mode assessment of background noise.
In the positive ion mode, the occurrence of solvent interference increases. A residual signal from previous analysis of rutin at m/z 611 was observed suggesting that not enough time between runs for removal of sample residue was provided. Residual sample may be removed by increasing the flow rate of the mobile phase through the mass spectrometer.

Closer inspection of the molecular ion peak at m/z 611.1 in the positive ion mode (Figure 10.15), showed signals at m/z 612.0 and 613.1 which represent carbon 13 and carbon 14 molecules respectively. At m/z 630 a sodium adduct may be observed.

![Figure 10.15. Electrospray mass spectra of rutin in the positive ion mode. The presence of C13 and C14 ions is observed.](image)

Apigenin was scanned in the negative ion mode at B₁ = 150 v. The deprotonated aglycone peak was observed at m/z 269.0 (Figure 10.16). A very low intensity peak at m/z 150.9 may represent an aglycone fragment C₇H₄O₄ following the
fragmentation pattern following the RDA pathway. This fragment was more readily observed at $B_1 = 80$ v (Figure 10.17).

Figure 10.16. Apigenin electrospray mass spectra in the negative ion mode at $B_1 = 150$ v. The molecular ion is observed at m/z 150.9.

Figure 10.17. Apigenin molecular ion observation at $B_1 = 80$ v.
A 1 mM solution of ferulic acid in methanol:water was injected and analysed at $B_1 = 40$ which enabled observation of the deprotonated molecular ion peak at m/z 192.9 (Figure 10.18). The increase of ionisation energy to $B_1 = 85$ v resulted in a fragment ion at m/z 177.9 suggesting the removal of an OH group, at m/z 148.8 the removal the OH and OCH$_3$ molecule leaving the benzene molecule with the carboxylic group attached (CH=CHCOOH) (Figure 10.10 B). The signal at m/z 78.8 may represent the basic benzene structure of the compound.

![Figure 10.18. Ferulic acid electrospray mass spectra in the negative ion mode at $B_1 = 40$ v showing the deprotonated molecular ion at m/z 192.9](image-url)
Figure 10.19. Ferulic acid at B1 = 85 v with fragment ions were evident at m/z 177.9, 148.8, 133.8 and 78.8.

When a background scan was performed with injection of the solvent system of 50 % aqueous methanol and 1 % acetic acid, it was established that high intensity peaks at m/e 118.9, 89.0 and 75.0 were the result of the solvents (Figure 10.20).

Figure 10.20. Electrospray mass spectra, negative ion mode. Background signals (noise) are evident with injection of the sample solvent 50 % aqueous methanol and 1 % acetic acid.
The alkaline extracted bran, Sample 1, was examined at $B_1 = 50$ v. This sample showed high molecular ions at low intensity and a strong signal at m/z 132.9 (Figure 10.21). The presence of flavonoid aglycone peaks at m/z 300.0 or 269 or ferulic acid peaks at m/z 192.9 were not observed.

Figure 10.21 Electrospray mass spectra in the negative ion mode of alkaline extracted Rosella bran.

Examination of the methanol extract ($B_1 = 40$ v) showed a high intensity signal at m/z 140.9 and many low intensity peaks (< 30 % I) between m/z 223 and m/z 952.1. The spectral patterns are not characteristic of flavonoids. It is interesting to note that a decrease of approximately 82 mass units between fragments was observed. There were no similarities between the spectra of the methanol and alkaline extracts.
Neither the alkaline or methanol Rosella extracts exhibited spectral patterns consistent with those observed for the standards examined or fragmentation patterns discussed in literature. It is suspected that the impurity of the samples hindered confirmation of the structure of the compounds. Further purification is required before information by EMS can be utilized to establish the molecular structure of the compounds isolated from the bran.

10.2.4 Conclusion

Electrospray mass spectroscopy of the flavonoid and phenolic standards was successful for the identification of molecular ion structure of standard compounds. The use of an EMS in the negative ion mode provided information on the deprotonated molecular ion and the aglycone structure and in some instances the fragmentation pattern of the purified compounds. The spectra of the Rosella bran
extracts was not of sufficient purity to facilitate the identification of flavonoid compounds within the bran. Further efforts to obtain sufficient amounts of purified samples did not result in attainment of individual compounds confirming the complexity and similarity of wheat flavonoid structure already observed in RP-HPLC chromatograms of extracts.

Further analysis using isopropyl alcohol and ammonium acetate was thought to be beneficial to facilitate and increase the ionisation of wheat flavonoid molecular ions, to obtain more information on the fragmentation patterns that would assist in their subsequent identification.

10.3 ELECTROSPRAY MASS SPECTRAL ANALYSIS OF FLAVONIODS ISOLATED FROM ROSELLA BRAN.

10.3.1 Introduction

Previous analysis of partially purified wheat bran flavonoid extracts using FAB and EMS with analytes dissolved in methanol water, with 1 % acetic acid in the negative ion mode, did not facilitate comprehensive identification of samples. It was thought that better sample ionisation may be achieved using a different solvent and by examining the compounds in the positive ion mode. In this experiment, isopropyl alcohol: water (50:50, v/v) and 10 mM ammonium acetate was used to enhance ionisation to facilitate greater signal intensity. To maximise sample ionisation, analytes were examined at various voltages in both the positive and negative ion mode.

10.3.2 Method and materials

10.3.2.1 Sample preparation

A methanol extract from Rosella bran (Section 3.6.3.2) was collected from open column purification on Sepadex G-15 between 40 mL to 200 mL of the methanol
eluate (Section 3.6.4.1) Further purification of the extract was conducted using fraction collection post RP-HPLC column separation, as described in Section 6.4.2.3.1. The sample solution was concentrated to dryness with heating and was redissolved in 50 µL deionised water (DiH₂O) and 50µL isopropyl alcohol with 10 mM ammonium acetate.

Flavonoid and phenolic standards were also analysed to verify procedure. The rutin standard (300µM) was prepared by combining 142.9 µL of a 1049 µM stock solution solubalized in methanol with 250 L water, 250 L isopropyl alcohol and 50 L ammonium acetate. The apigenin standard (300 µM) was prepared by combining 300 µL of a 500 µM stock solution in methanol with 250 µL water, 250 µL isopropyl alcohol and 50 µL ammonium acetate. Ferulic acid was prepared from 1000 µM stock solution in methanol in the same manner as apigenin but with 150 µL volume of the stock solution. The range of sample concentrations was therefore below 300 µM due to the sample volume exceeding 500 µL.

10.3.2.2 Instrumentation.

Mass spectra was obtained using the instrumentation described in Section 3.5.3.3.

10.3.3 Results and discussion

Negative ion spectrum of the rutin standard was obtained at B₁ = 115 to facilitate identification of the parent ion and the aglycone structure (Figure 10.23). At this voltage, the molecular ion peak was observed at m/z 609.1. The aglycone structure at m/z 300 was evident. The removal of the rhamnosyl sugar moiety was not detected at m/z 464. Detection of sugar groups and linkage sites to the base flavonoid structure has not been possible with EMS. Signals between relative intensity percentages 30-50 at m/z 126.8, 79.8 and 96.8 in order of increasing intensity were evident.
Closer inspection of the parent ion at m/z 609.1 showed the presence of C13 molecules at m/z 610.1 (Figure 10.24).

Figure 10.23. EMS spectra of rutin (300 µM). B_i = 115 v, ionisation solution isopropyl alcohol water (50:50, v/v) with 10 mM ammonium acetate.

Figure 10.24. EMS spectra of the rutin standard displaying the presence of C13 molecules at m/z 610.1.
The presence of C13 and C14 molecules were also observed at the aglycone fragment with m/z of 300.1 and 301.9 respectively (Figure 10.25).

Figure 10.25. EMS of the rutin standard were the C_{13} and C_{14} molecules are displayed at m/z 300.1 and 301.9.

Initially the apigenin standard was analysed at a voltage of B1= 150 v. The molecular ion was observed at m/z 268.8 (A-H) (Figure 10.26).

Figure 10.26. EMS of apigenin (300 µM) B_{1} = 150 v.
The base peak was observed at m/z 116.8 as was a second high intensity signal at m/z 162.9. The base peak may represent a fragment produced via RDA elimination, resulting from the fragmentation of the A ring.

Closer observation of the spectra between m/z 240-315 showed less intense signals at m/z 269.8 and 271.0 suggesting the presence of carbon 13 and 14 (Figure 10.27). A signal at m/z 300 may suggest the presence of the molecular ion with the addition of oxygen and hydrogen.

Figure 10.27. Carbon 13 and 14 signals observed for apigenin.

The apigenin standard was also analysed at B1 =115 (Figure 10.28). At this voltage, the spectral signal was observed at a higher intensity. At the lower voltage the signal at m/z 162.9 was represented, as the base peak, with the signal at m/z 116.8
occurring at 90 % relative intensity (RI) and the base peak at 48 % RI. At this voltage a possible fragment depicting B ring fragmentation at m/z 150.7 was observed.

Figure 10.28. EMS of apigenin (300 M) at b1=115 v.

The ferulic acid spectra was analysed at B1 = 85 v. A base peak at m/z 133.7 representing a fragment ion resulting form the removal of CHCOOH (m/z 58) from the parent ion (Figure 10.29).
A high intensity signal at m/z 255.1, 28.1 and 283.1 cannot be accounted for. A background scan was run to establish interfering peaks that may result from retention of previous sample molecules due to the high viscosity of the sample and propellant solvent (Figure 10.30). The high intensity peaks observed in the ferulic acid spectra were present. This therefore suggests that the high intensity peaks above m/z 205 are the result of compound retention in the system. Other low intensity signals at m/z 70.9, 81.8 and 96.8, 116.8, 140.9, 144.8, 156.7, 170.9 and 183.8 also appeared as background noise. Although a molecular ion peak was observed at m/z 192.9, its intensity was low.
A second scan of ferulic acid at B₁ = 40 was performed (Figure 10.31). The molecular ion peak was observed as the base peak at m/z 192.8. The fragment ion at m/z 133.7 was not evident which suggests that the higher voltage is necessary to facilitate detection of fragments ions. A signal at m/z 118.8 with a relative intensity of 38 % can not be conclusively accounted for, at this stage, but may represent a fragment of the parent ion.
Figure 10.31. EMS of Ferulic acid B₁=40 v.

The wheat bran sample was examined at a B₁ voltage of 45. The spectra showed a molecular ion peak at m/z 339.2 and a base peak at m/z 140.8 (Figure 10.32). The overall intensity of the signal was weak, which resulted in high intensity background noise, i.e. m/z 222.9 and 283.2. A more concentrated sample is required to facilitate analysis.
The bran sample was also analysed at $B_1 = 85$ v. A signal of 100 % relative intensity at m/z 339.2 signified the molecular ion that was also the base peak. This molecular ion was not found to be similar to other flavonoid standards. No additional information on the structure of the compound was provided at the higher scan voltage (Figure 10.33).
Figure 10.33. EMS of a wheat bran sample at $B_1 = 85$ v.

Because signal intensity is an arbitrary response dependent on the largest peak signal of each individual sample, comparison of spectra is not possible.

A more concentrated sample of the wheat bran extract was analysed at $B_1 = 45$ v (Figure 10.34). A molecular ion peak was observed at m/z 265 with a relative intensity of 42%. A base peak at m/z 140.8 alongside a high intensity signal of 90% relative intensity at m/z 126.7 was observed with an additional high intensity peak at m/z 118.8.
This sample was also analysed at a B\textsubscript{1} voltage of 85 v (Figure 10.35). A possible molecular ion peak at m/z 293.1 with 20 % relative intensity was observed. A higher relative intensity signal at m/z 265.1, RI 48 % was observed. The base peak occurred at m/z 126.6 and another signal at m/z 140.8 of 40 % RI. All the same signals were present in each of the scans at the two different B\textsubscript{1} voltages, however, the signal relative intensities were different. This cannot be explained, especially as a higher voltage should facilitate fragmentation and therefore lower intensity molecular ion signals would be observed rather than a higher one.
10.3.4 Conclusions

Using this method of analysis, it was challenging to confirm the identity of the standard compounds. In many instances, additional fragments not previously recorded in the literature were identified. In all spectra the molecular ion of the standards were readily observed along with base peaks representing fragmentation patterns. The wheat bran extract exhibited some of the characteristics of the flavonoid standards. In addition, the wheat bran extract exhibited a strong molecular ion peak at 339.2 m/z and a base peak at 140 m/z. No conclusive structural information for the flavonoid extracts was obtained. Sample identification was hindered by poor sample purity and by the limited amount of the sample available for analysis.
10.4 GC-MS OF FLAVONOIDS AND ALKALI BRAN EXTRACT

10.4.1 Introduction

To date the flavonoid components in wheat have not been fully characterized. Samples have not been of sufficient purity or concentration to facilitate structural identification using EMS and FAB mass spectrophotometry. Attainment of high purity samples using TLC and HPLC fraction collection has not been achieved. GC-MS has been reported to enable the separation and mass analysis of derivatised flavonoids. Flavonoids are non-volatile polar compounds and therefore required derivatisation to enable separation of individual compounds and detection of these via the GC system.

Furuya, (1965) used GC to separate and purify opium alkaloids and plant glycosides using a column packed with Chromosorb W (60-mesh) coated with 1.5 % silicone rubber SE-30. Samples were trimethylsilated with hexamethyldisilazane and trimethylchlorosilane after 1-2 mg of the flavonoid was dissolved in 0.1 mL pyridine. No information on the GC conditions was provided. GC-MS of perdeuteromethylated flavoniod aglycones was achieved by Schmid et al., (1973). Permethylation was carried out with CD$_3$I, DMF and NaH. For GC a 122 x 0.32 cm stainless steel column containing 5 OV17 on chromosorb W HP,80-100 mesh was used. The column flow rate was set at 30 mL.min$^{-1}$ and the compounds were separated either at 280°C or 260°C isothermal. The eluted compounds were detected with an FID. The GC was coupled to a Mass spectrophotometer. Trimethylsilylate-derivatives have been used previously by Schells and his colleagues (1977), as an alternative to the more expensive and less efficient methylation or perdeuteromethylation derivatisation method (Schels et al,1978). Schels et al, 1977, reported that the intensity of the M+ and M+ - 15 peaks are influenced by the molecular structure including the position of the sugar attachment, the kind of sugar, the type of glycosiolation. He reported that all flavonoid glycosides show the spectral fragments; 217, 204, 147, 117 and 73 which occur with intense signals.
The focus of this research was to identify and adapt a derivatisation and chromatographic methods for the separation and identification of wheat flavonoid compound. Several different methods of derivatisation were employed including using DMF, BSA, DMSO, hexamethyldisilazane and trimethylchlorosilane for the trimethylsilation of the flavonoids. Pure samples of flavonoids, apigenin, quercetin, rutin and morin, of the phenolic, ferulic acid and of the sugar compound rhamnose were used to ensure that interfering compounds did not influence results. A number of different chromatographic column and temperature programs were used to optimise separation and visualization of compounds.

10.4.2 Method and materials

For structural elucidation of flavonoids a Varian Star GC-MS system as described in Section 3.5.4.1 was employed.

Standard compounds were prepared and analysed using a number of derivatisation techniques. A Rosella wheat bran (Section 3.4.1) was extracted following the procedure described in Section 3.6.3.1 and purified following the process outlined in Section 3.6.4.1.

The GC system described in section 3.5.4.1 was used for analysis.

10.4.2.1 Chromatographic temperature program conditions

A number of different temperature programs were employed in an attempt to separate and identify wheat bran flavonoid compounds.

10.4.2.1.1 Method 1

Start column temperature: 60 °C
Hold time: 0 minutes
Rate of temperature increase: 5 °C.min⁻¹

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Final column temperature: 260°C
Temperature hold time: 20 minutes
End time: 60 minutes
FID/ Auxiliary Temperature: 280°C
Injection temperature: 280°C

![GC column temperature profile method 1](image)

**Figure 10.36. GC column temperature profile method 1**

**10.4.2.4.2 Method 2**

Start column temperature: 60°C
Hold time: 0
Rate of temperature increase: 2.5°C. min⁻¹
Final column temperature: 260°C
Temperature hold time: 5 minutes
End time: 85 minutes
FID/ Auxiliary Temperature: 280°C
Injection temperature: 280°C
10.4.2.4.3 Method 3

Start column temperature: 100°C
Hold time: 0
Rate of temperature increase: 10 C.min⁻¹
Final column temperature: 260°C
Temperature hold time: 5 minutes
End time: 21 minutes
FID/ Auxiliary Temperature: 280°C
Injection temperature: 280°C
10.4.2.4.4 Method 4

Start column temperature: 100°C
Hold time: 5 minutes
Rate of temperature increase: 5°C.min\(^{-1}\)
Final column temperature: 260°C
Temperature hold time: 0 minutes
End time: 37 minutes
FID/ Auxiliary Temperature: 280°C
Injection temperature: 280°C
10.4.2.4.5 Method 5

Start column temperature: 75°C
Hold time: 0 minutes
Rate of temperature increase: $10^\circ\text{C}.\text{min}^{-1}$ till 100°C
Rate of increase in temperature from 100°C: $5^\circ\text{C}.\text{min}^{-1}$
Final column temperature: 260°C
Temperature hold time: 0 minutes
End time: 34.5 minutes
FID/ Auxiliary Temperature: 280°C
Injection temperature: 280°C

Figure 10.39. GC column temperature profile method 4.
Temperature limits 35-260/280°C.

**Method 10.4.2.4.6 Method 6**

Isothermal method of separation with the column temperature maintained at 145°C for two minutes, compound detection at 260°C and the injection temperature at 240°C.

**10.4.3 Results and discussion**

Several different temperature program methods were trialled to ensure separation of the components in the samples. It was expected that a compound peak would be observed separately from the derivatising agent peak. To ensure visualization of the derivatising agent and the derivatised flavonoid, a sample of the derivatising agent only was applied prior to the analysis of the derivatised flavonoid sample (Figure 10.41). The solvent DMF was then used to solubilize the apigenin in the BSA (Figure 10.42). A large peak eluting over several minutes was observed. In fact, in all instances, no difference between the chromatograms of the derivatising agent and the flavonoid sample were observed (Figure 10.41), regardless of derivatisation
agent, the amount of standard used or the column temperature program. Operating temperatures started as low as 60°C to as high as 260°C.

Figure 10.41. GC chromatograms of A the derivatising agent DMF in BSA.
Figure 10.42 The apigenin standard solution in DMF and BSA. Separation conditions were as follows; Initial column temperature 100°C, rate of temperature increase to 260°C was 10°C/minute, temperature hold time 5 minutes, end time 21 minutes, Detector temperature 280°C, injector temperature 280°C.

At this point, the flow rate was calculated via a conversion of the linear flow velocity using methane as the un-retained sample (Section 3.3.7.3). The flow rate was determined to be sufficient at 2.57 mL.min⁻¹. Using the bubble flow meter the split ratio was measured and found to be 27.27 mL. Approximately 1 part of a 10 part solution of sample was being injected onto the column. To ensure that the amount of
analyte being detected was sufficient, the split flow ratio was increased to enable 9 parts of a 10 part solution injection for detection. Analysis of the apigenin sample resulted in an increase in the peak area of a compound. A greater volume of the sample was injected onto the column. Analysis of the derivatising agent and dilution solvent under these conditions resulted in a very similar chromatographic profile suggesting that apigenin may have been eluting too close to the DMF and BSA compounds. The amount of apigenin was increased from 4 mg.mL\(^{-1}\) to mg.mL\(^{-1}\). An increase in the size of the peak occurred from 3734 to 2720413 counts. Separation of the flavonoid from the derivatising agents was not achieved.

As it appeared that separation of the sample from the derivatising solution was not possible using the methods and column trailed so far a different column was installed. The BP1701 characterised as a moderately polar column was replaced with a highly polar BPX70 column. Different columns of varying polarity as mentioned in the methodology section were trailed with no improvements.

To facilitate separation, the back pressure was reduced from 15 psi to 7 psi to reduce the flow rate later determined to 1.16 mL.min\(^{-1}\). A 60 mg.mL\(^{-1}\) solution of apigenin was injected and run using the Section 10.4.2.4.3, temperature program. A marked in improvement in the peak resolution was observed, however, no difference between the sample and the derivatising agent, trisil Z, chromatograms was observed.

To ensure that the flavonoid sample was not being overlooked by high concentrations of the derivatising agent, chromatographic results were integrated to remove the derivatising agent and to visualize any possible flavonoid peaks. Sample concentrations from 1.6 mg.mL\(^{-1}\) to 16 mg.mL\(^{-1}\) of several different flavonoids were analysed. Split ratios and sample injection volumes were adjusted to ensure enough sample was being loaded onto the column and to ensure that overloading of the column was not occurring. Separation or visualisation of the flavonoid compounds was not achieved.
As a last resort, an isothermal method of separation was employed (Section 10.4.2.4.6), the chromatographic conditions where start column temperature, 145°C, run time 20 minutes, final column temperature 145°C, detector temperature 260°C, injector temperature 240°C. Compound separation was achieved with the flavonoid sample (Figure 10.43) displaying a different profile from the derivatising agent, trisil Z (Figure 10.44).

Figure 10.43. Separation of the flavonoid morin from the derivatising agent, following an isothermal temperature program set at 145°C for 20 minutes using a BPX70 column.
Next a mixture of derivatised flavonoids, catechin and morin, was injected to establish their retention times. Separation of the two flavonoids was not been achieved even with adjustment of column temperatures. Other flavonoids such as rutin were analysed and their retention times were no different to those of catechin and morin.

10.4.4 Conclusion

Throughout this analysis, compound derivatisation was assumed to have occurred if the sample was soluble with the derivatising solution and if colour was evident, both of which occurred. From the lack of success separating the derivatised flavonoids from each other under a number of chromatographic conditions with all instrumental checks ensuring no instrument faults, it was assumed that derivatisation was not occurring. To confirm this NMR analysis was conducted.
10.5 NMR examination of compounds to confirm derivatisation

10.5.1 Introduction

To ensure that the compounds were derivatising under the conditions applied using trimethylsilation with BSA and DMF the samples were analysed by NMR. A derivatised sample was expected to show the same number of protons and C13 carbons however a shift in the field should be observed.

10.5.2 Methods and materials

10.5.2.1 Sample preparation

Several approaches were employed to encourage derivatisation of compounds. Initially the standards catechin and morin was derivatised DMF and BSA as described in Section 3.6.8.1. A second approach is described in Section 3.6.8.2 and unmodified sample preparation is Section 3.6.8.3.

10.5.2.2 Instrumentation

To establish if flavonoid derivatisation had occurred, proton and C13 NMR scans where obtained as described in Section 3.5.5 with a Bruker NMR.

10.5.3 Results and discussion

Comparison of acetone in duetarised acetone of underivatised catechin did not result in the observation of a difference in proton configuration. The same was observed with a morin sample prepared and analysed as for catechin. This suggested that no derivatisation of the hydroxy compounds on the base structure had occurred. It was noted however that the morin sample had not completely dissolved in the NMR acetone solution as some precipitate remained.
The unmodified proton structure of the morin sample displayed 5 protons that represented the $H_a$ and number 1,3 and 4 carbons on the morin structures. Impurities were evident at 8.18, 8.15 and 7 ppm of approximately 15% (Figure 10.45).

Figure 10.45. Morin in deuterated acetone. Five protons were observed with impurities evident at 8 and 7 ppm.

A C13 scan of unmodified catechin was performed (Figure 10.46). Seven carbons with OH groups were recognized, 7 CH were evident in the lower field. The carbon in the C ring was identified at 27.3 ppm.
A proton scan was conducted on catechin (Figure 10.47) and compared to catechin derivatised with DMF (Figure 10.48). The proton NMR of the DMF modified catechin exhibited little difference from unmodified catechin, suggesting poor silation. Little evidence of DMF in the trace was expected and was not observed (Figure 10.48).
Figure 10.47. Unmodified proton NMR of catechin
To the modified morin sample (after removal of the DMF under nitrogen), 300 µl of BSA was added and the reaction process was performed with shaking and heating at 70°C for 15 minutes. This sample did not derivatised. The use of BSA was not successful in the derivatisation of the flavonoids morin and catechin.

A 50 mg sample of morin was derivatised with 1 mL of dichloromethane and 0.5 ml of trifluoroacetic acid. The sample was shaken and left to react for 30 minutes at room temperature or until complete solubility. A C13 scan revealed a definite shift in
spectra, of the modified morin with trifloroacetamine when compared with the unmodified morin C13 NMR trace. Flourine was present at 77.165 ppm (Figure 10.49). The change in spectra suggests that all of the OH groups on the flavonoid were modified.

Figure 10.49. Observation of fluorine at 77.16 ppm.

A sample of catechin was prepared in the same way as the morin with triflouroacetamide. The reaction took 45 minutes at room temperature to achieve
solubility. The solution was dried under nitrogen and was redissolved in acetone $D_6$. A white solid formed which was latter discovered to be a contaminate present in the reaction vessel. The unmodified C13 scan (Figure 10.50) was compared to the modified (Figure 10.51). Differences in the C13 spectra of the modified catechin where evident. A CH$_2$ had moved downfield from the deuterated acetone around 27 ppm. The same number of carbons in the lower field were observed.

Figure 10.50. Unmodified catechin C 13 spectra.
Evidence of the reaction with TFA was observed when fluorine signals were observed (Figure 10.52).
10.5.4 Conclusion

Derivatisation of the morin and catechin flavonoids with BSA was not achieved as observed by NMR spectra of the sample that showed no alteration. With the addition of TFA to the standard flavonoids, a difference in the spectra to those of the unmodified samples were observed suggesting that the OH groups on the flavonoids had been altered. Different signals were also evident suggesting the presence of TFA. Additionally, evidence of reaction with the TFA was observed when it was found that the flavonoids did not dissolve in the DCM until the addition of TFA and reaction at room temperature for 30-45 minutes.

Figure 10.52 Catechin derivatised using TFA.
With successful derivatisation using TFA, flavonoid standards and a wheat flavonoid sample were analysed using GC. GC analysis of standards was conducted following the method described in Section 10.4.2.4.6 with a BPX 70 column. Reproducibility of results was not achieved. Further analysis of flavonoids using GC and derivatised compounds was abandoned.

10.6 OVERALL CONCLUSION

Unfortunately, though several different mass spectral methods were employed and a number of different experts in mass spectral analysis of compounds assisted with the identification of the flavonoids, conclusive identification of the major pigments in wheat bran was not achieved. At the time of this analysis no compound libraries were used to assist in identification, as they were not available. It is now an entirely different situation with compound libraries routinely provided with analysis software or readily available for purchase separately.

The biggest challenge in the identification of flavonoids during this study was the difficulty found when trying to purify individual flavonoid compounds. This was due to the similarity of the flavonoids in their polarity (established by RP-HPLC) along with the large number of different phenolic and flavonoid compounds within the wheat extracts.
CHAPTER 11.
GENERAL DISCUSSION, CONCLUSIONS AND FURTHER WORK

In this study, the challenge has been to develop methodologies for the assessment and characterisation of the individual pigment components that influence wheat colour. Traditionally, methods for the analysis of wheat have focused on measurement of total pigment content. With advancements in analytical instrumentation, it has become possible to identify and further characterise the compounds that contribute to wheat flour and wheat based product end colour.

Prior to this research, little was understood about the number of and complexity of the types of pigments within Australian Standard White wheat flours that contribute to noodle colouration. For the first time individual carotenoid and flavonoid pigments have been assessed with semi quantification of these pigments achieved. The flavonoids within wheat have been further characterised with the detection of the phenolic compounds, ferulic acid and chlorogenic acid along with several different types of the flavonoid apigenin. Different wheat varieties contain different phenolic and flavonoid compounds and vary in the concentration of these compounds. Wheat varieties contain five major carotenoid compounds that were observed in all seventeen varieties of wheat analysed. Lutein was consistently observed with variation in its concentration across varieties. For the first time it has been established that lutein is not always the predominant carotenoid pigment in wheat.

The focus of this study was to identify and design accurate and objective measures for the assessment of pigment type and concentrations in wheat. Different extraction processes were tried and modified to suit the extraction of flavonoids from wheat bran and flour. Assessment of extraction success in isolating pigment types was conducted using RP-HPLC analysis. A number of different solvent types and combinations were employed to identify and optimise a method that would facilitate the high extraction rates, ease of extraction and reproducibility of extraction. Methods were also trialed to assist in the isolation of individual pigment groups and the simplification of extracts, however, no one method was found that achieved this.
RP-HPLC separation was identified as the most successful method for the separation of pigment when compared to traditional TLC and paper chromatography techniques. Its use resulted in excellent characterisation and observation of the many different flavonoid and phenolic compounds in wheat. Methods examined included; a methanol, formic acid and water gradient elution system, an acetonitrile-water-acetic acid isocratic system, a methanol and acetic acid gradient elution, acetonitrile-sodium ortho phosphate-tetraethylamine gradient elution, and methanol-chloroform-acetonitrile isocratic elution. For separation and semi quantification of phenolic and flavonoid pigments, a Varian 9100 autosampler, 9010 Varian solvent delivery system and a Varian 9050 UV-vis detector interfaced to 9021 Varian liquid chromatography Star workstation was used. For the UV visible characterisation of flavonoids a 9065 Photodiode array detector interfaced to 9021 Varian liquid chromatography Star Workstation was used. A Varian PolyviewYM spectral processing application interfaced with the PAD provided post run analysis of data, in the time, absorbency and wavelength domain.

The predominant method used for the separation and semi-quantification of flavonoid and phenolic compound was a gradient elution system using formic acid-water and methanol with separation facilitated using a reverse phase C18 column and guard column. The elution system was 0-2 minutes 7 % B (formic acid-water (5:95,v/v)) in A (Methanol), isocratic, 2-8 minutes 7-15 % B in A (linear gradient), 8-25 minutes, 15-75 % B in A (linear gradient), 25-27 minutes, 75-80 % B in A (linear gradient), 27-29 minutes, 80 % B in A (isocratic) 30 minutes. The column temperature was ambient and the flow rate was at 0.7 ml.min\(^{-1}\) to suit the chromatographic system and to maintain pressure within the optimum column pressure range (<300 atm).

Wheat flavonoid and phenolic compound semi quantification can be achieved using catechin as either an internal or external standard. Catechin was found to elute early within the chromatographic profile of wheat extracts and separately from other components within the wheat using the formic acid: water and methanol gradient elution system. Tentative identification of the aglycone structure of flavonoid and
phenolic compounds was achieved using RP-HPLC analysis with diode array detection enabling characterisation of the UV-visible spectral characteristics of these wheat compounds.

It has been recognized that wheat flour contains many different flavonoid and phenolic compounds and for the first time, the complexity of these compounds has been reported. Different wheat flours contain varying concentration of flavonoid and phenolic compounds with as many as 56 different compounds observed in some of the flours. This suggests that variation in colour response between different varieties of wheat for alkaline and salted noodles is probably due to the differences in pigment type and concentration. In the chromatograms of the wheat samples, eight major compounds were consistently observed across the varieties and were characterised by UV visible detection using a PAD. Quantification of these compounds within the different varieties showed considerable variation in amounts of these components with no predominant pigment identified within the sixteen varieties of wheat examined.

Consistently observed in low concentrations was ferulic acid, with concentrations ranging from 0.01 µg.g⁻¹ to 0.13 µg.g⁻¹. Chlorogenic acid was detected in Trident, Cadoux, Goldmark, Vectis, Suneca, Janz and Katunga at concentrations of 0.11 µg.g⁻¹ to 0.51 µg.g⁻¹. The most predominant form of apigenin detected at a retention time of 21.37 minutes, was observed in all wheat varieties at concentrations ranging from 0.2 µg.g⁻¹ for Rosella wheat to 1.57 µg.g⁻¹ for Katunga. Some varieties contained as many as three forms of apigenin observed as compounds with different retention times with the same absorption spectra. Structural differences would result in changes to compound polarity and therefore retention time. The three different types of apigenin were observed in Euradu, Cadoux, Machete, Vectis and Katunga. The identity of the apigenin forms and other major compounds could not be confirmed due to compound separation challenges. Further research on flavonoid pigment characterisation should focus on the development of methodology for the separation and definitive identification of these compounds using LC-MS technology.
Carotenoid compounds can be successfully characterised using a Varian 9100 autosampler, 9010 Varian solvent delivery system and a Varian 9050 Ultra violet-visible detector interfaced to 9021 Varian liquid chromatography Star workstation. Several different solvent delivery systems were trialed and included acetone-water gradient elution, methanol-dichloromethane-acetonitrile isocratic elution, acetonitrile-water-ethyl acetate gradient elution, methanol-acetonitrile-chloroform isocratic elution and methanol-chloroform gradient elution.

The most successful method for carotenoid pigment separation was achieved using a reverse phase C18 column and guard column. A linear gradient from 60% acetonitrile-water (90:1, v/v) and 40% ethyl acetate, at time zero to 100% ethyl acetate over 25 minutes, and held for 15 minutes, for a total 40 minute run time. Compounds were detected at 450 nm and the solvent flow rate was 0.7 mL min⁻¹. Quantification was achieved using β-apo-8'-carotenal, which elutes separately from the carotenoid pigments identified in flours and either the external or internal standard method. Either the IS or ES method may be employed for quantification.

Carotenoid characterisation of wheat showed the consistent observation of lutein with β-carotene. For the first time, the presence of five other major compounds within wheats has been observed in thirteen different varieties of Australian Standard White wheat including; Meering, Machete, Vectis, Janz, Eradu, Hartog, Yanac, Suneca, Goldmark, Rosella, Batavia, Trident and Cadoux. Lutein is not always the predominant compound within wheat flour with some compounds observed in concentrations greater than lutein dependent on the variety.

Each wheat variety displayed a unique pigment profile shown by differences in the concentration of these major compounds. High total pigment concentrations did not necessarily reflect a high yellow colour value (b* value) reading for the flours. Wheats with high total pigment contents tended to have a high a* value (hue reading) and L (lightness) reading. Further work is required to statistically validate these observations and to understand the influence of the individual compounds. For
this to be possible, methodologies for the separation of individual compounds would need to be developed with the limiting factor here being available technology. Advancements in online MS after RP-HPLC separation of compounds could, as with the flavonoids, facilitate conclusive structural identification of the carotenoid pigments in wheat.

By assessing the colour impact of pigments on noodle colour it was confirmed that both these pigment groups do indeed influence wheat product end colour. Using colour space analysis, the influence of these pigment groups was readily observed when the flour of Batavia wheat was extracted for flavonoids, extracted for carotenoids and extracted for both pigment groups. Flavonoid pigment appears to have more influence on hue and carotenoid on the lightness and yellowness of flour, noodle sheets and noodles. The removal of both pigment groups caused a reduction in yellow coloration confirming that both pigment types influence the yellow colouration of wheat. To understand individual pigment contribution to noodle and flour colour more sophisticated separation techniques than those used in this study are required and is dependant on the development of such equipment and methods in the future. How each individual compounds within the wheat influences final product colour is yet to be determined and would be of benefit to plant breeders and processors.

In the investigation of pigment influence on noodle colour different processors for the preparation of alkaline and salt prepared noodles where carried out. Colour expression changed with the production of a darker noodle colour with alkaline treatment of the flour. This was attributed to pH changes influencing the expression of colour for flavonoid compound. How wheat flour is processed will therefore influence final production colouration and this may be of significance for plant geneticists, millers and processors.

For pigment quantification the traditional approach developed by the AACC was compared to the RP-HPLC methodology of ES and IS quantification. Differences in total concentrations between the different approaches were observed. The HPLC
method resulted in higher total carotenoid concentrations compared to the AACC method. Concentrations values, obtained using the ES method ranged from 1.8-4.1 µg.g\(^{-1}\) depending on the wheat variety. Concentrations determined using the AACC method showed a range from 1.4 µg.g\(^{-1}\) to 2.9 µg.g\(^{-1}\). The higher concentration values of the HPLC method are attributed to the greater accuracy and sensitivity of the HPLC analysis process were the contribution of each individual pigment separated can be calculated relative to the standard. A poor relationship between the two approaches was observed with low correlation (r\(^2\) = 0.0498). Further analysis is required to validate this information with a greater number of sample points required to better understand the correlation between the AACC and HPLC ES method.

Methods for the isolation of individual flavonoid compounds were extensively examined. The complexity of flavonoid extracts from wheat ruled out the use of TLC methods for separation and collection of individual flavonoid compounds present in wheat. The flavonoid and phenolic compounds in wheat are very similar in polarity to each other making separation of individual compounds very difficult. Post column fraction collection of compounds after separation by RP-HPLC offered a degree of success. This approach enabled simplification of the flavonoid extracts but did not facilitate collection of individual compounds despite many efforts and the investigation of many different collection programs.

Different flavonoid extraction methods were also examined, in an attempt to identify both a highly efficient extraction methods and a method that would facilitate the separation of flavonoid and phenolic compounds. Each of the different extraction methods employed, including an alkaline extraction and a methanol soxhlet extraction approach, resulted in the removal of similar compounds. Variations that occurred tended to be related to concentration rather than compound type with no further success achieved in separating individual compounds.

Conclusive structural elucidation of flavonoids using both traditional and more advanced methods including EMS, FAB, GC-MS and NMR was not achieved. Identification of flavonoids and carotenoids in wheat was not achieved due to the
lack of success in isolating individual compounds using TLC and RP-HPLC with fraction collection. For full characterisation, the challenge for the researcher is the complexity of the wheat extracts, which in some instances contained as many as 56 closely eluting phenolic and polyphenolic compounds: for carotenoids as many as 20 different compounds, observed within wheat flours. Analysis of synthesised standard compounds using these structural identification methods was successful. This highlighted the importance of compound purity for identification using these methods.

With advancements in instrumentation it is now possible to use HPLC with mass spectral analysis using gradient elution mobile phases to separate compounds. Unfortunately this type of equipment was not available at the time of this study. It is recommended that a separate investigation be undertaken to develop methodology to conclusively indentify the individual flavonoid and carotenoid compounds characterised in this study using LC-MS. Further research is required to isolate and individually separate these compounds, to clearly understand the impact on flour and noodle colour that these compounds may have individually and in combination.

This research has show that different varieties of wheat have different concentrations of the same pigment type as well as different pigment combinations. Research to show the impact of individual variety on end product colouration may be of further assistance to wheat breeders and processors and may highlight the importance of further segregation between Australian Standard White wheats (and other classes of wheat), to better select wheats for specific end product use.
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Figure 9.7. Batavia wheat flour extracted for flavonoid pigments and examined at 280 nm using a methanol formic acid water gradient elution system.

Figure 10.1. Chromatographic representation of alkaline extracted Rosella bran using a methanol and formic acid-water gradient elution system and a C-18 column and guard column with compound detection at 280 nm.

Figure 10.2 Chromatographic representation of methanol extracted Rosella bran purified by RP-HPLC post column fraction collection. Compounds were separated with a methanol and formic acid-water gradient elution system and a C-18 column and guard column, with compound detection at 280 nm.

Figure 10.3. Positive ion FAB mass spectra of the flavonoid standard rutin.

Figure 10.4. Positive ion FAB mass spectra of the flavonoid standard quercetin.

Figure 10.5. Positive ion FAB of the alkaline extract of bran.

Figure 10.6. The positive ion FAB mass spectra of methanol extracted bran.

Figure 10.7. Proposed fragmentation of alkaline extracts via the retro-diels-alder scheme.

Figure 10.8. Electrospray mass spectra of rutin in the positive ion mode at B1=75 v.

Figure 10.9. Electrospray mass spectra of rutin in the positive ion mode at B1 = 150 v.

Figure 10.10. Electrospray mass spectra of rutin in the positive ion mode at B1 = 115 v.

Figure 10.11. Positive ion mode of rutin at a voltage of B1 = 75 v.

Figure 10.12. Positive ion mode of rutin at a voltage of B1 = 115 v.

Figure 10.13 Positive ion mode of rutin at a voltage of B1 = 33 v.
Figure 10.14. Positive ion mode assessment of background noise.

Figure 10.15. Electrospray mass spectra of rutin in the positive ion mode. The presence of C13 and C14 ions is observed.

Figure 10.16. Apigenin electrospray mass spectra in the negative ion mode at B1 = 150 v. The molecular ion is observed at m/z 150.9.

Figure 10.17. Apigenin molecular ion observed at B1 = 80 v.

Figure 10.18. Ferrulic acid electrospray mass spectra in the negative ion mode at B1 = 40 v showing the deprotonated molecular ion at m/z 192.9.

Figure 10.19. Ferulic acid at B1 = 85 v with fragment ions were evident at m/z 177.9, 148.8, 133.8 and 78.8.

Figure 10.20. Electrospray mass spectra, negative ion mode. Background signals (noise) are evident with injection of the sample solvent 50 % aqueous methanol and 1 % acetic acid.

Figure 10.21 Electro spray mass spectra in the negative ion mode of alkaline extracted rosella bran.

Figure 10.22. Electrospray mass spectra in the negative ion mode of methanol extracted Rosella wheat bran.

Figure 10.23. EMS spectra of rutin (300 mM). B= 115 v, ionisation solution isopropyl alcohol water (50:50, v/v) with 10 mM ammonium acetate.

Figure 10.24. EMS spectra of the rutin standard displaying the presence of C13 molecules at m/z 610.1.

Figure 10.25. EMS of the rutin standard were the C13 and 14 molecules are displayed at m/z 610.1.

Figure 10.26. EMS of apigenin (300 mM) B1 = 150 v.

Figure 10.27. Carbon 13 and 14 signals observed for apigenin.

Figure 10.28. EMS of apigenin (300 M) at B1=115 v.

Figure 10.29. EMS of ferulic acid.

Figure 10.30 Background scan.

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REFERENCES


Anderson, J. A. 1932. The yellow colouring matters of Khalpli wheat. II. The 334


J&W Scientific. 1996. GC column installation manual 10/93. USA.


Markley, M. C., and Bailey, C. H. 1935. The pigments of the dilute alcohol or acetone extract of whole wheat. Cereal Chem. 12: 40


National Scientific Instrument training Laboratory, 1994


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Personals communications, Wootton, M. University of NSW. 1995

Personal communications, Wootton, M. University of NSW .1996

PIE Newsletter of Australia’s international and national primary industries research and development organization 1994.

Pierce 1996. Chromatography catalogue and handbook. Lab supply PTY TLT. Melb


SGE capillary column installation validation procedure. Publication No. 0047-C Rev0012/95


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APPENDIX 1. STANDARD UV VISIBLE AND MOLECULAR CHARACTERISTICS

Xanthone $C_{13}H_8O_2$ Mw 208

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Kampferol $\text{C}_{15}\text{H}_{10}\text{O}_{6}$ Mw 286

![Spectrum plot of kaempferol](image)

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<td>84.686</td>
<td>311</td>
<td>32.341</td>
<td></td>
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Other compounds without UV spectra

Rutin $C_{27}H_{30}O_{16}$ MW 610.51
Rhamnosse $C_{6}H_{12}O_{5}$ Mw 164.16
Quercetin $C_{15}H_{10}O_{7}$ Mw 302.23
Myricetin $C_{15}H_{10}O_{8}$ Mw 318.2
Epicatechin $C_{25}H_{14}O_{6}$ Mw 290.28
Caffaic acid $C_{9}H_{6}O_{4}$ Mw 180.15
Chalcone $C_{15}H_{12}O$ Mw 208.25