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TOTAL PHENOLIC CONTENT AND ANTIOXIDANT CAPACITY OF A SELECTION OF SOUTH AFRICAN INDIGENOUS FRUITS

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Declaration

I, Daniela Amalia Kucich, declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Signed **Date**

Abstract

It has recently been confirmed that people consuming 7+ portions of fruit and vegetables daily have a lower risk of mortality from any cause. With a fifth of the population of South Africa falling below the poverty line, it has been found that rural adults have a very low daily intake of fruit and vegetables; at the same time rural children are consuming a primarily maize-based diet. This low dietary diversity translates into a higher level of infectious diseases in children younger than five years. Interventions at national level included promoting the growing of underexploited traditional indigenous vegetables and fruits in home gardens, in the hope that rural households would help themselves in diversifying their cereal-based diet, while using crops they are accustomed to in their environment.

Ten indigenous South African fruits found in the Western Cape were evaluated for their potential to make a positive contribution to the diet of rural communities and were compared with Blueberry and Cranberry, the North American 'gold standards'. The following determinations were carried out on 12 samples: Total Phenolic Content, Total Flavanols and Total Monomeric Anthocyanins were analysed using the Folin-Ciocalteu, Mazza and pH Differential methods. Total Antioxidant Capacity was assessed using the Trolox Equivalent Antioxidant Capacity (TEAC), DPPH and Molybdenum Reduction assays. The Oxygen Radical Absorbance Capacity ($ORAC_{FL}$) was also determined. Iron Chelating Activity, one of the methods recommended to reflect other antioxidant mechanisms, was also investigated.

The fruits possessing the highest concentration of Total Phenolic Content (Mazza) were Christmas berry, Bietou, Wild Olive and Wild Plum, at levels significantly higher than those of the two control berries, Blueberry and Cranberry. The fruits yielding the highest results for the TEAC assay were Wild Plum, Wild Olive, Tortoise berry, Christmas berry and Colpoon. The fruits giving the highest results for the DPPH assay were Wild Plum, Colpoon, Wild Olive, and Christmas berry. The fruits showing the highest results for the Molybdenum Reduction assay were Wild Olive, Wild Plum, Christmas berry, and Tortoise berry. The fruits yielding the highest results for the ORAC Total Antioxidant Capacity assay were Colpoon, Christmas berry, Wild Olive, Crossberry, Wild Plum, Waterberry followed by Blueberry and Cranberry. The results from the Iron Chelating Activity assay revealed a ranking of Christmas berry, Blueberry, followed by Num-num.

On combining the results of eight assays, namely TPC (Mazza), TF, TA, TEAC, DPPH, TAC, TPC (FCR), ICA to give an Antioxidant Potency Composite Index, the fruits with the highest

rankings were (1) Wild Plum, (2) Wild Olive, (3) Colpoon, and (4) Christmas berry. By comparison the northern hemisphere control berries ranked (5) Blueberry and (9) Cranberry.

These findings show that by introducing even small servings of indigenous fruits into the diet, an important and inexpensive source of natural antioxidants could be accessed and the mean daily ORAC intake could thereby be boosted significantly by about 4,000 μmol Trolox Equivalents to bring the Total ORAC consumed to within optimum levels (6,000 μmol Trolox Equivalents and above). These bioactive plant compounds have the potential to deliver immense benefits to health to impoverished South African adults, as well as rural children, well beyond basic nutrition.

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Dedication

For Roberto, my champion…

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Glossary

AO	Antioxidant
AOC	Antioxidant Capacity
TAC	Total Antioxidant Capacity
ROS	Reactive Oxygen Species
RNS	Reactive Nitrogen Species
TEAC	Trolox Equivalent Antioxidant Capacity
Trolox	(6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) a water-soluble analogue of vitamin E
TPC	Total Phenolic Content
FCR	Folin Ciocalteu Reagent
$ORAC_{FL}$	Oxygen Radical Antioxidant Capacity (using Fluorescein as probe)
L-$ORAC_{FL}$	Lipophilic Oxygen Radical Antioxidant Capacity
H-$ORAC_{FL}$	Hydrophilic Oxygen Radical Antioxidant Capacity
FRAP	Ferric Reducing Ability of Plasma
TAP	Total Antioxidant Potential
$ABTS^{\cdot+}$	2, 2'-azinobis (3-ethyl-benzothiazoline-6-sulfonate)
ICA	Iron Chelating Activity
SET	Single Electron Transfer
HAT	Hydrogen Atom Transfer
GC-MS	Gas Chromatography – Mass Spectroscopy
GIT	Gastro Intestinal Tract
AUC	Area under the Curve
LDL	Low Density Lipoprotein

CHAPTER 1 INTRODUCTION

'When there are blueberries, we shall not need a doctor' Proverb popular in Northern countries.

Very few scientific studies have been carried out on South African indigenous fruits. This is because, with a few exceptions, the fruits of indigenous plants have not generally been used in traditional medicine; rather only the bark, leaves and roots have been seen to have medicinal value. Therefore, with the scientific examination of South African indigenous plants having been approached historically mainly from an ethnopharmacological viewpoint, (with guidance sought from traditional medicine healers as to the uses of the various species), the result has been that very few studies have been carried out on South African indigenous fruits *per se*.

The ethnopharmacological approach is demonstrated, for example, in the study by van Staden of 12 medicinal plants traditionally used for treating gastro-intestinal ailments in South Africa [(1), (2), (3)]. Ben-Erik van Wyk has extensively documented and reviewed indigenous plants that were commercialized for medicinal use during the last century; he has also examined the potential of South African plants in the development of new medicinal products [(4), (5)]. Springfield has attempted to establish quality standards for traditional medicines using HPLC fingerprinting of *Chironia baccifera* (Christmas berry), which has proved useful where botanical identification has not been possible [(6)]. Essential oils of indigenous plants have been evaluated for anti-microbial activity [(7)]. The active compounds of *Sutherlandia frutescens (L)* (Cancerbush) have been determined using LC-MS (8). South African teas, including Rooibos and Honeybush, have undergone in-depth assessment both locally and abroad [(9), (10), (11)]. Extracts from the leaves of *Carpobrotus edulis L*. (Sour Fig) have been examined to identify potential antimicrobial agents, revealing five bio-active flavonoids that exhibit strong anti-bacterial activity [(12), (13)].

In 1966 a seminal work was published, which documented the basic nutrient composition (carbohydrates, fats, proteins, minerals and vitamins) of some edible wild fruits found in the 'Transvaal'. Nine fruits were examined, including Wild Plum, Marula, Wild Apricot, Monkey Orange, Amatungulu, Baobab, Sour Plum, Kei Apple and Red Gherkin. It was found that the nutrients in these fruits did not differ substantially from that of domestic fruits, except that their ascorbic acid content was considerably higher [(14)]. During 1984, as climate change projections heightened interest in the commercialization of totally new fruit crops, totally wild or neglected crops known only in their country of origin were selected for testing. Trials on 45 fruit tree species were carried out in the Negev desert, Israel. Among these were the following Southern African native species: *Harpephyllum caffrum* (Wild Plum), *Sclerocarya birrea* (Marula),

Strychnos cocculoides (Monkey orange) [15]. Wild Plum was rated as a species which performed very well in most tested ecozones.

A literature search revealed that the following fruits have been scientifically examined: In 2006, *Dovyalis caffra* (Kei- Apple) was examined for its polyphenol composition and antioxidant activity [16]. In this study Total Polyphenols, ascorbate determination, ORAC, FRAP and GC-MS analysis was carried out. This was followed in 2008 by the determination of the Total Phenolic Content, Flavanols and Proanthocyanidin content of a selection of wild fruits sold in market places and along road sides in urban areas of Zimbabwe, including *Ximenia caffra* (Sourplum), *Artobotrys brachypetalus* (Mukusvo in Shona) and *Syzygium cordatum* (Waterberry or Waterbessie) [17]. In 2011, the leaves, bulbs, roots, flowers and the 'highly fragrant edible fruit' of the Kukumakranka (*Gethyllis multifolia L. Bolus* and *G.villosa Thunb*. species) were assayed for Total Polyphenol, ORAC, FRAP, flavonol and flavanone contents [18]. Unfortunately, some of the species of this particular genus have been classified as endangered, threatened or vulnerable [19].

In 2006, with a view to improving on present Western diets, researchers in the field of pharmacognosy raised the issue of whether certain kinds of food contain pharmacologically active substances in concentrations that are high enough to have drug-like effects when consumed. They also raised the question as to whether biologically active compounds present in foods really are indicative of therapeutic value [20].

Data from the recently released Health Survey for England [21] shows a robust inverse association exists for the consumption of 7+ portions of fruit and vegetables daily and all-cause mortality. No significant association was found between consumption of fruit and deaths from cancer and cardiovascular disease; however a significant association was found between vegetable consumption and cancer and cardiovascular mortality. This study does, however, underscore the fact that the amount and type of fruit and vegetables with the greatest benefits to health are still not known.

A recent review article by Ronald Prior [22] once again makes a strong case for a direct relationship between the prevention of adverse health outcomes and the level of consumption of antioxidants. This is backed up by the results released from recent clinical trials [23].

Within the context of the South African medical insurance market, eating more fruits, vegetables and whole grains has been recognized as contributing to improved health and lower medical costs. It is for this reason that the largest medical scheme operating in the country (serving two and a half million South Africans), has been rewarding its members with 25% cash back for

making healthier dietary choices. Between 2009 and 2012, members received over R210 million in cash back on healthy food items. Recognized internationally as a leader in incentive-based wellness programmes, the success of this program has been built on a clinical understanding of the factors that influence health risk. Studies have shown that good nutrition and regular exercise are key factors in reducing the incidence of illness – and it has been clinically proven that engaged members are healthier, live longer and have lower healthcare costs [(24)].

However, access to medical insurance and healthy food benefits eludes the majority of South Africans. The South African National Department of Agriculture [(25)] reported in 2002 that as many as 14.3 million South Africans were unemployed and as a result were experiencing continued food insecurity and malnutrition. Coupled with this, South Africa has been exposed to intermittent rapid food inflation for over a decade. Year-on-year food price inflation reached 20% in the last quarter of 2002, driven by sharp increases in international commodity prices and a significant depreciation in the Rand/USD exchange rate. Between 2005 and 2008 inflation was again significant due to increased bio-fuel production, droughts in key grain-producing regions, and the rapid growth in developing countries such as India and China, all of these contributing to high commodity prices. In 2011 local food inflation reached 8.9 %, driven by higher international commodity prices, with steep increases in local electricity prices increasing cost throughout the value chain. During 2011 and early 2012, rapid food price inflation was observed for maize meal, margarine, coffee, bread and chicken. Maize meal and bread are the two staples consumed by the poorest households in South Africa. Therefore the rapid price increases in these products had a direct influence on food security in terms of food affordability.

Regarding food affordability, it was found that for low-income households specifically, increased wages negotiated by trade unions resulted in improved nutrition. However, poor households receiving minimum wages were still unable to afford the ideal option in terms of dietary diversity and energy intake [(26)]. By 2014, it was estimated that the impact of a continued rise in food prices had left half of the population struggling to put food on the table. A report by Pali Lehohla, Statistician General of Stats SA released in February 2015 stated that between 2010 and 2014 the percentage of South Africa's population falling below the poverty line had increased from 20% to 21.5%. Whereas in 2010 it required R321 per month to purchase food with the recommended energy requirements, by 2014 this had increased to R355 per month [(27)].

It has been pointed out that even in First World countries consumption of fruit and vegetables is related to household income. As a result of the Health Survey for England study [(21)] in the United Kingdom it was recommended that not only should health education be put in place for

the socioeconomically disadvantaged in that country, but even fiscal policy could be used to promote increased fruit and vegetable consumption.

Historically, South Africa's long standing problem has been one of chronic rather than acute malnutrition [28]. According to the 1999 National Food Consumption Survey [29], from infancy rural South African children in particular consume a primarily maize-based diet that results in micronutrient malnutrition. Monotonous diets linked to food insecurity do not provide the requisite nutrients to sustain optimal health. This low dietary diversity therefore translates into a higher level of infectious diseases in children younger than five years, including measles, acute or chronic diarrhoea, acute lower respiratory infection and severe protein-energy malnutrition. In the long run children are affected through poor development, stunting, as well as decreased academic ability. One study revealed that 40% of adult South Africans surveyed had consumed food from only 1-3 different groups on the day prior to the survey (a cereal, meat/chicken and a vitamin A poor vegetable), with the most neglected food groups being fruit and vegetables [30].

The reality is that the dietary pattern of indigenous peoples of South Africa changed for the worse as a result of colonization. Nutritionally superior indigenous crops have been gradually displaced by cash crops that do not serve poor rural communities well, placing rural children at a higher risk of malnutrition. In determining rural household dietary diversity, a study carried out in two districts of the Eastern Cape showed that sugar, tea, coffee, grains, and potatoes were among the food groups most frequently consumed, while only 5% and 3% reported consuming from the vegetable and fruit groups [31].

In 2004 the WHO revised the minimal quantity of fruit and vegetables that should be consumed per day for optimal health benefits to 600g [32]. However, on average South African adults consume 115g of vegetables and 91g of fruit per day, with the combined fruit and vegetable intake of rural adults reaching only 141g per day [33]. South African children aged 1-5 years consume on average 52g of vegetables and 48g of fruit per day [34], which is ⅓ the recommended amount. Availability issues due to absence of supermarkets in rural areas further limits healthy food choices, with fruit and vegetable intake among one adult rural group calculated at ⅓ of the previously recommended WHO minimum of 400g per day [35]; however even among the urban population, 25% of urban black adults consumed zero fruits and vegetables [36] in a 24 hour recall study. Only 16% of children sampled in a National Food Consumption Survey [29] had partaken of any fruit or vegetable in a 24-hour recall period. And although locally grown wild fruits, loquats and guavas were consumed, the number of respondents consuming was below 0.5%.

The clear association between fruit and vegetable intake and household income is shown in the more limited choice of nutrient dense fruit by lower socio-economic groups, with apples, bananas and oranges being the preferred choice [37]. A nutrient dense diversified diet may cost 69% more on average, so where households are reliant on grants and pensions, meeting this extra cost becomes prohibitive [38].

A measure of whether fruit and vegetable intake is adequate can be ascertained from the total antioxidant capacity consumed per day as measured by Oxygen Radical Antioxidant Capacity (ORAC). A study carried out by Prior on participants consuming 5 fruits and vegetables per day determined their daily plasma ORAC to be around 1,670 µmol Trolox Equivalents (TE). Increasing the intake of fruits to 10 a day increased the plasma ORAC to 3,300-3,500 µmol TE [39]. However, the choice of seven fruits with low ORAC values would yield only 1,300 µmol TE, whereas the choice of seven fruits with high ORAC values could yield up to 6,000 µmol TE, with 1 cup of blueberries alone supplying 3,200 µmol TE.

A WHO funded study found that approximately 91- 94g of fruit is consumed (average per capita per day) by 1-5 year olds in South Africa. The diversity is limited to Apple (26.1g), Banana (17g), Pear (10g), Orange (8.3g) and Grape (7.3g) [40], [29]. From the South African ORAC database [41], the calculated average Total Antioxidant Capacity (TAC) consumed per capita from these fruits is 1,600 µmol Trolox Equivalents per day. This compares with the average intake of ORAC in the U.S., calculated as 1,500 µmol TE /day. A high intake is considered to be 6,000 µmol TE/day and above [42].

The question that arises is: To what extent can our freely available local indigenous fruits provide a source of phenolics and antioxidants which will positively contribute to the nutritional status of these children, despite their otherwise impoverished diet?

Particularly when food is scarce, wild resources take on an important role in the diet of people in rural areas. Among 10 South African rural villages sampled, it was reported that families collected up to 104.2 kg ± 15.6 kg of wild fruits per year [43].

Eighty lesser-known fruits commonly utilized in the rurals include the following varieties, six of which were used in this study: *Carissa macrocarpa* (Num-num), *Carpobrotus edulis* (Sour fig), *Dovyalis caffra* (Kei-apple), *Grewia flava* (Velvet raisin bush), *Harpephyllum caffrum* (Wild Plum), *Nylandtia spinosa* (Tortoise berry), *Olea africana* (Wild Olive), and *Syzygium cordatum* (Waterberry) [44].

A study of indigenous edible plant use by contemporary Khoe-San, revealed that of the 58 indigenous edible plant species collected, over 40% were collected for their fruits, among them *Osyris compressa* (Colpoon)*, Carissa bispinosa* (Num-num)*, Carpobrotus edulis* (Sour fig) *and Muraltia spinosa* (Skilpadbessie), *Chrysanthemoides monilifera* (Bietou), *Grewia occidentalis (Crossberry)*, *Olea europaea* (Wild Olive) and *Chironia baccifera* (Christmas berry) [(45)].

A further question is: What interventions have been put in place to address this micronutrient malnutrition at national level?

Interventions at national level have included supplementation, food fortification, biofortification (genetic breeding of crops to enhance mineral and vitamin content), and dietary diversification/modification (which includes promoting the growing of underexploited traditional indigenous vegetables and fruits in home gardens) [(28)]. It was felt that promotion of the growing of home gardens could overcome the twin hurdles of affordability and accessibility and thereby increase household consumption of fruits and vegetables [(46)]. In this way it was hoped that rural households would help themselves in diversifying their cereal-based diet to address the issues of under-nutrition, using crops they are accustomed to in their environment. Endemic crops are generally better adapted to the harsh conditions of the South African climate and therefore require less input agriculturally. Also, being already known by the community, their acceptance is a given, with the added bonus of their having a higher nutrient content [(47)].

In addition, Arbour Day was instituted in South Africa in 1975. Under the auspices of the Department of Water Affairs and Forestry, it was extended to a national Arbour Week in 1996. This takes the form of an annual national campaign to promote environmental awareness. Annually the department nominates an indigenous tree to be planted throughout South Africa, especially in previously disadvantaged communities. Projects aimed particularly at schools in rural districts encourage pupils to appreciate the value of trees [(48)]. In 1988 the tree of the year was *Olea europaea* subsp. *Africana* (Wild Olive); in 2008 it was *Harpephyllum caffrum* (Wild Plum) [(49)]; in 2012 the tree of the year was *Syzygium cordatum* (Waterberry) and in 2013 it was *Grewia occidentalis* (Crossberry) [(50)]. These are among the species bearing the native fruits known and traditionally consumed by rural children in South Africa, the most popular being the *Harpephyllum caffrum* (Wild Plum) and the *Carissa macrocarpa* (Num-num), both high in ascorbic acid content. These fruits can provide a source of vitamins and phenolics which will positively contribute to the nutritional status of these children, despite their otherwise impoverished diet.

What interventions have been put in place to encourage higher household consumption of fruits and vegetables, particularly among children? An MRC intervention study was carried out to try to facilitate both the availability and access to Vitamin A-rich vegetables in rural communities. It made use of an existing program monitoring the growth of 2-5 year old children to encourage planting of vegetable home-gardens in a village in KwaZulu-Natal. Four vitamin A rich vegetables were planted and the recommendation was made to mothers to include a pawpaw tree, to encourage consumption of Vitamin A-rich fruits [(51)].

In reviewing the outcomes of this project, it was noted that this nutrition education program had empowered communities with the knowledge as to which nutrients are required for healthy children, as well as how to produce vitamin A dense foods in home gardens. Compared to those without homestead food production, 1-3 year olds in households with food gardens had significantly increased energy and micronutrient intakes. However, a criticism of this intervention was that the focus was placed exclusively on vitamin A-rich vegetables, whereas a range of nutrients is required for good health [(52)]. But the evidence is compelling that this type of intervention can address micronutrient deficiencies.

One means by which an attempt has been made to increase the fruit and vegetable content of children's diets is the HealthKick programme, instituted in 2007. It is a nutrition education intervention (introduced as part of the Life Orientation course), initiated at several schools in the Western Cape. It encourages the involvement of both teachers and parents in planting a school vegetable garden, while teaching the children how to grow vegetables. 6,503 schools across South Africa had set up food gardens by 2008.

In this study, the aim was to estimate, by comparative means, the potential health benefits that might accrue from the consumption of the following ten indigenous fruits:

1. *Syzygium cordatum* (Waterberry)
2. *Osyris compressa* (Colpoon)
3. *Harpephyllum caffrum* (Wild Plum)
4. *Nylandtia spinosa* (Tortoise Berry)
5. *Carissa macrocarpa* (Num-Num)
6. *Chironia baccifera* (Christmas berry)
7. *Chrysanthemoides monilifera* (Bietou)

8. *Grewia occidentalis* (Crossberry)
9. *Carpobrotus edulis* (Sour Fig)
10. *Olea europaea* subsp. *Africana* (Wild Olive)

This comparison involved determining the Total Phenolic Content and Antioxidant Capacity of these species. For control purposes, two Northern Hemisphere berry species *Vaccinium corymbosum* (Blueberry) and *Vaccinium macrocarpon* (Cranberry) were included in the evaluation, as these have been extensively studied [53], [54], [55], [56] and recommended for their healthful properties.

CHAPTER 2 SELECTED INDIGENOUS BOTANICAL SPECIES

The greatest service which can be rendered any country is to add a useful plant to its culture. Thomas Jefferson, 1821

The trees or shrubs of the species from which our samples were drawn are found on the Cape Town campus of CPUT and at Kirstenbosch. All were verified by Dr Nicolette Louw in conjunction with SANBI. Of the ten indigenous fruits examined, the following five have been used traditionally for medicinal purposes:

1. *Chironia baccifera* (Christmas berry)

A decoction of the fruit, leaves and flowers has been used to treat stomach ulcers, diarrhoea, syphilis, kidney and bladder infections, diabetes, haemorrhoids, acne, boils and sores [57], [58].

2. *Nylandtia spinosa* (Tortoise Berry)

The leaves together with the fruit have been used to treat stomach complaints (acidity, flatulence); also sleeplessness and nervous disorders [58].

3. *Carpobrotus edulis* (Sour Fig)

The fruit has been used as a purgative and by the Khoi to facilitate the birth process [59].

4. *Chrysanthemoides monilifera* (Bietou)

The fruit has been used to treat impotence, intestinal ailments and acne [60].

5. *Olea europaea* subsp. *Africana* (Wild Olive)

The fruit has been used to treat diarrhoea [61]. The medicinal herb is available in the marketplace as bundles of fresh or dried material comprising mainly leaf, with some smaller stems and occasional flowers and fruit [58].

A short synopsis of each of the ten selected species follows:

Syzygium cordatum

Waterberry, Waterbessie Zulu: Umdoni Family: Myrtaceae

Figure 2.1 *Syzygium cordatum*

Waterberry trees are evergreen, growing up to 20m in height. They occur from the Eastern Cape to Kenya and are found in forests and other wooded areas, almost always near water. The fruit grows in clusters and appears from November to March. They are about 20 × 10 mm in size, deep purple when ripe and usually one seeded. Although the fruit is edible it is not particularly tasty, unless fully ripe, when it has a sweet faintly resinous taste. A good quality jelly can be prepared from the fresh fruit and intoxicating beverages can be produced from fermented fruit [62], [63], [64], [65].

Only the bark, leaves and roots were used in traditional medicine by the Zulu for treating diarrhoea and dysentery [57], [66]. Recently, evaluations regarding the anti-diabetic activity of the leaf extracts have been carried out [67]. It has been noted that the presence of phenolics most probably accounts for the anti-diarrhoeal activity [68].

Osyris compressa

Cape Sumach, Colpoon, Wildepruim, Pruimbas Family: Santalaceae (Sandalwood family)

Figure 2.2 ***Osyris compressa***

Colpoon is a shrub or small tree that can grow 3-5m tall. It is restricted to coastal dunes and occurs from the South Western Cape to the southern part of Mozambique. It bears fruit from November to April; the fruits are ovoid, fleshy and turn from shiny red at first to purplish-black when ripe. The fruit is edible, favoured by birds [(63), (64)]. The fresh leaves were used for tanning leather [(62)].

Harpephyllum caffrum

Wild Plum, Wildepruim Zulu: Umgwenya Family: Anacardiaceae (Mango family)

Figure 2.3 *Harpephyllum caffrum*

The Wild Plum is evergreen and grows up to 20m in height. It occurs from the Eastern Cape through to Mozambique and Zimbabwe. Fruiting from December to May, the fruits are oblong, 20-25 mm long, thinly fleshed and bright red when ripe. Some trees have sweet tasting fruit whilst others bear fruit that is sour. The fruits are well known and commonly used for eating. A drink, similar in taste to lemonade, can be made by the addition of water and sugar to peeled fruit. The sour, juicy pulp is also excellent for making jams and jellies. Fruits have also been used for making rosé wine [(62), (64), (65)].

Bark decoctions were used as blood purifiers, to treat acne and eczema [(57), (66)]. The polyphenolics and flavonoids of the leaves and bark of this species were examined by El Sherbeiny in 1976 [(69)]. This study resulted in the isolation of protocatechuic acid, gallic acid, methyl gallate, kaempferol–3–rhamnoside, kaempferol–3–galactoside, apigenin–7–glucoside, quercetin–3–rhamnoside, quercetin–3–glucoside, and quercetin–3–arahinoside, as well as the free aglycones, quercetin and kaempferol.

Chironia baccifera

Christmas berry, Bitterbos, Aambeibossie Family: Gentianaceae

Figure 2.4 *Chironia baccifera*

This is a fast growing shrub that can grow up to 1m tall. It is usually found in dry, sandy soil or on sand dunes, growing in the shade of other plants. It occurs from KwaZulu-Natal, along the coastal belt south through the Eastern Cape and Western Cape to as far north as Namaqualand in the Northern Cape. It bears small red fruit which ripen around Christmas - hence the common name.

The berries have been used as a remedy for piles [(57), (64)]. This species has been an important medicine in South Africa, being sold as bundles of dried herb in the informal markets. It was traditionally used by the Khoi as a bitter tonic. Infusions and tinctures have been used to treat stomach ulcers, diarrhoea, syphilis, kidney and bladder infections, diabetes and haemorrhoids. The recommendation for its use in the treatment of syphilis dates back to 1868 [(70)]. It has also been used as a blood purifier for skin conditions including acne, sores and boils [(62), (57)]. Taken as an infusion in combination with *Leonotis leonurus* and *Helichrysum petiolare,* it has been used to treat hypertension [(71)].

Although dried plants have caused death in experimental trials with sheep and rabbits [66] , there are no reports of human toxicity or contraindications. This was confirmed *in vitro* for the aqueous extracts of the whole plant in a recent anti-diabetic screening study [72] .

The following monoterpene secoiridoids (intermediates in the synthesis of bio-active alkaloids) have been isolated from four species from southern Africa: Chironioside, Eustomoside, Gentiopicroside, Sweroside and Swertiamarin [73]. Beuscher *et al* (1994) [74] examined *Chironia baccifera* among other African medicinal plants for activity against Poliovirus, *herpes simplex* and Rhinovirus using Plaque Reduction assays. Their general toxicity and effects on Interferon production were also studied. The leaves of *Chironia baccifera* were assessed for antimicrobial activity, the ethanol and ethyl acetate extracts showing some measure of growth inhibition activity against *Mycobacterium smegmatis* [75].

SANBI, in their assessment of the species, report not having found any cardiac, cyanogenic or anthraquinone glycosides present; however, tannins and saponins were found, including oleanolic acid in the leaves [64]. Their comment is that little information is available regarding the pharmacology of this herb.

Carissa macrocarpa

Big Num-num, Grootnoemnoem Zulu: Amathungulu Family: Apocynaceae

Figure 2.5 *Carissa macrocarpa*

The Big Num-num is a spiny shrub or small evergreen tree that grows up to 4m tall. It is a common species on sand dunes and on the edges of coastal forest from the Eastern Cape to Mozambique. It bears delicious large (up to 50 mm), ovoid, bright red fruit, with a milky latex. Fruiting is from September to January. All the parts of the fruit are edible (pips and milky juice included). They have a sweetish taste and are rich in vitamin C, calcium, magnesium and phosphorus. The fruits can be eaten raw or made into an excellent jam. Raw fruit can be marketed locally and abroad as it has a long shelf life. It has been suggested that this fruit should be promoted much more than it is at present [(62), (65), (64)].

Among the Swazi, unspecified parts are said to have aphrodisiac properties [(66)].

Nylandtia spinosa

Tortoise berry, Skilpadbessie Family: Polygalaceae

Figure 2.6 *Nylandtia spinosa*

This spiny shrub grows up to 1m high and is widespread throughout the Cape along the coastal belt. It bears a profusion of pink-purple flowers followed by small fruit from May, which ripen in June, turning from orange to red when ripe. Rich in vitamin C, the fruits are eaten by tortoises and birds and are also a popular snack with children [(63)].

Leaves and stems have been used as a digestive tonic and for treating abdominal pain and tuberculosis. It was first illustrated in 1685 in Simon van der Stel's record of his expedition to Namaqualand where the fruit was reported to be thirst quenching [(62), (64)].

Carpobrotus edulis

Sour fig, Suurvy, Hotnotsvy Khoi: Ghaukum Zulu: Umgongozi

Family: Mesembryanthemaceae (Vygie or Mesemb family)

Figure 2.7 *Carpobrotus edulis* flower

Figure 2.8 *Carpobrotus edulis* fruit

The Sour Fig is a robust creeping succulent, low growing to a height of 10-12cm, with trailing stems 2m in length. Seven *Carpobrotus* species are found in the Cape coastal region and along the East Coast to Southern Mozambique. It bears large flowers between August and October followed by conical, fleshy fruit capsules which turn reddish brown when ripe. The fruit contains an edible sweet-sour slimy pulp with shiny brown seeds. The base of the fruit is bitten off and the pulp sucked out - a popular snack amongst Cape children. The dried fruits are regularly sold on local markets in and around Cape Town, mainly to be processed into jam but also as an important ingredient of oriental cooking [(62), (63), (64)].

Sap from the plant is mildly antiseptic and syrup made from the fruit is said to have laxative properties. The Khoi and San were the first to make use of the plant, hence its name, and generations have made use of its remarkable medicinal properties. The three angled succulent leaves contain an astringent juice which is antiseptic and can be mixed with water and taken internally for treating tuberculosis, diarrhoea, dysentery. It is also used as a gargle for treating sore throats, mouth ulcers and gum infections; and as a lotion for bruises, scrapes, cuts, grazes and sunburn. It is also effective as a daily application for ringworm, and in the treatment of infantile eczema. The figs also act as a purgative [(57), (58), (66), (12)]. Extracts of the leaves have been shown to have antimicrobial properties [(13), (76)].

Chrysanthemoides monilifera

Bush-tick Berry, Bietou Zulu: Inkhupuyana Family: Asteraceae (Daisy family)

Figure 2.9 *Chrysanthemoides monilifera*

The Bietou is a fast growing shrub or small spreading tree up to 2 m tall. It is widely distributed along the coast from the Northern Cape through the Western and Eastern Cape and up along the Drakensberg escarpment in KwaZulu-Natal, through Swaziland, Mpumalanga and further into Zimbabwe and to the northern Eastern Africa. It occurs in both Fynbos and Strandveld in the Western Cape. It bears attractive yellow flower-heads; the small fruit is fleshy, egg-shaped, sweet, brown/ black at maturity and up to 6 mm in diameter. The fruits appear shortly after flowering, which can be right through the year, but mainly from late autumn to winter, April to June.

Today the fruit is mainly eaten by children although it was important to the Khoi and San as a food source [(62)]. The ripe berries can be made into a delicious jam or nourishing syrup. Small frequent doses of juice from the fruit were reported to be administered by the Zulu, Xhosa or Sotho for blood strengthening or purification to men suffering from impotence or weakened by intestinal ailments. The ripe berries are added to their porridge or the juice taken in water or tea, sometimes combined with other herbs that also give strength. It was also used by adolescents to clear up pimples [(66)].

Grewia occidentalis

Crossberry, Kruisbessie Zulu: Iklolo Family: Tiliaceae

Figure 2.10 *Grewia occidentalis*

The Crossberry is an evergreen to semi-deciduous tree that can grow up to 10m tall. It occurs from Zimbabwe in the North to the Western Cape in the South and grows in a variety of habitats. The Crossberry flowers throughout the year with a peak during spring and early summer. Fruits are 4-lobed drupes, up to 25mm in diameter, reddish brown and slightly fleshy and shiny when mature. Fruiting occurs from January to May. The ripe fruits are eagerly eaten by humans for their sweet fruity taste. In certain areas where the sugar content of the fruits is high, fruits are collected and dried for later use. Dried fruits are sometimes boiled up in milk as a flavouring [(65), (64), (65), (66)]. Beer is brewed from the ripe fruit in certain areas. Ripe fruits are also eaten by various birds.

Olea europaea subsp. africana

Wild Olive, Olienhout Zulu: Isadlulambazo, umquma Family: *Oleaceae*

Figure 2.11 *Olea europaea* subsp. *Africana*

The Wild Olive is an evergreen tree growing up to 14m in height. It is found in almost any type of habitat, especially in woodlands, along stream banks and on rocky slopes. It bears small, spherical, thinly fleshy fruits (either sweet or sour) from March to August that ripen purple-black. The fruits are edible and the early Cape settlers used the fruits to treat diarrhoea. Leaves, roots and bark were used by the Zulu to treat sore throats, high blood pressure, and urinary tract infections [(57), (65), (66), (64)].

Research carried out on the leaves of this species revealed the active compounds to be the secoiridoids, oleuropein and oleacein. These showed promise of reducing blood pressure through coronary flow increase and by inhibiting angiotensin converting enzyme (ACE) [(77)]. Cardiotonic and antidysrhythmic effects of the extracts were shown by Somova *et al* [(78)].

CHAPTER 3 THE LINK BETWEEN DIET, ANTIOXIDANTS AND HEALTH

"Blueberries best be eaten because they taste good, not because their consumption will lead to less cancer."
James Watson, Nobel Prize winner for the double helix structure of DNA [79].

How did we arrive at the concept of 'oxidative stress'?

The definition of an antioxidant has come to include "Any substance that delays prevents or removes oxidative damage to a target molecule" [80]. The remarkable preservation of the Egyptian mummy on display in the British Museum bears testimony to the technical expertise in the use of antioxidants derived from plants which was available to the ancient Egyptians as far back as 3,500 BC [81].

In our modern times the impetus to gain an understanding of the mechanisms by which free radicals are formed gained momentum from the 1870's onwards. It was driven initially by the need to overcome the industrial problem of the perishability of rubber. By the 1940's the auto-oxidation mechanism had been elucidated, and chain-breaking antioxidants were identified [82]. Other important industrial processes involving oxidation that needed to be addressed at the time included metal corrosion, and with the advent of internal combustion engines, polymerization of fuels, resulting in the fouling of engine parts. The food industry, in trying to curb rancidity in unsaturated fats, began to research the role of antioxidants in biology [83]. This resulted in the identification of the antioxidant vitamins A, C and E. Soon their place in biochemical pathways involving oxidation was being elucidated.

In the 1950's Denham Harman (widely known as the "father of the free radical theory of aging"), began to research the effects that exposure to radiation had on animals. The results obtained after exposure to radiation appeared similar to what occurs in aging. Because ionizing radiation generates free radicals, Harman hypothesized that radicals were also generated as a by-product of metabolism, as well as by copper and iron containing enzymes present within the cells, and that these contributed to aging or were the actual cause of the aging process. In 1957 Harman carried out his first antioxidant study using a radio-protective compound. He obtained a 20% increase in the lifespan of the mice studied, which suggested a connection between free radicals and aging. This marked the beginning of a generation of studies on longevity and its relationship to oxidative stress.

In 1968 Harman used BHT (Butylated Hydroxy Toluene), the food preservative, in a dietary lifespan study and demonstrated that antioxidants affect maximum species lifespan. In 1972 he put forward the idea that free radicals were being generated within the cell, and that free radical

damage was the cause of aging, thereby proposing the “mitochondrial free radical theory of aging”. This “free radical theory of aging [84]” postulated that a chronic state of oxidative stress is found in the cells of aerobic organisms under normal physiological conditions due to an imbalance of antioxidants and pro-oxidants. As the oxidative damage accumulates, it contributes to a decline in the function of cellular processes with age. This theory fuelled the drive to begin research on the links between oxidative stress, longevity and age-related disease in humans. And thus began the search for the “elixir of youth” in the form of antioxidants believed to slow the aging process [81]. This “free radical theory of aging” has since been modified to the “oxidative stress theory of aging” as it was realized that molecules other than free radicals e.g. peroxides and aldehydes, are also involved in oxidative damage to cells [85].

The epidemiology puzzle

In 199I, Serge Renaud presented intriguing epidemiological evidence showing a correlation between the high consumption of flavonoids from red wine and a lower risk of cardiovascular disease. In his treatise the “French Paradox” [86] he noted that the French suffer a relatively low incidence of coronary heart disease despite eating a diet relatively rich in saturated fats. From this observation an assumption was drawn up that consumption of red wine decreases the incidence of cardiac diseases. What followed was ‘an explosion’ of epidemiological studies interrogating the protective role food polyphenols might play in human health [87].

These studies analysed the health implications of dietary phenolic intake on various pathological situations. However, a major limitation of the use of component-based epidemiological studies is their inability to distinguish between the effects of various flavonoids, for example, catechins, from flavonols and flavones. It should be remembered that a particular food contains a large number of compounds; and while some of these may be known and even quantified, others are unknown and therefore not measurable [88]. In fact, it has been estimated that a plant-based diet comprises over 25,000 phytochemicals, with 4,000 to 8,000 of these believed to be flavonoids [89] [90] [91].

Therefore, when such studies are used to try to ascertain the independent effects of dietary components, the problem of ‘multicollinearity’ may arise, often making the task an impossible one. While the reliability of the model as a whole is not compromised, it becomes difficult to assess the relative importance of the independent variables in explaining any variation caused by the dependent variable. In other words, a multiple regression model with correlated predictors could indicate how well the entire bundle of data forecasts the outcome variable, but it

may not give valid results about any individual variable, or about which variables are redundant with respect to others [92].

Between 1993 and 2005, twelve cohort studies on flavonoid intake and the risk of coronary heart disease and five cohort studies on flavonoid intake and the risk of stroke (involving over 250,000 people) were published [88]. Among these were the following:

- Zutphen Elderly study (part of the Seven Countries study)
- Finnish Mobile Clinic Health Examination Survey
- Iowa Women's Health Study
- Alpha -Tocopherol, Beta-Carotene Cancer Prevention Study
- Rotterdam Study
- The Male US Health Professionals Study
- Caerphilly Study

These studies required, firstly, an estimation of flavonoid intake and then the analysis of their protective effects for risk of chronic disease. Most focussed only on flavonols (quercetin, myricetin, kaempferol) and flavones (apigenin, luteolin). This was because, at the time, these were the only flavonoids reported in nutrient databases. Even the most up to date (2013) databases for flavonoids in food report only 26 predominant flavonoids in five subclasses found in 506 items, namely four flavonols, two flavones, three flavanones, eleven flavan-3-ols (catechins) and six anthocyanidins [93]. To compound matters, a wide variation in estimates of flavonoid intake was found, from between 23 - 26 mg/day [94] to 50 mg/day [95] in the two Dutch studies.

The Major Classes of Plant Phenolics

Phenolics and polyphenolic compounds are present in all plant tissues and frequently form the most abundant secondary metabolites in fruits, where they sometimes reach high concentrations. In fact, the total phenolic content of certain fruits can reach 1g /100g, such as in persimmons, *Diospyros virginiana*, and certain plums, *Prunus domestica.*

It is estimated that plants produce more than 25,000 terpenoids, 12,000 alkaloids and 8,000 phenolic compounds. The phenolics form a vast group of substances that cannot be defined in

simple terms. The fundamental structural element that characterizes this group is the presence of at least one aromatic ring, substituted by at least one hydroxyl group. This group is either free, or engaged in another function: ether, ester, or glycoside [96]. Only plants and microorganisms are capable of biologically synthesizing the aromatic nucleus. Animal organisms are almost always dependent on either their nutrition, or a symbiosis, to obtain the indispensable metabolites that comprise this structural element [68]. A classification of secondary metabolites according to David Still [96] follows. Secondary metabolites have been divided into three groups, based on their biosynthesis:

1. Terpenes and terpenoids
2. Alkaloids
3. Phenylpropanoids

Most phenolic compounds are derived from phenylpropanoids and include lignins, lignans and flavonoids**.** These are derived from phenylpropanoid (**C_6-C_3**) and phenylpropanoid acetate skeletons (**C_6-C_3-C_6**). The several thousand polyphenols that have been described in plants can subsequently be grouped into several classes. Distinction between these classes is drawn, first of all, on the basis of the number of constitutive carbon atoms, and then on the structure of the basic skeleton. Besides simple soluble forms, there are also polymerized forms of varying solubility (tannins), or completely insoluble forms (lignins) [97]

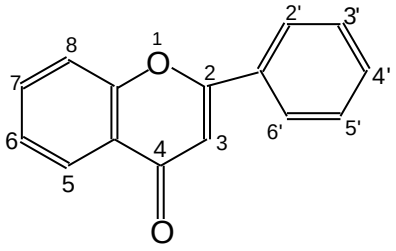
Major classes of plant phenols are given below:

Table 3.1 Major classes of plant phenols

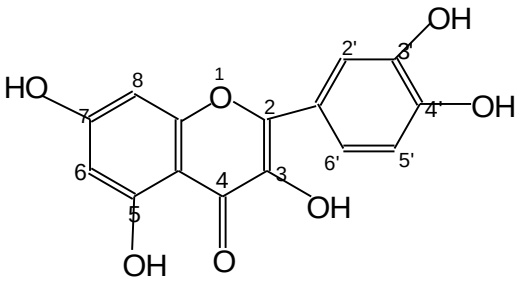
# C atoms	Class	Example
C_6	Simple phenols	Catechol
C_6-C_1	Phenolic acids	p-hydroxybenzoic acid
C_6-C_2	Phenylacetic acids Acetophenones	2-hydroxyphenylacetic acid
C_6-C_3	Cinnamic acids Phenylpropenes Coumarins Isocoumarins Chromones	Caffeic acid Myristin 6,7-dimethoxycoumarin Hydrangenol Eugenin
C_6-C_4	Naphthoquinones	Juglone
C_6-C_1-C_6	Xanthones	Mangiferin
C_6-C_2-C_6	Stilbenes Anthraquinones	Resveratrol Emodin
C_6-C_3-C_6	Flavonoids	Flavones Flavanones Catechins Flavonols Isoflavones Flavanonols Anthocyanins Chalcones Aurones
C_{18}	Lignans Neolignans	Pinoresinol Eusiderin
C_{30}	Bioflavonoids	Amentoflavone
C_n	Tannins Lignins	

As seen from Table 3.1, flavonoids can be regarded as C_6-C_3-C_6 compounds in which each C_6 moiety is a benzene ring. The properties and class of each such compound is determined by the oxidation state of the connecting C_3 moiety [98].

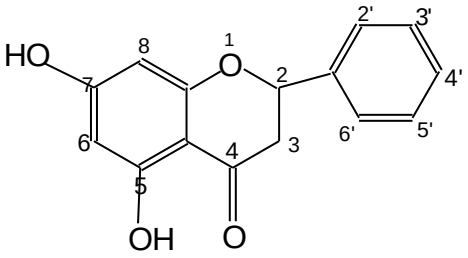
The classes are represented graphically by the following structures:



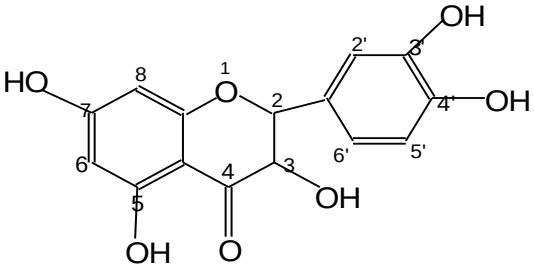
Flavone



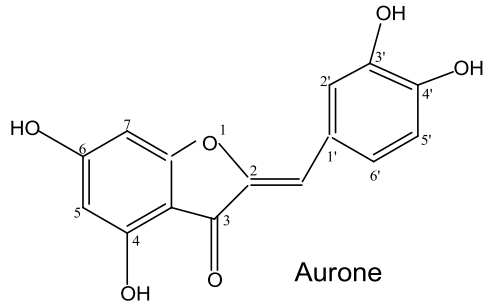
Flavonol



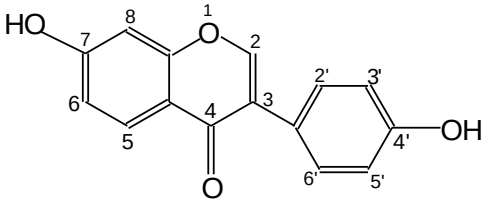
Flavanone



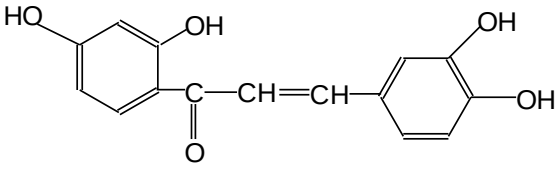
Flavanonol



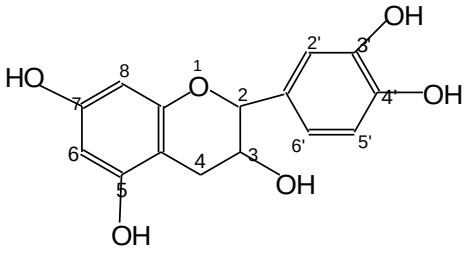
Aurone



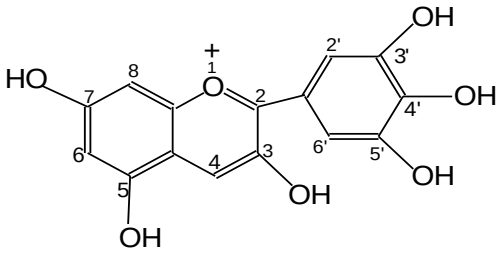
Isoflavone



Chalcone



Catechin



Anthocyanidin

Figure 3.1 Representative Structures of Major Classes of Plant Phenolics

What the Epidemiology studies revealed

From the findings of the meta-analysis of the twelve aforementioned cohort studies, seven of these epidemiological studies suggested an inverse association between increased consumption of fruits and vegetables and risk of cancer and cardiovascular diseases. Two out of five of the epidemiological studies on flavonoid intake and risk of stroke found an inverse association [88]. An explanation was sought. Chronic diseases are associated with increased oxidative stress. It was found that consumption of fruits and vegetables leads to large transient increases in total antioxidant capacity of plasma in humans [99]. The hypothesis was therefore put forward that fruits and vegetables contain compounds (specifically plant polyphenols) that exert protective effects due to their natural antioxidant ability [100].

Using animal models and *in vitro* systems, it had been shown that polyphenols trap and scavenge free radicals. As a natural progression, it was believed that these effects contributed to their protective role in cardiovascular disease and cancer. It is true that flavonoids in particular are strong antioxidants *in vitro*. This is due to their low redox potential and their capacity to donate several electrons or hydrogen atoms. The one-electron reduction potential of quercetin (+0.33V) and catechins (+0.53 – 0.57 V) allows for the protection of urate (+0.59 V) from oxidation by peroxyl radicals (+1.06 V). Quercetin (+0.33 V) and (-)-epigallo-catechin gallate (+0.43 V) have redox potentials similar to ascorbate which allows them to regenerate α-tocopherol (+0.48V) [87]. They are unable to recycle ascorbyl radicals (+0.28 V). However, despite their strong antioxidant capacity *in vitro,* it was found that the antioxidant efficacy of flavonoids *in vivo* is limited by the following factors:

- The absorption of flavonoids in humans is very low compared to other dietary antioxidants, plasma concentrations reaching between 0.06 - 7.6 μM for flavonols, flavonols and flavanones and less than 0.15 μM for anthocyanidins between 1 - 3 hours after consumption of flavonoid rich foods, compared to 30-150 μM for ascorbate and 15-40 μM for α- tocopherol [101].

- Human exposure to flavonoids is chronic and at relatively low concentrations [102]. With the body's endogenous plasma Total Antioxidant Capacity (TAC) usually in the range of 1 mM or more (due to urate, reduced glutathione, redox proteins, hormones, and enzymes [100]), the idea was put forward that the addition of 1 μM polyphenols could hardly exert a powerful antioxidant effect *in vivo.*

- The half-life of flavonoids in human plasma is very short, in the region of a few hours, which limits their capacity to act as antioxidants in plasma *in vivo* [99].

- Even with long term consumption of flavonoid rich foods, significant amounts of flavonoids are not accumulated in plasma. Steady state concentrations of quercetin in human plasma were found to be less than 1 μM [(81)].

- Also, because of the extensive metabolism which flavonoids undergo in the intestine and liver, their *in vivo* form becomes quite different from the glycosidic form present in fruits and vegetables. With their conversion by glucuronidation, methylation and sulfation, they become more water soluble and often less potent as antioxidants in the process [(103)].

- Lastly, flavonoids are degraded by intestinal bacteria, with the breakdown products being far removed from the original form [(87)].

Support for the 'oxidative stress theory of aging' had come mainly from correlative research, the fact that oxidative damage to lipids, DNA and proteins increases with age, while manipulations that increase lifespan reduce oxidative damage. In the case of longer lived animals, decreased oxidative damage (or conversely, increased resistance to oxidative stress) was seen. However, it was pointed out that the manipulations that lead to increased lifespan could very likely alter processes other than oxidative stress, which begged the question as to whether there are other mechanisms leading to the increase in longevity. This hypothesis was tested with the use of transgenic mice, those whose genes influencing the oxidative defence system had been knocked out. The fact that lifespan was not altered in almost all of these models provides strong evidence against oxidative stress playing a major role in the aging of mice [(104)].

Subsequently, an investigation to determine the difference between the *in vitro* versus *in vivo* effects of polyphenols in human plasma was carried out. Addition of apple extracts to human plasma *in vitro* protected endogenous urate, α-tocopherol and lipids from free radical-mediated oxidation brought about by a constant flux of aqueous peroxyl radicals. However, these effects could not be reproduced *ex vivo* following apple consumption by six human subjects [(105)]. The explanation put forward to account for this anomaly includes poor absorption and metabolic conversion of the absorbed polyphenols. The fact that flavonoids are poorly absorbed (being found at micromolar or submicromolar levels in human plasma), and yet after consumption of flavonoid rich foods a large increase in plasma antioxidant capacity occurs in humans, also proved to be a conundrum. In addition, this increase in plasma antioxidant capacity often greatly exceeds what could be expected from the increase in plasma flavonoids.

This was the subject of a research paper published by Balz Frei of the Linus Pauling Institute at Oregon State University in 2004 [(106)]. In this study, six subjects serially consumed five apples; then they consumed bagels and water matching the carbohydrate content and mass of the

apples; and finally they consumed fructose in water matching the fructose content of the apples. The FRAP or 'Ferric Reducing Antioxidant Potential' of their plasma was then measured, with an acute transient increase noted after apple consumption, as well as after fructose consumption. Plasma urate levels were also found to increase significantly after both apple consumption as well as fructose consumption. This was reported to be the well-documented effect of 'fructose-mediated increase on endogenous urate levels', urate being the main contributor to FRAP of human plasma. It had previously been suggested that the cardioprotective effect of red wine might be due in part to the effects of urate, as 30 minutes after ingestion of port wine, both plasma urate and antioxidant capacity increased by 25% [(107)]. Frei concluded that "the purported antioxidant health benefits of flavonoids from fruits are confounded by the metabolic effect of fructose on urate" [(94)].

Flavonoids, as complex molecules, may however exert multiple actions besides antioxidation, including inhibiting the activities of the following enzymes:

- Telomerase
- Glutamate dehydrogenase
- Cyclooxygenase
- Lipoxygenase
- Xanthine oxidase
- Matrix metalloproteinases
- Angiotensin-converting enzyme
- Proteasome
- Cytochrome P450
- Sulphotransferase

They may also affect signal transduction pathways, interact with sirtuins[1], inhibit protein glycation, increase endothelial nitric oxide production, and affect platelet function. However, once again, their low bioavailability should be borne in mind [(108)].

1 Sirtuins are a class of proteins that are involved in the regulation of important biological pathways. Thus far seven subcellular sirtuins have been discovered to be present in mammalian nuclei, cytoplasm and mitochondria. These are implicated in a wide range of cellular processes (cell cycle, metabolism, insulin secretion, tumorigenesis, DNA repair, rDNA transcription, ammonia detoxification, inflammation, apoptosis, aging) as well as degenerative diseases (cancer, diabetes, Alzheimer's, gout, atherosclerosis)

Regarding the routes by which dietary polyphenolics are absorbed in the human body as shown in Fig 3.2, it was found that those reaching the small intestine undergo extensive microbial and enzymatic bio-transformations. Phase II metabolism may yield methoxylated, glucuronidated and sulphated compounds. Products of these conjugation reactions have an increased molar mass and tend to have a decreased bioactivity compared with their substrates [103].

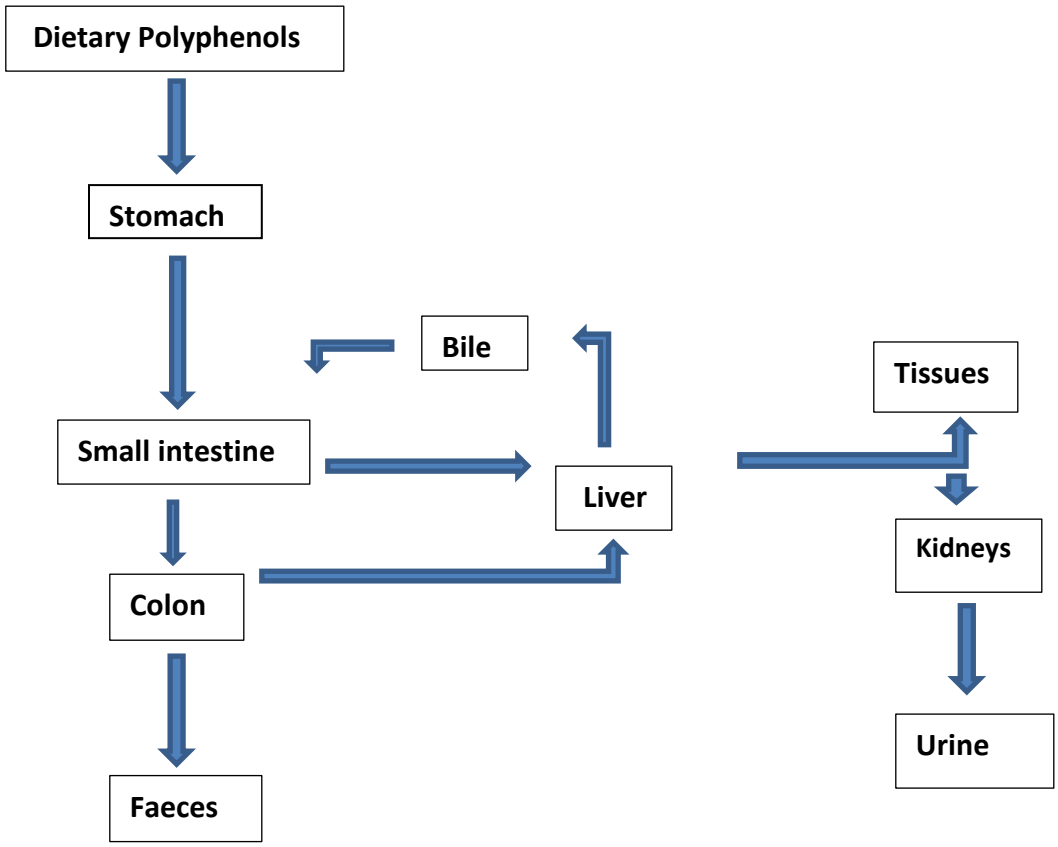
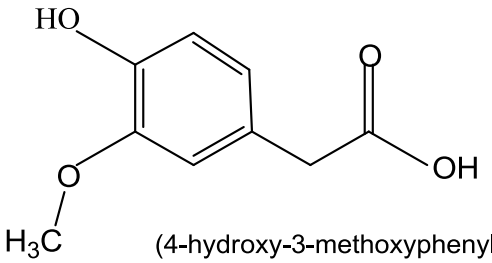
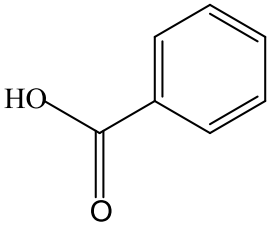


Figure 3.2 Possible routes for polyphenols through the human body.

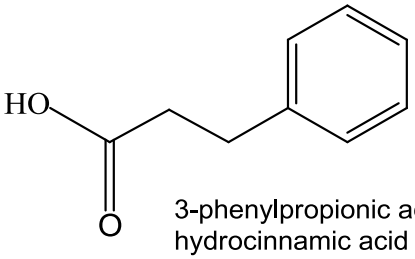
These metabolites are then transported to the liver, where further enzymatic bio-transformations may occur. By means of the blood, these metabolites are delivered to somatic tissues and then to the kidneys for excretion in the urine. They may however be returned to the small intestine through the bile [109]. Those polyphenols that pass through to the colon undergo changes effected by the micro flora of the colon and may, through dissolution into the water fraction of the faeces, influence the epithelial walls of the colon, or be absorbed or excreted in the faeces (see structures of these compounds in Fig 3.3) [110]. Metabolism of polyphenols by intestinal bacteria yields high concentrations of phenolic acids as a major product, for example, homovanillic acid, a metabolite found in human urine [111].



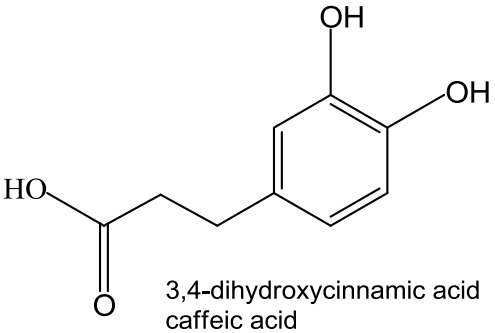
(4-hydroxy-3-methoxyphenyl)acetic acid
Homovanillic acid



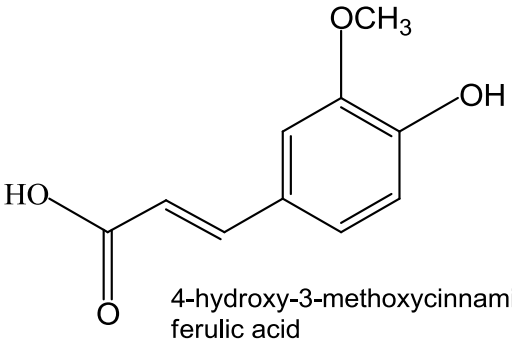
benzoic acid



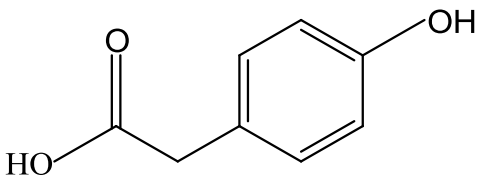
3-phenylpropionic acid
hydrocinnamic acid



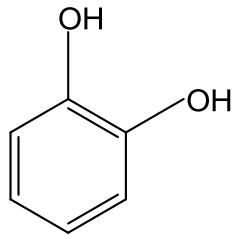
3,4-dihydroxycinnamic acid
caffeic acid



4-hydroxy-3-methoxycinnamic acid
ferulic acid



4-hydroxyphenylacetic acid



catechol
1,2-dihydroxybenzene

Figure 3.3 Major aromatic components of human faecal water [(110)].

While the GI tract is the major site of microbial biotransformation, and enzymatic biotransformation occurs in the liver [(109)] it has been postulated that polyphenols may exert their greatest protective effects before absorption. This is because they are found in higher concentrations in the stomach (at around ≥ mM concentrations), and epidemiological evidence associates a lower risk of gastric, colon and rectal cancer with diets rich in fruits and vegetables [(112)]. The exposure of the gastro intestinal tract (GIT) to lipid hydroperoxides, isoprostanes, iron, nitrating agents, cytotoxic aldehydes, and carcinogens from the diet, coupled with the high rate of cell turnover in the GIT, places this organ at higher risk for the development of carcinoma [(113)]. This has resulted in colorectal cancer becoming the third most frequent cancer and the second leading cancer killer in the USA [(110)].

It is believed that the epithelium of the colon is influenced more by the water phase than the solid phase of human faeces. The analysis of human faecal water yielded total polyphenol mean concentrations of 2.67 μM, with 1.20 μM for naringenin and 0.63 μM for quercetin, respectively. However, total monophenolic mean concentrations were 317.9 μM, with total non-phenolic aromatic acid concentrations 614.2 μM, thus making this level of phenolic acids and aromatic acids up to two orders of magnitude higher than the most abundant polyphenols [(110)]. These levels may therefore contribute significantly to the antioxidant activity available to the colonic epithelium, so that although monophenols have less antioxidant activity, their presence

(in considerable excess compared to polyphenols) may have more influence on the development of colonic disease.

From the foregoing historical background to 'antioxidant status' it can be seen that the problem of unravelling the association between the consumption of specific food items and risk of degenerative disease revealed by epidemiology has proved far more complex than had initially been anticipated by the research community. As stated previously [(88)], given that "each food contains a large number of different compounds, some known and quantified, others less well characterized, and some unknown or unmeasurable", the task becomes almost impossible. Despite the numerous assertions drawn by researchers from several epidemiological studies, it has been said that the actual evidence supporting the cause-effect relationship of increased flavonoid intake (and by extension Total Antioxidant Capacity (TAC)), and decreased cardiovascular disease (CVD), as well as cancer, has proved to be scanty [(114)].

From the above arguments, it follows that while the concept 'Total Antioxidant Capacity' as taken from chemistry may be useful in the comparison of different food items, it cannot be meaningfully applied to *in vivo* situations. It has been suggested that it may possibly be extrapolated to the provision of antioxidant defence and protection of the lining of the GIT by reaction with other dietary pro-oxidants, like iron and copper.

However, in a recent review article by Ronald Prior [(22)] a strong case has once again been made for a relationship between the prevention of adverse health outcomes and the level of consumption of antioxidants, which is backed up by the results released from recent clinical trials [(23)]. The flavonoid/ORAC database from the USDA (available up to 2010 and subsequently withdrawn) has been used in assessing health outcomes in these epidemiology studies. Furthermore, postprandial oxidative stress that has been implicated in inflammatory responses and increased risk for cardiovascular disease has been shown to be mediated by dietary antioxidant components [(23), (115)].

CHAPTER 4 CHOOSING FROM A PLETHORA OF ANTIOXIDANT CAPACITY ASSAYS

"If we knew what it was we were doing, it would not be called research, would it?"

Albert Einstein, Physicist.

It goes without saying that oxygen is fundamental to the support of life. Paradoxically though, due to its reactivity, it is recognized as one of the most toxic chemicals to biological systems. In 1954 it was proposed that oxygen poisoning and radiation injury both lead to the production of oxidizing free radicals [116]. An estimated 1-5% of oxygen consumed by living organisms ends up as Reactive Oxygen Species (ROS). In generating energy, the mitochondria in each cell use a 4-electron chain reaction which reduces oxygen to water [117].

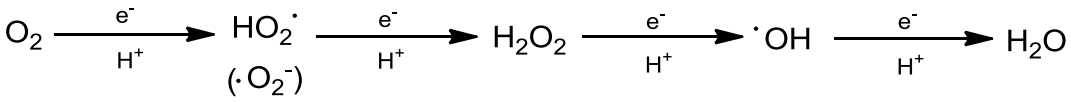


Figure 4.1 Reduction of oxygen to water

Any electrons that escape during these reductions react with oxygen to form superoxide anions $^{\cdot}O_2^-$. These in turn may form further reactive species like hydrogen peroxide H_2O_2, hydroxyl radical $^{\cdot}OH$ or singlet oxygen $^1O_2$. If superoxide reacts with nitric oxide, peroxynitrite $ONOO^-$ is formed, which in turn may generate hydroxyl radicals $^{\cdot}OH$ or nitric dioxide $^{\cdot}NO_2$.

O_2 in its ground state is a free radical, having the electronic configuration ($1s^2\ 2s^2\ 2p^4$). In its orbital diagram [2] it possesses two unpaired electrons of parallel spin in a π * anti-bonding orbital (making it actually a bi-radical). Singlet oxygen $^1O_2$ has paired electrons and is therefore not a radical. However, it is very reactive and can exist in isolation for over an hour at ambient temperature. It has been implicated in the oxidation of LDL cholesterol with resulting cardiovascular disease [118].

2 See Figure 4.2 showing the possible electronic configurations of molecular oxygen O_2: Singlet oxygen $^1\Delta_g$ excited state. Singlet oxygen $^1\sum_g^+$ excited state. Triplet ground state: $^3\sum_g^+$. The 1s molecular orbital is omitted for simplicity [186].

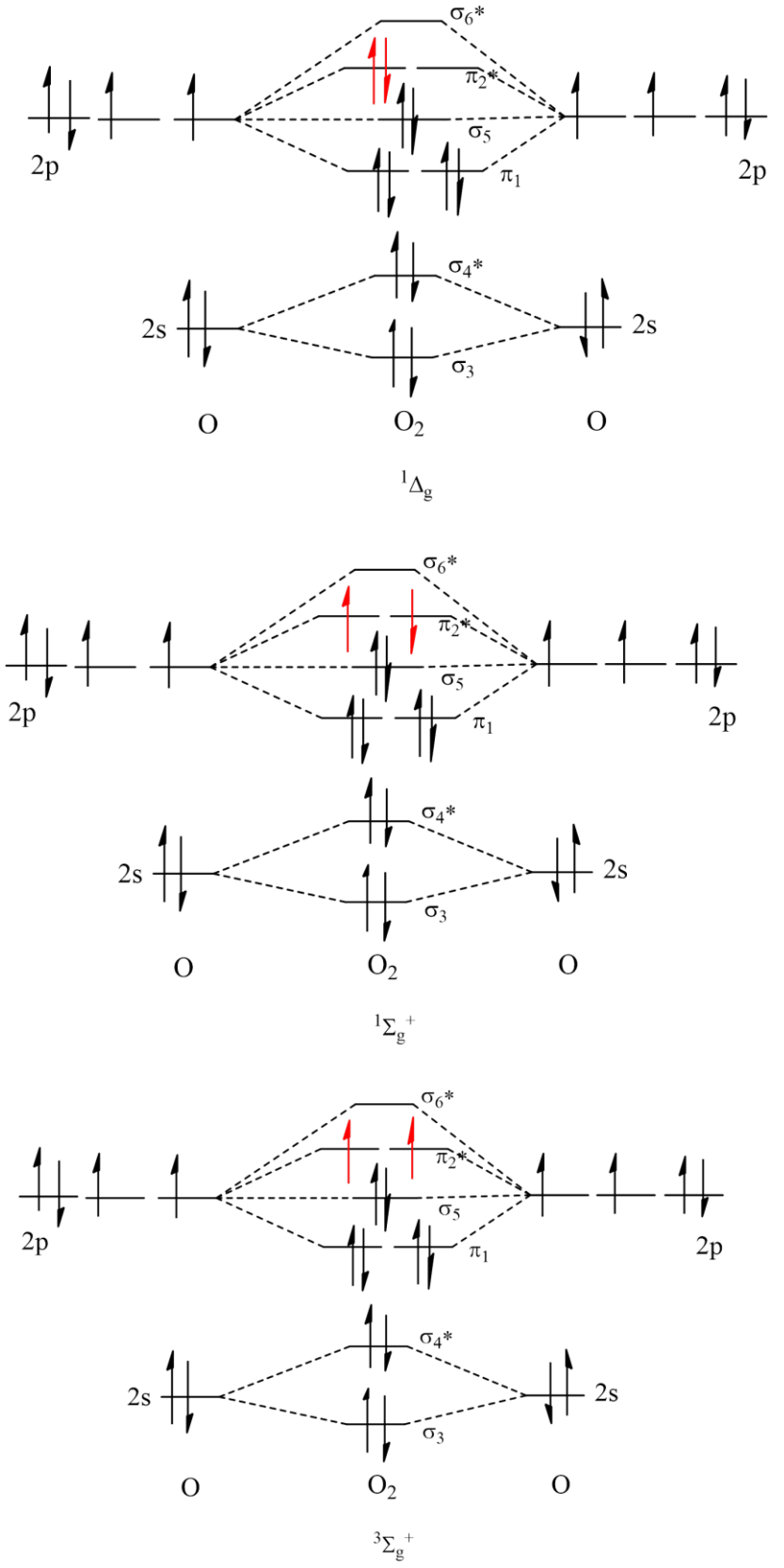


Figure 4.2 Molecular orbital diagram

The superoxide radical $O_2^{\bullet -}$ (see Figure 4.3) forms readily from the one-electron reduction of dioxygen, the second electron filling one of its degenerate molecular orbitals, giving a charged ionic species with a single unpaired electron and a net negative charge of -1. It occurs widely in nature, with hydrogen peroxide H_2O_2 (see Figure 4.5) resulting from the two electron reduction and addition of two hydrogen ions. These species have limited reactivity; however they are able to generate more dangerous hydroxyl radicals $^{\bullet}OH$ (see Figure 4.4) which are highly reactive with a half-life of 10^{-9} seconds and are able to do much damage in biological systems. The hydroxyl radical is not eliminated by any endogenous enzymatic system.

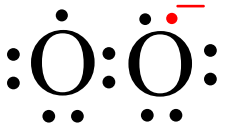


Figure 4.3 Superoxide radical [119]

Figure 4.4 Hydroxyl radical

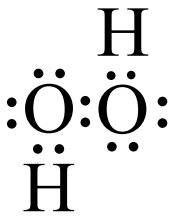


Figure 4.5 Hydrogen peroxide

Free radicals have been defined as 'any chemical species (capable of independent existence) possessing one or more unpaired electrons (one that is alone in an orbital)'. Electrons become more stable when paired together in orbitals, each having a different direction of spin. The radical dot indicates the presence of one or more unpaired electrons. Hydrogen, the simplest free radical, contains one proton and a single unpaired electron [120]. The sources of free radicals and oxidants include the following:

$O_2^{\bullet -}$	superoxide anion
$^1O_2$	singlet oxygen (not a free radical)
HO·	hydroxyl radical
NO·	nitric oxide
ONOO-	peroxynitrite (not a free radical)
HOCl	hypochlorous acid (not a free radical)

RO(O)· peroxyl radical

RS· thiol radical

LO(O)· polyunsaturated fatty acid peroxyl radical

H_2O_2 hydrogen peroxide (not a free radical)

O_3 ozone (not a free radical)

Although most biological molecules are non-radicals, having paired electrons, the following five free radicals wreak the most havoc in living organisms: hydroxyl, superoxide, nitric oxide, thiol, and peroxyl. Other species that are not free radicals, for example peroxynitrite, hypochlorous acid, hydrogen peroxide, singlet oxygen and ozone can however lead to free radical reactions [(120)].

The body's defence system against ROS

It has been estimated that adults at rest inhale approximately 1.8g – 2.4g of oxygen per minute [(119)]. When oxygen is partially reduced in the mitochondria superoxide O_2·- is produced. Under normal respiration, each cell is exposed to 10^{10} molecules of superoxide daily, or approximately 2 kg per annum in a normal weight individual [(121)]. Given that exposure to oxygen is life-long, coupled with the hazard, requires that the endogenous antioxidant system should offer high protection in order to sustain life [(122)]. As a consequence, the body's antioxidant defence system is a multi-pronged network involving prevention, interception and repair. It comprises mainly enzymatic mechanisms, with back-up systems to regenerate these enzymes, and transport systems to remove any detoxification products. Small molecules play a critical role here, including urate, ascorbate, thiol groups, and selenium. The body's *in vivo* defence network against ROS consists of the following:

1. 'Preventing antioxidants' to suppress the formation of ROS/RNS.
2. 'Scavenging antioxidants' to remove ROS rapidly before they can attack.
3. Enzymes to repair, clear and reconstitute.
4. The 'adaptation mechanism' to generate appropriate antioxidants at the right time, transporting them to the right position, in the correct concentration.
5. The 'cellular signalling messengers' to regulate the level of antioxidant compounds and enzymes in the system [(123)].

At least four general sources of antioxidants, both endogenous and exogenous [124] are present in the human body:

- Enzymes: Superoxide dismutase, Glutathione peroxidase, Catalase
- Large molecules: Albumin, Ceruloplasmin, Ferritin, other proteins
- Small molecules: Ascorbic acid, Glutathione, Uric acid, Tocopherol, Carotenoids, Polyphenols
- Some hormones: Estrogen, Angiotensin, Melatonin

It therefore becomes apparent that any assessment of 'Antioxidant Capacity' AOC would present a challenge due to the complexity of these biological systems [120]. Matters are complicated even further by the introduction of the variety of exogenous dietary antioxidants into the system in addition to the endogenous antioxidants. These dietary antioxidants may act either sacrificially or preventatively, by scavenging the reactive oxygen or nitrogen species (ROS/RNS), thereby stopping radical chain reactions; or they may act by inhibiting the reactive oxidants from being formed in the first place.

However, even where a dietary extract may be active *in vitro*, this is no guarantee that its metabolism products are active, or that they are absorbed or become bioavailable *in vivo* [114]. Adding to the complexity of the above systems, individual antioxidants can act by multiple mechanisms within a single system [125]; or under the influence of the reaction system, they may act by a different single mechanism. Among the mechanisms of action involved are free radical chain breaking, oxygen scavenging, singlet oxygen quenching, metal chelation and inhibition of oxidative enzymes [126]. The defensive mechanisms listed include prevention of oxidant formation, scavenging of activated oxidants, and reduction of active intermediates, along with induction of repair systems.

Evaluation of natural antioxidants

From the foregoing, it is evident that natural antioxidants, being multifunctional, cannot be fully evaluated by means of one-dimensional methods only. So, as it is impossible for any one single assay to accurately reflect all of the radical sources, as well as all of the antioxidants in a complex system, a valid evaluation demands the use of several assay methods which allows for the inclusion of different mechanisms of inhibition. It was in trying to address this problem of evaluating these multiple antioxidant activities in natural products that researchers put forward 38 different methods (*in vitro*, *ex vivo* and *in vivo*) between the years 1983 – 1998. These made use of many different free radical initiators, substrates, methods of inducing oxidation, and

oxidation end point measures. However, in the end these methods yielded inconsistent results. This may have been due to the fact that the assay models did not take into account the complexity of the antioxidant activity. As some assays measure Hydrogen Atom Transfer (HAT) activity, while others measure Single Electron Transfer (SET), it stands to reason that activities measured on the same sample extract by different assays would show a lack of correlation [127]. Eventually the need was seen for the application of more valid and rigorous guidelines to bring some uniformity to the methodology [128].

Frankel [128] recommended considering the following six parameters when setting up a protocol:

- Use of substrates relevant to biological systems
- Selection of conditions to simulate biological systems closely
- Analyses that allow for the measure of initial products and secondary decomposition products of oxidation at detection levels of below 1 %
- Similar molar concentrations of structurally related compounds used as reference materials
- Total Phenol Contents and compositional data used to compare natural products
- Quantification on the basis of % Inhibition, Rates of Hydroperoxide formation/decomposition, I_{50} (Antioxidant Concentration to achieve 50% Inhibition), T_{50} (Time to reach 50% Inhibition), Induction Period.

In trying to achieve a comprehensive evaluation of each natural product, in addition to the above recommendations it was also advised that various oxidation conditions be used, as well as several methods of measuring the different products of oxidation. By the careful design of these protocols, as well as the use of relevant substrates and mild conditions of oxidation, the elucidation of the mechanisms of antioxidation could be achieved [129].

In June 2004, the First International Congress on Antioxidant Methods was held to address the analytical issues surrounding the determination of AOC 'Antioxidant Capacity' and to propose the use of one or more methods for standardization. It was pointed out that the selection of an appropriate AOC assay method requires matching the radical source and system characteristics

to the antioxidant reaction mechanisms. From this congress, the following recommendations were put forward for a standardized method for AOC [124]:

- Measure the actual chemistry occurring
- Use a biologically relevant radical source
- Be simple
- Use a method with a well-defined end point and chemical mechanism
- Use readily available instrumentation
- Have good within-run and between-day reproducibility
- Be adaptable to lipophilic and hydrophilic antioxidants
- Use of different radical sources
- Be adaptable to high through-put analyses for routine QA

It was also recommended that the following performance characteristics be scrutinised:

- Analytical range
- Recovery
- Repeatability
- Reproducibility
- Recognition of interfering substances

With reference to the above criteria, following the Congress it was proposed by Prior [124] that three methods be standardized for the measurement of AOC in natural products:

1. Folin-Ciocalteu Phenolics assay
2. TEAC
3. ORAC

The above methods were chosen to involve the use of both reaction mechanisms, HAT and SET - with the $ORAC_{FL}$ assay using the biologically significant peroxyl radical in the HAT mechanism (measuring both the lipophilic as well as the hydrophilic AOC) and the TEAC method using the $ABTS^{\cdot+}$ radical with the SET mechanism. The Folin-Ciocalteu assay has traditionally been used

to measure total phenolics, but it is actually a redox reaction (and therefore another AOC method), reducing phenols by means of the molybdotungstate reagent [124]. The possible future development of new methods using other radical sources such as hydroxyl, superoxide, and peroxynitrite to form an arsenal of pertinent assays provides a challenge for researchers working in this field.

Jimenez-Alvarez [130] in developing standard procedures for the measurement of AOC of food extracts for a food multinational, selected the following assays: $ORAC_{FL}$ (hydrophilic and lipophilic), FRAP, and in addition, ICA (Iron Chelating Activity), as both iron and copper have been shown to be catalysts of lipid oxidation in foods [131]. By this means it was believed that all the relevant antioxidant mechanisms were being targeted, namely radical scavenging, reducing capacity, and metal chelating properties. In addition, Total Phenol Content, as measured by the FCR method, was included in this battery of assays.

The radicals used in the TEAC and FRAP assays, namely Fe^{3+}-TPTZ and $ABTS^{+}\cdot$ have very similar redox potentials (0.70 and 0.68 V respectively), thus providing similar results. However, the FRAP assay uses iron as the oxidant, which is considered to be more biologically relevant in the lipid oxidation process than the artificially generated $ABTS^{+}\cdot$ radical [130].

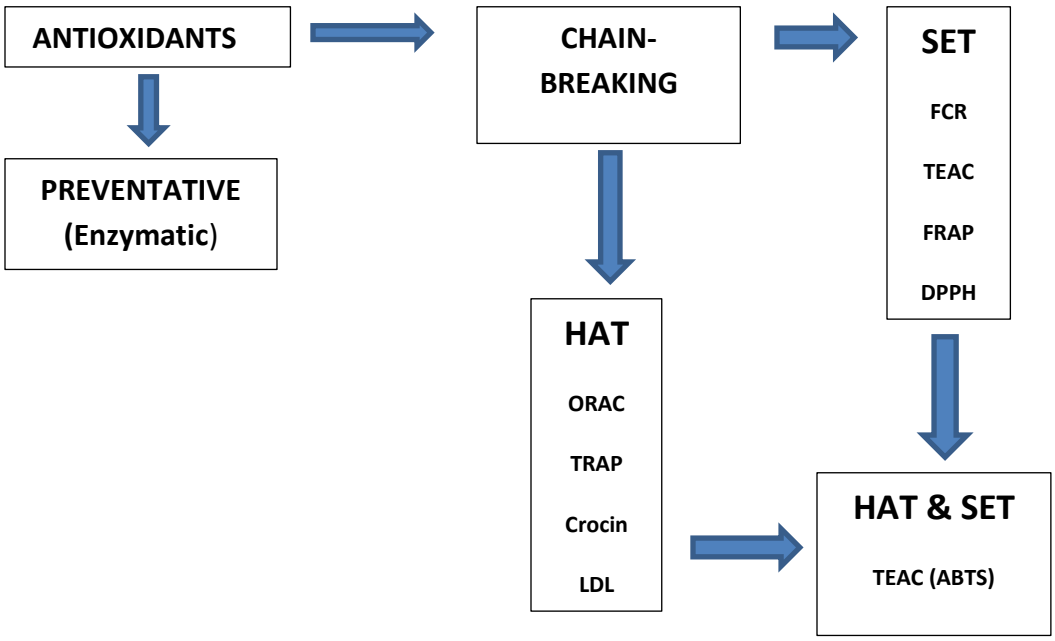


Figure 4.6 Classification of antioxidant assay methods

Classification of antioxidants by reaction mechanism

Antioxidants have been classified into two categories by their mechanism of action:

- Preventative antioxidants
- Chain-breaking antioxidants

Preventative antioxidants (mainly the enzymatic systems) work to inhibit the formation of ROS. Chain-breaking antioxidants (which include the small molecules like uric acid, ascorbic acid, tocopherols, bilirubin and also polyphenols) work by scavenging oxygen radicals, thereby breaking the radical chain sequence. Chain-breaking antioxidants can follow two possible pathways, hydrogen atom transfer (HAT) or single electron transfer (SET). It follows then that AOC Antioxidant Capacity assays are classified, according to the reactions involved, as the following [(132)]:

- HAT – hydrogen atom transfer
- SET – single electron transfer

HAT-based assays quantify any hydrogen donating capacity, making this method more relevant to the radical chain-breaking antioxidant capacity; whereas SET-based assays measure the antioxidant's reducing capacity. From empirical evidence gathered to date, it appears that any radical trapping capacity relates directly to the hydrogen atom donating ability of the compound. It is not correlated to redox potentials alone. Unfortunately, HAT assays do not mimic real life situations in that they use concentrations of antioxidants in excess of the concentration of substrate (in the case of fluorescence studies, the substrate is the probe), whereas in real life the concentration of the antioxidant is always far below that of the substrate.

The information regarding the reducing capacity of a sample provided by SET-based assays is useful, because, while not directly related to radical scavenging capacity, it remains an important parameter of antioxidants. Peroxynitrite and hypochlorite, for example, can be readily reduced to harmless species.

Classification of AOC assays by HAT or SET reaction mechanisms [(124)]

HAT (hydrogen atom transfer) assays include:

- LDL Low Density Lipoprotein oxidation
- ORAC Oxygen Radical Absorbance Capacity

- TRAP Total Radical Absorption Potentials
- Crocin bleaching by LOO ·

SET (single electron transfer) assays include:

- FCR Total Phenols assay by Folin-Ciocalteu reagent
- TEAC Trolox Equivalent Antioxidant Capacity (oxidant= ABTS+ ·)
- FRAP Ferric ion Reducing Antioxidant Power (oxidant = Fe(III))
- TAP Total Antioxidant Potential (oxidant = Cu(II)complex)
- DPPH assay

HAT-based methods:

HAT-based methods measure the ability of an antioxidant to quench free radicals by hydrogen donation [124]. In the case of the ORAC assay, a radical initiator generates the peroxyl radical ROO· which preferentially abstracts an H atom from the antioxidant (AH), resulting in the formation of a stable antioxidant radical, which allows the target molecule probe (FL) to be spared.

$$X^{\cdot} + AH \rightarrow XH + A^{\cdot} \qquad \text{(AH= any H donor)}$$

$$ROO^{\cdot} + AH \rightarrow ROOH + A^{\cdot}$$

$$ROO^{\cdot} + FL\text{-}H \rightarrow ROOH + FL^{\cdot}$$

(FL= fluorescent probe; FL· = oxidized probe, resulting in loss of fluorescence)

In the case of phenolic antioxidants, a relatively stable phenoxy radical ArO· is produced , which reacts slowly with lipid substrates, yet rapidly with peroxyl radical ROO· [133] .

$$ROO^{\cdot} + ArOH \rightarrow ArO^{\cdot} + ROOH$$

All of the above reactions constitute classical radical chain-breaking activity by hydrogen atom transfer. The relative reactivity of the antioxidant is determined by the bond dissociation energy (BDE) of its hydrogen donating group, and its ionization potential (IP). Compounds with Δ BDE of ca. -10 kcal/mol and Δ IP of ≤-36 kcal/mol would dominate in this category. The kinetics of HAT reactions is dependent on solvent and pH; they occur rapidly, being complete within

minutes. It should be borne in mind that the presence of reducing agents and metals may result in high 'apparent activity'.

SET-based methods:

SET-based methods measure the ability of a potential antioxidant to transfer a single electron from the antioxidant molecule to the oxidant to reduce a radical or metal. This results in a change in the spectrophotometric absorbance of the antioxidant/oxidant, the measurement of which quantifies the reducing ability [124), (133)].

$X^{\bullet} + AH \rightarrow X^- + AH^{\bullet+}$ (AH=antioxidant)

$AH^{\bullet+} + H_2O \leftrightarrow A^{\bullet} + H_3O^+$

$X^- + H_3O^+ \rightarrow XH + H_2O$

$M(III) + AH \rightarrow AH^+ + M(II)$ (M= metal ion)

$ROO^{\bullet} + ArOH \rightarrow ROO^- + ArOH^{+\bullet}$ (ArOH= antioxidant)

$ROO^- + ArOH^{+\bullet} \rightarrow ArO^{\bullet} + ROOH$ ($ROO^{\bullet}$ = peroxyl radical)

SET reactions are pH dependent, the charged species being stabilized by the solvent. The relative reactivity of SET reactions is affected by the deprotonation and Δ IP of the reactive functional group. As pH increases, Δ IP of the antioxidant decreases, and, as deprotonation increases, the electron-donating capacity of an antioxidant increases. SET mechanisms predominate if Δ IP > -45 kcal/mol. SET reactions are characteristically slow, taking long to reach completion; therefore it is more convenient for the antioxidant capacity to be calculated as % decrease of product.

Here again, secondary reactions can cause significant interference, the method being sensitive to both ascorbic and uric acids. Also the presence of trace metals can lead to high variability and poor reproducibility. It should be borne in mind that antioxidant capacity measurements are based on competition kinetics. As both HAT and SET occur simultaneously during the reaction, their balance is determined by the antioxidant structure and the pH of the system. It should also

be noted that deactivation of radicals by either HAT or SET gives the same end result, whichever mechanism occurs. The difference lies only in the kinetics and the potential for side reactions that might occur [124].

In biological redox reactions, oxygen is reduced to H_2O by change of the oxidation state of oxygen from 0 to -2 with transfer of hydrogen atoms, the reductants being dehydrogenated while oxygen is hydrogenated [124]. In the case of polyphenols, with their multiple activities, certain mechanisms become dominant, depending on the medium and the substrate of testing. Therefore, their assessment should always include the measurement of more than one property.

Detailed discussion of the recommended AOC methods using HAT reaction mechanisms

ORAC: Oxygen Radical Absorbance Capacity

The ORAC method is a so-called 'competition method' used to measure the capacity of antioxidants for scavenging radicals in competition with a probe. Fluorescence is used to measure the resulting suppression of decay of the probe. In the ORAC assay, artificially generated peroxyl radicals react with fluorescein. As the reaction progresses the fluorescein is consumed and subsequently the fluorescence decreases. In the presence of an antioxidant, a competitive reaction takes place between the fluorescent probe and the antioxidant, retarding the consumption of fluorescein. Because different antioxidants show different reaction curves, taking a fixed time point or inhibition degree may yield quite different AOC values, changing the ranking between compounds.

This is overcome by using the area under the curve (AUC) method of quantitation, as this method uses the baseline as the starting and endpoint. By using inhibition time and degree, the different reaction kinetics can be accounted for, thus providing better data [124]. The AOC in this case is a measure of the area under the time-recorded fluorescence decay curve and can be calculated by the difference in the area under the curve (AUC) between the blank and the sample. It may also be evaluated at the midpoint of the assay. The relationship between decrease in fluorescence and time is not linear [130].

As vitamin E and carotenoids found in natural products are not accounted for by the hydrophilic $ORAC_{FL}$ assay [134] an assay to measure the ORAC of lipophilic components was developed by Huang [135] using randomly methylated β-cyclodextrin, which allows the lipophilic components to be solubilized. The lipophilic and hydrophilic components can thus be measured separately using the same peroxyl free radical source. By combining the lipophilic (L-$ORAC_{FL}$) and

hydrophilic (H-ORAC$_{FL}$) values, the total antioxidant capacity (TAC) can be obtained [136]. In most cases, it is the H-ORAC$_{FL}$ that accounts for >90% of the TAC [134].

Put another way, ORAC measures the inhibition of peroxyl radical induced oxidations. The peroxyl radical reacts with a fluorescent probe to form a non-fluorescent product, which is quantitated using fluorescence. The fluorescence decay is therefore an indication of the damage inflicted by the peroxyl radical.

Initiation Step:

$$R\text{-}N{=}N\text{-}R \rightarrow N_2 + 2R^{\cdot} \qquad (R_2N_2 = \text{azo compound})$$

$$R^{\cdot} + O_2 \rightarrow ROO^{\cdot}$$

$$ROO^{\cdot} + LH \rightarrow ROOH + L^{\cdot} \qquad (LH = \text{substrate})$$

Propagation Step:

$$L^{\cdot} + O_2 \rightarrow LOO^{\cdot}$$

$$LOO^{\cdot} + LH \rightarrow LOOH + L^{\cdot}$$

$$ROO^{\cdot} + \text{probe (fluorescent)} \rightarrow ROOH + \text{oxidized probe (loss of fluorescence)}$$

Inhibition Step:

$$ROO^{\cdot} + AH \rightarrow ROOH + A^{\cdot} \qquad (AH = \text{antioxidant})$$

Termination Step:

$$ROO^{\cdot} + A^{\cdot} \rightarrow ROOA$$

$$(n\text{-}1)\ LOO^{\cdot} + A^{\cdot} \rightarrow \text{nonradical products}$$

$$LOO^{\cdot} + LOO^{\cdot} \rightarrow \text{nonradical products}$$

In this assay the relative fluorescence intensity is plotted against time, with the antioxidant capacity being determined by a decreased rate and amount of product over time. The area under the fluorescence decay curve (AUC in the absence of antioxidant) is calculated and

subtracted from the area under the curve (AUC in the presence of antioxidant) to give the net increase in AUC area under the curve. These net integrated areas under the fluorescence decay curves (AUC) are used in the calculation, which takes into account the lag time, initial rate and total extent of inhibition in a single value.

Net increase in AUC = {(AUC in presence of AOX) – (AUC in absence of AOX)}

A standard curve for 5 concentrations of Trolox (between 0.78 and 12.6 µM) is plotted versus the AUC, and the ORAC values obtained are reported as Trolox equivalents. These values are calculated using linear ($y = a + bx$) or quadratic ($y = a + bx + cx^2$) relationships between Trolox concentration (y) (µM) and the net area under the FL decay curve (x) (AUC sample – AUC blank). The dynamic range of the assay is slightly extended by the use of quadratic regression. The data are reported as micromoles Trolox equivalents (TE) per litre or g of sample (µmol of TE/g or µmol of TE/L).

Advantages:

- ORAC uses the $ROO^{\cdot}$ peroxyl radical which is biologically relevant, and although slightly less reactive than $HO^{\cdot}$ has a half-life of seconds rather than nanoseconds [137]
- The method can be adapted by the use of different radicals, HORAC utilising peroxynitrite radicals and NORAC utilising hydroxyl radicals [138]
- The AOC of many foods and compounds have been measured by ORAC and their rankings can be used for comparative purposes [133]

Disadvantages:

- The original ORAC assay could only measure the hydrophilic chain-breaking antioxidant capacity against peroxyl radicals. Through the use of an acetone: water (1:1) solution containing 7% randomly methylated β- cyclodextrin, the method was adapted so that lipophilic antioxidants could be solubilized to allow for their inclusion in the assay. However, the lipophilic and hydrophilic components must be selectively extracted before assay [134]

- The value obtained depends on which probe is used, fluorescein tending to overestimate the capacity of a weak antioxidant. It has been recommended therefore that two probes be used, one with low and one with high reactivity toward free radicals [123]

- Interference by sample matrix may occur, especially where the antioxidant activity of the sample is low
- Whether the antioxidant mechanism and the protection of fluorescein by antioxidants can really mimic critical biological substrates is merely an assumption [129]
- The temperature sensitivity of the assay reduces its reproducibility
- The equipment for this assay is not standard in many laboratories
- The analysis time is long (ca.1 hour)
- The values obtained, being a combination of both inhibition time and extent of inhibition, cannot be compared with singular values from any other methods [129]

Detailed discussion of recommended AOC methods using both HAT and SET reaction mechanisms

TEAC and DPPH methods neutralize radicals both by direct reduction via electron transfers, and by radical quenching via H-atom transfer.

TEAC or other $ABTS^{\bullet+}$ assays [139]:

This method has been used to assess the AOC of various fruits, including blueberries, blackberries and Goji berries [140], [141]. 2, 2'-azinobis (3-ethyl-benzothiazoline-6-sulfonate) ($ABTS^{\bullet+}$) is prepared in situ by direct oxidation of $ABTS^{\bullet+}$ with potassium persulphate [126]. The sample to be tested is added after the generation and quantification of the ABTS·+ so as to overcome any possibility of the antioxidant reacting directly with the oxidizing agents and the subsequent overestimation of the AOC. The antioxidant is added and after a fixed time period (4-6 min) the remaining $ABTS^{\bullet+}$ is quantified spectrophotometrically, the absorbance being measured at 734 nm, with a decrease indicating antioxidant activity in scavenging this radical. In other words, at a fixed time point of 4-6 min, the decrease of absorbance of $ABTS^{\bullet+}$ in the presence of the sample, or of Trolox, is measured and the antioxidant capacity is calculated as Trolox equivalents.

Trolox Equivalent Antioxidant Activity (TEAC) is the amount of 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox) (mM) required to produce the same activity as 1mM of the test compound. The reduction in the concentration of $ABTS^{\bullet+}$ by a certain concentration of antioxidant is related to that of Trolox and gives the TEAC value of that antioxidant. The AOC is therefore the ability of the sample to decrease the colour by reacting

directly with the $ABTS^{\bullet+}$ radical. Limitations of this method include underestimation of the TEAC of natural products due to the reaction not reaching completion within the incubation time allowed (4-6 min).

It has been suggested that the exponential function

$$y = C\{1 - e^{(-bx)}\}$$

be extrapolated to conditions where the reaction between the antioxidant and $ABTS^{\bullet+}$ has reached completion (ie to an infinite excess of $ABTS^{\bullet+}$) so as to give the maximum concentration of $ABTS^{\bullet+}$ that can be scavenged by the antioxidant at the concentration employed [124), (142)].

In the above function

y = the reduction in $ABTS^{\bullet+}$ scavenged by the antioxidant

x = the initial $ABTS^{\bullet+}$ concentration

C = the maximal amount of $ABTS^{\bullet+}$ scavenged by the antioxidant at the concentration tested

In this method, an assumption is being made that this mechanism for protection of the fluorescent protein reflects the complex interactions occurring in biological systems. It has also been pointed out that protection of the photoreceptor portion of the protein by the antioxidant is not necessarily a reflection of protection of the protein itself. A further weakness of the method comes from the fact that it does not distinguish between the rate and amount of radical scavenging. However, this method can be used to rank the order of antioxidant activity.

Advantages:

- a wide pH range is tolerated (allowing for the study of pH effects on antioxidant mechanisms)
- the reaction is rapid, ca. 30 min
- $ABTS^{\bullet+}$ is soluble in both aqueous and organic solvents
- $ABTS^{\bullet+}$ is not affected by ionic strength

- $ABTS^{\bullet+}$ can be used to determine both hydrophilic and lipophilic antioxidant capacity of extracts and body fluids

Disadvantages:

- $ABTS^{\bullet+}$ is a non-physiological radical source [128]
- It can be reduced by compounds with redox potentials of ≤0.68V (ie many phenolics)
- Using an endpoint of 4-6 min may result in taking a reading before the reaction is completed, resulting in low TEAC values, and giving poor quantitation
- It is an end-point assay therefore the values obtained cannot reflect reaction rate differences between antioxidants and oxidants [132]

DPPH [123]:

This is one of the oldest and simplest assays [143] used to determine relative capacity for scavenging radicals. It makes use of a stable organic nitrogen radical which on being reduced loses its deep purple colour. Its stability is due to delocalisation of the extra electron over the entire molecule [144].

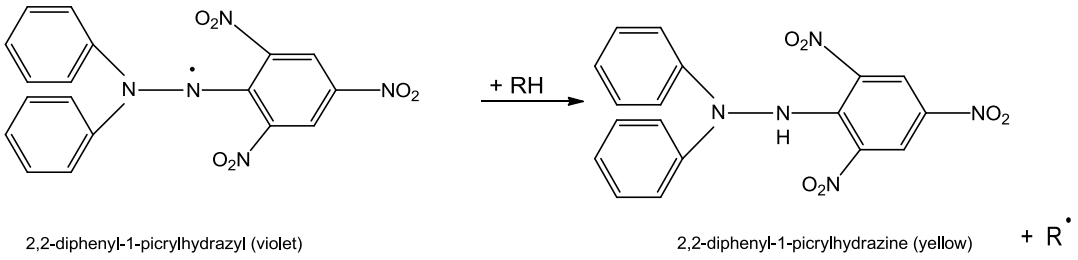


Figure 4.7 DPPH Radical mechanism

Because of the extra electron DPPH shows strong absorption in the visible range along with a high molar extinction coefficient; therefore the reducing ability of antioxidants towards DPPH can be measured by the decrease of its absorbance at 515nm as the reaction proceeds. 2, 2-diphenyl-1-picrylhydrazyl is both the oxidising radical as well as the indicator in the following reaction:

$$DPPH^{\bullet} + AH \rightarrow DPPH\text{-}H + A^{\bullet}$$

While able to accept an electron or hydrogen atom (thereby becoming a stable, diamagnetic molecule) it is oxidized with difficulty (ie irreversibly) [143]. The time lapse before the absorbance level reaches steady state varies from 20 min to a few hours. Use of different antioxidants at

different concentrations can result in varying decay slopes and absorbance levels of remaining DPPH radicals. Use of differing ratios of antioxidant to DPPH can result in non-linear time response curves before steady state is reached [132]. The loss of colour of DPPH allows the % DPPH remaining to be calculated. This is proportional to the antioxidant concentration [132]. The mechanism uses mainly a SET reaction, with H-atom abstraction a marginal reaction pathway.

$$\%\ DPPH_{REM} = \frac{100\ [DPPH\cdot]_{REM}}{[DPPH\cdot]_{T=0}}$$

Where **% DPPH$^{\cdot}$ REM** $\propto$ **[antioxidant]**

EC_{50} = Antioxidant concentration that causes a decrease in initial DPPH$^{\cdot}$ by 50%

TEC_{50} = Time needed to reach the steady state with **EC_{50}**

AE = Antiradical efficiency = $\frac{1}{EC_{50} \cdot T_{EC50}}$

Advantages:

- The method is simple and rapid, using a UV/VIS spectrophotometer

Disadvantages:

- DPPH, as a stable nitrogen radical, is totally dissimilar to the peroxyl radical involved in lipid peroxidation, which is highly reactive and transient. Antioxidants that react quickly with peroxyl radicals react slowly, if at all, with DPPH. This could be due to steric hindrance, as this determines reaction rate. Therefore, small molecules with better access to the radical site have higher apparent antioxidant capacity with this test [124]
- The absorbance spectra of the test compounds may overlap that of DPPH at 515nm (for example, carotenoids), making interpretation difficult [124]
- The assay is not a competitive reaction because DPPH acts as both a radical probe and an oxidant [129]
- Since there is no oxygen radical present in the assay, relating the results to AOC becomes questionable [132]
- The reaction range is only linear over 2 – 3 fold [124]
- Limitations include non-specificity, the fact that DPPH radicals can also react with alkyl radicals [126]
- DPPH is a synthetic radical that bears no relation to any biological ROS [132]
- This method is limited in that DPPH is not water soluble [145]

Total Phenolic Content - Folin Ciocalteu Reagent:

This method was originally developed in 1927 for the determination of the amino acid tyrosine in proteins (within its structure tyrosine has a phenolic group) [146]. The reagent consists of a mixture of sodium molybdate and tungstate which reacts with phenols to give an intense blue colour with an absorbance maximum at 765 nm. The advantage of the long-wavelength absorption is that it minimizes sample matrix interference. However, at the time of development, the lack of specificity of the method was noted by Folin who found that tryptophan (a non-phenolic) also gave a positive reaction. Several other compound classes have shown reactivity, including nitrogen containing compounds [147], vitamins and thiols. Of the vitamins, ascorbic acid and retinoic acid proved to be the most reactive [148].

This assay has been used to measure Total Phenolics in natural products, but its basic mechanism is a redox reaction, so it may therefore be considered another AOC method [124]. The reaction involves a single electron transfer SET from the substrate to the complexed Mo (VI) in the reagent, Mo (VI) reducing to Mo (V). Various authors have recommended that this assay be seen as a measure of the total antioxidant capacity rather than the phenolic content of a sample. However, because antioxidants in plants consist mainly of phenolics, it may be used as an approximation of the Total Phenol Content of the sample. As ascorbic acid and some reducing sugars are the main reducing agents which can interfere in the Folin-Ciocalteu reaction, correction factors for ascorbic acid may be applied, or SPE extraction methods may be used to remove most of the ascorbic acid and sugars [149].

General Chemistry: [124]

$$Na_2WO_4/\ Na_2MoO_4 \rightarrow (phenol\text{-}MoW_{11}O_4)^{-4}$$

$$Mo\ (VI)\ (yellow) + e^- \rightarrow Mo\ (V)\ (blue)$$

An improved method by Singleton and Rossi [150] recommended a molybdotungstophosphoric heteropolyanion reagent to improve specificity towards phenols with the absorbance reading being taken at 765 nm. It consists of:

$$3H_2O - P_2O_5 - 13WO_3 - 5MoO_3 - 10H_2O \text{ and } 3H_2O - P_2O_5 - 14WO_3 - 4MoO_3 - 10H_2O$$

A further modified method, developed in 2013, allows for the lipophilic components as well as the hydrophilic antioxidants to be measured simultaneously. The F-C reagent is diluted with isobutanol-water and an alkaline medium is provided by NaOH, so that both a lipophilic and a hydrophilic phase can be supplied simultaneously [151].

Advantages: [124]

- The simplicity, sensitivity, as well as precision of this assay make it a useful method
- It is a fast and robust method, not requiring specialized equipment
- It may be used in comparing botanical samples if conditions are carefully controlled
- Absorbance reading at 765 nm cuts out sample matrix interferences

Disadvantages:

- The reaction is slow at acid pH
- The reaction lacks specificity
- Correction for interfering substances should be made. These include sugars, aromatic amines, sulphites, EDTA, ascorbic acid, enediols and reductones, organic acids, Fe (II) and other inorganic substances [124]

Lack of standardization of FCR methods can result in a difference of several orders of magnitude in detected phenols, therefore the following conditions have been recommended for reliable and predictable data:

- The proper volume ratio of alkali to Folin-Ciocalteu reagent should be maintained
- Optimal reaction time and temperature should be adhered to for colour development
- Absorbance should be monitored at 765 nm
- Use of gallic acid as the reference standard phenol [124]

Iron Chelating Activity ICA:

This assay has been included in the recommended battery of assays in an effort to overcome the limitations brought about by non-reflection of other antioxidant mechanisms like metal chelation. Iron can both initiate and amplify lipid peroxidation according to the following reactions: The initiation step may be induced through two different mechanisms, either HO· hydroxyl radical dependent or hydroxyl-independent, where iron-oxygen complexes initiate the lipid peroxidation. In the first mechanism, iron participates in the Fenton reaction, forming the HO· radical which initiates the lipid peroxidation. In the second, iron-oxygen complexes such as perferryl ion (a resonance hybrid of Fe^{3+} - O_2 $^{\bullet-}$ and Fe^{2+} - O_2 $^{\bullet-}$) or ferryl ion FeO_2+ initiate the lipid peroxidation.

Qian [152], by means of electron paramagnetic resonance spin, studied the radical yield in an aerobic Fenton (Fe^{2+} + H_2O_2 + O_2) and an aerobic (Fe^{2+} + O_2) system. He showed that the Fenton reaction with pre-existing H_2O_2 is only a minor initiator of free radical oxidations in the cellular environment since the physiological ratio of [O_2] / [H_2O_2] is high at ≥ 103. Because of this, the Fenton reaction becomes suppressed and the radical yield from the Fe^{2+} + O_2 reaction becomes predominant. The oxidizing species formed by the reaction of Fe^{2+} and dioxygen are therefore the major initiators of biological free radical oxidations, with reactivities similar to that of HO·

Metal chelation may possibly be another route by which antioxidant activity is achieved. [131] Flavonoids readily chelate metal ions such as Fe (II), Fe (III) and Cu (I) which catalyse free radical reactions. And chelated flavonoids are more effective free radical scavengers than flavonoids alone [153]. Optimal pH for complex formation is ~6, as the structure of flavonoids is that of a weak polybasic acid. If the pH of the system is below 3, they remain undissociated which does not favour complex formation; above 7 they are deprotonated, which leads to the formation of more complex species.

The hydrophilic Metal ion Chelating Capacity assay ICA is an estimate of the ability of the sample to chelate Fe^{2+}. Ferrozine forms a magenta-coloured Fe (ligand)$_3^{2+}$ species with divalent iron in aqueous solution between pH values of 4 and 9 which can be measured at 562 nm [154]. According to the reaction stoichiometry of this mechanism, 3 moles of ferrozine complexes with 1 mole of Fe^{2+}. In the presence of a co-existing chelator, complex formation is disrupted with fading of the red colour. Measurement of the colour reduction allows for estimation of the co-existing chelator that competes with ferrozine for the ferrous ions. The difference in absorbance between the blank and the sample can be used to calculate the amount of iron chelated by the extract [130]. The absorbance at 562 nm is measured against a calibration curve prepared from standard solutions of di-Sodium Edetate (used as a positive control) and Trolox treated in the same manner.

$$\text{Chelating effect (\%)} = \left\{1 - \frac{A_{sample}}{A_{control}}\right\} \times 100 \quad (141)$$

$$\%\ Iron\ chelated = \frac{(1 - Absorbance\ of\ sample\ at\ 562nm)}{Absorbance\ of\ blank\ at\ 562nm} \times 100 \quad (130)$$

Results are expressed as micromoles of EDTA equivalents per gram of sample on a dry mass basis (µmol of EDTA equiv/g DW).

Validation issues:

As pointed out by Prior [124], problem areas overlooked in collaborative studies may include sample homogeneity, as well as failure to maximize extraction efficiency, identify critical control points and adhere to good QC procedures. Suggested protocols for standardization include:

- Detailed sampling and extraction procedures
- Critical handling considerations – Interference identification (with procedures for elimination)
- Storage procedures
- Detailed procedures for the analysis, as well as the statistical analysis

Often a compromise must be reached as no single solvent system is suitable for the diversity of antioxidant phytochemicals in botanicals.

Standardization of AOC methods:

- A sample's 'Total Antioxidant Capacity' cannot be characterized by only one AOC assay
- Both lipophilic and hydrophilic capacity should be measured
- To properly reflect physiological activity, hydrogen atom transfer (radical quenching) and electron transfer (radical reduction) should be measured
- A full profile of antioxidant capacity requires testing against various reactive oxygen species/ reactive nitrogen species, for example, $O_2\bullet^-$, HO•, and $ONOO^-$
- Prior [124] therefore recommends the following three methods: ORAC, FCR and TEAC, this choice utilizing two methods with differing reaction mechanisms. The $ORAC_{FL}$ assay, using the peroxyl radical, mimics a biologically relevant mechanism, and is able to measure the lipophilic as well as hydrophilic AOC. The TEAC assay uses the SET mechanism with the $ABTS^{\bullet+}$ radical. The FCR assay is a simple and robust, requiring only standard UV/VIS equipment

Summation: Generating an 'Antioxidant Profile' for natural materials:

Extracts of natural materials comprise many different polyphenols with varying solubilities, redox potentials, and mechanisms of action. As no single assay is able to furnish all the information required for an 'antioxidant profile', evaluation of the 'overall antioxidant activity' of an extract may require multiple assays to generate a so-called 'antioxidant profile'. Included in this profile should be the antioxidant's direct reactivity towards aqueous and lipid organic radicals by means

of radical quenching and reducing mechanisms, as well as its indirect reactivity through the complexing of metals [124].

Some researchers have combined the results from multiple assays to arrive at a ranking that more accurately represents a sample's overall antioxidant potency [155]. In this case, eight of the test results were combined into a single Antioxidant Potency Composite Index (APCI) by the following procedure: All eight assays were equally weighted (TPC, TF, TA, TEAC, DPPH, TAC, TPC (FCR) and ICA). An index value of 100 was assigned to the best score for each test. The index score of the samples in each assay was calculated as follows:

$$Antioxidant\ Index\ score\ = \left[\left(\frac{Sample\ score}{Best\ score}\right) \times 100\right]$$

An average of all eight assays for each sample was calculated giving an overall mean index value for APCI. A simple rank order is reported.

CHAPTER 5 RESEARCH METHODOLOGY

Sampling of fruits:

Hillcrest frozen Blueberries and Cranberries were purchased from Pick n Pay supermarket. Indigenous fruits were collected from Kirstenbosch Botanical Gardens or from the Cape Town campus of the Cape Peninsula University of Technology. On returning to the laboratory, these were immediately rinsed with deionised water, air dried, packed into Ziploc bags and purged with nitrogen before freezing at -18°C.

Extraction of fruits:

The method of Sellapan [140] was followed for all assays carried out except the ORAC assay. All berry extractions were done in triplicate. Indigenous berries were deseeded (except for Bietou and Sour Fig) and 1g of frozen sample was ground with 10 ml of 4% v/v acetic acid in acetonitrile in a pestle and mortar. The sample was transferred to a 25 ml volumetric flask and made up to volume with the washings from the mortar. The flask was shaken for 1 hour and then sonicated for 10 min. The extracts were then filtered through 0.22 micron nylon syringe filters and stored in cryo vials in a freezer at -18°C.

For the ORAC assay the following extraction method was followed [156]. A mass of 1 g of each berry was ground and extracted with 20 ml hexane for the lipophilic fraction and then with 30 ml of an acetone: water: acetic acid mixture (70:29.5:0.5, v/v/v) for the hydrophilic fraction. All the fractions were centrifuged for 10 min at 4° C and stored at -80° C until analysis. On the day of analysis, a portion of the lipophilic fraction was dried under nitrogen and re-dissolved in water: acetone (1:1) containing 7% m/v β-cyclodextrin.

For the Metal Chelating Activity assay extraction of the fruit was carried out as follows [141]: A 50mg/ml extract solution was prepared by weighing 2.5 g of frozen fruit and then crushing in a mortar in 10 ml 4% v/v acetic acid in acetonitrile. The mixture was homogenized with a Kinematica Polytron fitted with a medium head at low/medium speed (setting 4) for 3 min. The solutions were purged with nitrogen, sonicated 5 min, and centrifuged for 5 min at 3000 g. The supernatant liquid was taken up to 50 ml, and then stored in cryotubes purged with nitrogen at -4° C.

Chemicals and apparatus:

Acetonitrile, Gallic Acid, Quercetin, Caffeic Acid, 4-dimethylaminocinnamaldehyde (DAC), Trolox (6-hydroxy-2,5,7,8 – tetramethylchroman-2-carboxylic acid), Potassium Persulphate ($K_2S_2O_8$),

2,2-diphenyl-1-picrylhydrazyl (DPPH), 3',6' – dihydroxy-spiro[isobenzofuran-1[3H],-xanthen]-3-one (Fluorescein), 2,2'-azobis (2-amidino-propane) dihydrochloride (AAPH), Ascorbic Acid and Butylated Hydroxy Toluene was purchased from Sigma (South Africa). Folin Ciocalteu reagent, ABTS (2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonate)) was purchased from Fluka Chemicals. Acetic Acid glacial, Sodium Carbonate, Hydrochloric Acid 32%, Methanol, 95 % Ethanol, tri-Sodium Orthophosphate dodecahydrate, Ammonium Molybdate tetrahydrate and Sulphuric Acid, all of analytical reagent grade, were obtained from Merck (South Africa). Cyanidin-3-glucoside was obtained from Extrasynthese (Genay Cedex, France)

Milli-Q water was used.

A Pharmacia LKB Ultrospec II E Spectrophotometer was used.

A Sigma centrifuge 2-16 was used.

ORAC absorbance was read on a Fluorescent plate reader (Fluoroskan Ascent, Thermo Electron Corporation, USA).

Methods

1. Total Phenolic Content (Folin Ciocalteu Reagent):

The method of Singleton was followed [157], [158]. For the calibration curve a 100 ppm gallic acid stock standard solution was made by dissolving 10 mg gallic acid in a few drops of 95% ethanol in a 100 ml volumetric flask and then taken to volume using water. Amounts between 0 – 800 µl were pipetted into 10 ml volumetric flasks containing 2 ml of water, 0.5 ml Folin Ciocalteu reagent was added, and after 3 min 1.5 ml of Na_2CO_3 (20 % m/v) was added. The flasks were taken up to volume to give final concentrations of 0 – 8 ppm and allowed to stand for 30 min at ambient temperature. The absorbance was then read on the spectrophotometer at 765 nm. Samples were analysed by taking 100 µl of sample in place of the standard.

Concentrations of the samples in mg Gallic Acid Equivalents/L were obtained from the following equation:

$$conc\left(\frac{mg\ GAE}{L}\right) = \frac{Absorbance - intercept}{Slope} \times dilution\ factors \quad \text{.....(Equation 5.1)}$$

This value was converted to mg Gallic Acid Equivalents/100g FW by the following equation:

$$conc\left(\frac{mg\ AE}{100gFW}\right) = conc\left(\frac{mg\ AE}{L}\right) \times \frac{dilution\ factor \times 100}{40 \times sample\ mass} \quad \text{.....(Equation 5.2)}$$

2. Total Phenolic Contents (Mazza method):

The method of Mazza was followed [159]. A stock standard of 1000 ppm Gallic Acid was prepared in a 25 ml volumetric flask by dissolving 25 mg gallic acid in 10% v/v ethanol. A dilution range of 0 – 200 ppm was made. Each standard was then diluted 20 times to give a range consisting of 0 – 10 ppm gallic acid. 0.50 ml sample (or standard) was placed in a 10 ml volumetric flask and 0.50 ml 0.1% v/v HCl in 95% v/v ethanol was added. The contents were taken to volume using 2% v/v HCl. The mixed solution was allowed to stand for 15 min at ambient temperature. The absorbance was read at 280nm.

The concentration of the sample in mg antioxidant equivalents per litre was calculated using Equation (1). It was converted to mg antioxidant equivalents per 100g fresh weight by means of Equation (2).

See Appendix for examples of calculations.

3. Total Flavonols:

The method of Mazza was followed [159]. A stock standard of 1000 ppm of Quercetin was prepared in 25 ml volumetric flasks by dissolving 25 mg Quercetin in 95 % v/v ethanol. A dilution range of 0 – 300 ppm was prepared. Each standard was then diluted 20 times to give a final concentration range of 0 – 15 ppm. 0.5 ml sample (or standard) was pipetted into 10 ml volumetric flasks, followed by 0.1% HCl in 95% ethanol. The flask was taken to volume using 2% v/v HCl. The solution was allowed to stand for 15 min before the absorbance was read at 360 nm.

The concentration of the sample in mg Antioxidant Equivalents/L was calculated using Equation (1). It was converted to mg Antioxidant Equivalents/100g Fresh Weight by Equation (2).

See Appendix for examples of calculations.

4. Total Monomeric Anthocyanins:

The pH differential method was followed [140], [159], [160]. Two buffer solutions were made, a Potassium Chloride buffer solution of pH 1.0 and a Sodium Acetate buffer solution of pH 4.5. An appropriate dilution of the sample was determined to give an absorbance reading between 0.1 – 1.2 at 510 nm. 0.2 ml of the diluted sample was added to 1.8 ml of each buffer and these solutions were allowed to equilibrate for 15 min. The absorbance was then measured against an instrument blank (Milli-Q water) at 510 nm and 700 nm. The absorbance was calculated using the following equation:

$$A = (A_{510nm} - A_{700nm})_{pH1.0} - (A_{510nm} - A_{700nm})_{pH4.5}$$ .....(Equation 5.3)

Monomeric anthocyanins were calculated as cyanidin-3-glucoside equivalents by the following equation:

$$Monomeric\ Anthocyanin\ Equivalents\ \left(\frac{mg}{L}\right) = A \times MW \times DF \times \frac{1000}{(\varepsilon \times 1)}$$ .....(Equation 5.4)

Where *A*= Absorbance

MW= Molecular Weight Cyanidin-3-Glucoside (449.2g.mol^{-1})

DF= Dilution Factor

ε = Molar Absorptivity (26,900 Lcm^{-1}mol^{-1})

The concentration of anthocyanins (mg/100gFW) was calculated from the total volume of extract and the mass of the sample.

See Appendix for examples of calculations.

5. TEAC ($ABTS^{\cdot+}$) Assay:

The method of Re [161] was followed. A 7mM ABTS solution was prepared by dissolving 0.019g ABTS in a 5 ml volumetric flask using water. A 140mM $K_2S_2O_8$ solution was prepared by dissolving 0.189g in a 5 ml volumetric flask using water. A 2mM stock standard Trolox solution was prepared by dissolving 0.025g in a 50 ml volumetric flask using 99% ethanol. The $ABTS^{\bullet+}$ radical solution was generated by adding 88µl $K_2S_2O_8$ solution to 5 ml $ABTS^{\bullet+}$ solution and mixing well. The solution was left in the dark at ambient temperature for at least 16 hours (it remains stable for 2 -3 days under these conditions).

The spectrophotometer was calibrated with 99% ethanol at room temperature. The radical solution was cooled on ice, then diluted with cooled ethanol to give an absorbance of 0.7 (±0.1) at 734 nm using a 10mm path length. This solution was kept on ice. The calibration standards were prepared for reading by adding 20 µl of each standard to 980 µl ABTS radical solution in a 2 ml Eppendorf tube. The Trolox dilution series was made from the 2mM Trolox stock solution diluted with 99% ethanol to give a range of 0 - 0.8 mM.

The berry extracts were analysed using 20 µl sample (or standard) added to 980 µl ABTS$^{\bullet+}$ solution in a 2 ml Eppendorf tube. For the blank, 20 µl 99% ethanol was used. The mixture was vortexed for 30 sec, placed in a dry bath at 30°C and exactly 4 min after the initial mixing the absorbance of the solution was read at 734 nm.

The ABTS$^{\bullet+}$ radical concentration remaining after 4 min versus Trolox, in µM, was calculated (after reaction with the standard) from the molar extinction coefficient of ABTS$^{\bullet+}$ at 734 nm (ε, 1.6 x 10^4 L $mol^{-1}cm^{-1}$). The difference of the absorbance reading is plotted against the antioxidant concentrations. The concentration of antioxidant giving the same percentage change of absorbance of the ABTS$^{\bullet+}$ as that of 1 mM Trolox is regarded as TEAC (Trolox Equivalent Antioxidant Capacity).

$$TEAC\ \left(\frac{\mu mol}{L}\right) = \frac{(Conc\ ABTS\ remaining\ (\mu M) - Intercept)}{Slope} \quad \text{.....(Equation 5.5)}$$

Conversion of the above value to µmol TEAC/g FW is by the following equations:

$$TEAC\ of\ original\ sample\ \left(\frac{\mu mol}{L}\right) = TEAC\left(\frac{\mu mol}{L}\right) \times dilution\ factor \quad \text{.....(Equation 5.6)}$$

$$conc\ \left(\frac{\mu mol\ TEAC}{g\ FW}\right) = conc\left(\frac{\mu mol\ TEAC}{L}\right) \times \frac{dilution\ factor \times 100}{40 \times sample\ mass} \quad \text{.....(Equation 5.7)}$$

Note: Samples Wild Olive and Wild Plum were diluted 5 times.

See Appendix for examples of calculations.

6. DPPH Assay:

The method of Brand-Williams [162] was followed. A 1000µM DPPH stock standard was prepared by dissolving 4 mg DPPH in methanol in a 10 ml volumetric flask. This stock was kept at 4°C in the dark until required. A calibration range spanning 0 - 80 µM DPPH was prepared in 10 ml volumetric flasks. The absorbance was measured at 515 nm using methanol as the blank.

A 1000 ppm stock standard of Ascorbic Acid was prepared by dissolving 10 mg of Ascorbic Acid in methanol in 10 ml volumetric flask. A dilution series was prepared in 10 ml volumetric flasks ranging from 0 – 80 µM. A 0.1 ml aliquot of sample extract (or Ascorbic Acid standard) was added to 3.9 ml of DPPH solution. In order to measure the absorbance at steady state, the mixture was allowed to react for 17 hours in the dark before the absorbance was measured at 515 nm.

The slope and intercept from the DPPH calibration curve were used to determine the DPPH remaining from the absorbance after the samples and standards reacted with the DPPH. The

slope and intercept were then determined from the ascorbic acid versus DPPH Remaining calibration curve for each sample. This information was then used to determine the ascorbic acid equivalents for each sample (mg/L). Finally the concentrations were converted from mg Ascorbic Acid Equivalents per litre to µmol Ascorbic Acid Equivalents/g FW.

$$\%DPPH\bullet_{REM} = 100 \times \frac{[DPPH]_{REM}}{[DPPH]_{T=0}}$$

The %DPPH• $_{REM}$ is proportional to the antioxidant activity and the antioxidant concentration.

$$DPPH\ remaining = \frac{(Absorbance\ of\ sample - DPPH\ intercept)}{DPPH\ slope}$$ .....(Equation 5.8)

$$Ascorbic\ Acid\ Equivalents\left(\frac{mg}{L}\right) = \frac{(DPPH\ remaining - AA\ intercept)}{AA\ slope}$$ .....(Equation 5.9)

$$Ascorbic\ Acid\ Equivalents\left(\frac{\mu mol}{g\ FW}\right) = AAE\left(\frac{mg}{L}\right) \times \frac{40(dilution) \times 1000(conversion\ to\ \mu mol)}{40(extract\ volume) \times 176.13\ g/mol}$$

..... (Equation 5.10)

See Appendix for examples of calculations.

7. Molybdenum Reduction assay:

The method of Prieto [163] was followed. A freshly prepared 1000 ppm Ascorbic Acid stock standard was made daily by weighing 20 mg Ascorbic Acid and dissolving it in water in a 20 ml volumetric flask. A 1000 ppm Gallic Acid stock standard was prepared by weighing 20 mg of Gallic Acid and dissolving it in water in a 20 ml volumetric flask. This solution was stored in a sealed vessel under nitrogen at 0°C. A dilution series of each of the above was prepared in the range 100 – 500 ppm by pipetting the standards into 10 ml volumetric flasks and diluting with water.

Ammonium Phosphomolybdate reagent was made up using two solutions, A and B, mixed in the ratio 1:2 as required. Solution A consisted of 1.597g Sodium Orthophosphate + 0.742g Ammonium Molybdate taken to 50 cm^3 with water. Solution B consisted of 0.6 M Sulphuric Acid. 2 ml of 0.6 M Sulphuric Acid was pipetted into a capped test tube. 1 ml of Ammonium Phosphomolybdate reagent was slowly added with stirring. 100µl of standard, control or sample extract solution was now added; the test tube was closed and the mixture incubated in a water bath at 95°C for 90 min. After cooling, the absorbance was read at 695 nm versus a blank. Wild Olive, Waterbessie, Wild Plum and Bitterbessie extracts were diluted 5 times.

The concentration of the sample in milligrams Antioxidant Equivalents per litre was calculated from the slope and intercept of the calibration curve for the Molybdenum Reduction assay. This concentration is then converted to milligrams Antioxidant Equivalents per gram of fresh weight by the following equations:

$$mg\,\frac{GAE}{L} = \frac{(Absorbance\ of\ sample - intercept)}{slope} \quad \text{.....(Equation 5.11)}$$

Conversion to Gallic Acid Equivalents is as follows:

$$\mu mol\,\frac{GAE}{g\ FW} = mg\,\frac{GAE}{L} \times \frac{Dilution\ factor \times 1000}{40 \times 170.12\ g/mol} \quad \text{.....(Equation 5.12)}$$

Similarly, to Ascorbic Acid Equivalents, the equation is as follows:

$$\mu mol\,\frac{AAE}{g\ FW} = mg\,\frac{AAE}{L} \times \frac{Dilution\ factor \times 1000}{40 \times 176.13\ g/mol} \quad \text{.....(Equation 5.13)}$$

For the concentration of Antioxidant Equivalents in indigenous fruits, the mass of sample in grams replaces the molar mass of the Gallic Acid or the molar mass of the Ascorbic Acid in the above equations.

See Appendix for example calculations.

8. ORAC method:

The method of Prior [156] was used for both H-ORAC$_{FL}$ and L-ORAC$_{FL}$. Phosphate Buffer (0.075 M pH 7.0), Fluorescein stock solution (final concentration 14 µM), Peroxyl radical solution (25 mg/ml, prepared freshly each day) and Trolox stock solution (500 µM stock solution) were prepared. The peroxyl radical (ROO•) was generated from 2,2'-azobis (2-amidino-propane) dihydrochloride, which forms on breakdown of the AAPH at 37°C . The peroxyl radical oxidizes Fluorescein (3',6' – dihydroxy-spiro[isobenzofuran-1[3H],-xanthen]-3-one, resulting in a product with no fluorescence. Any antioxidants present in the sample inhibit this degradation of the fluorescence. The measurement of the signal was carried out at excitation wavelength 485 nm and emission wavelength 538 nm over a period of 2 h.

The strength of the antioxidant capacity of the sample is directly proportional to the fluorescence intensity and can be related to the net area under the curve given by the standard Trolox. The ORAC value is obtained by dividing the area under the sample curve by the area under the Trolox curve and correcting for both values by subtracting the area under the Blank curve; it is expressed as µmol Trolox equivalents/g FW.

To provide calibration standards, a standard stock solution of 500 µM trolox was diluted with phosphate buffer for the H-ORAC$_{FL}$ assay, whereas for the L-ORAC$_{FL}$ assay the standard was diluted with acetone: water (1:1) containing 7% m/v β-cyclodextrin. The standard curve showed a linear relationship in the range 0 – 25 µM Trolox.

The Fluoroskan Ascent plate was set at 37°C. The fluorescence conditions were set at 485 nm excitation and 530 nm emission. The fluorescence intensity was measured every minute under simulated biological conditions (pH 7.4, 37° C). As the reaction progressed, fluorescein was consumed and the fluorescent intensity decreased. In the presence of antioxidant, the fluorescein decay was inhibited and an ORAC$_{FL}$ assay kinetic curve was obtained. The ORAC$_{FL}$ values were calculated using the regression equation

$$y = a + bx + cx^2$$

between Trolox concentration (µM) and the area under the kinetic curve. All samples were assayed in triplicate and the mean and standard deviation values were calculated using Microsoft Excel. The results were expressed as µmol Trolox equivalents per g fresh weight (µmol TE/g FW).

See Appendix for examples of calculations.

9. Iron Chelating Activity ICA:

The method of Le [141] was followed. A 5 mM (0.005 M) solution of Ferrozine reagent was prepared by dissolving 0.123 g anhydrous Ferrozine in Milli-Q water in a 50 ml volumetric flask. A 2 mM ferrous chloride solution was made up using 0.0397 g ferrous (II) chloride tetrahydrate in a 100 ml volumetric flask; this was then diluted to give a working solution of 0.6 mM ferrous chloride. A 50 mg/L $Na_2EDTA.2H_2O$ solution was made up by dissolving 0.01 g of $Na_2EDTA.2H_2O$ in a 200 ml volumetric flask and taking it up to volume with water. Stock solutions of the fruit extracts (50 mg/ml) were prepared and stored in cryotubes in the refrigerator at 4°C.

Dose response curves were prepared for each extract and $Na_2EDTA.2H_2O$ by diluting the stock solutions serially in separate cuvettes from 0 to 500 µl with the addition of 900 µl methanol, 100 µl ferrozine and 100 µl ferrous ions. The reagent blank consisted of all the reagents but no EDTA or sample extract. After the addition of Ferrozine solution, the mixture was shaken and allowed to react at ambient temperature for 30 min before the absorbance was read at 562 nm against a methanol blank. The percentage Chelating Effect was plotted against the

concentration of sample in mg/ml. A decrease in absorbance of iron (II) - Ferrozine complex was noted with increasing sample concentration.

The % Chelating Activity value reported was for 15.3 mg/ml sample.

The following equations were used to obtain the Percentage Chelating Effect:

$$Chelating\ Effect\ (\%) = \left(1 - \frac{A_{sample}}{A_{control}}\right) \times 100 \quad \text{.....(Equation 5.14)}$$

$$\%\ Iron\ chelated = \frac{(1 - Absorbance\ of\ sample\ at\ 562nm)}{Absorbance\ of\ blank\ at\ 562nm} \times 100 \quad \text{.....(Equation 5.15)}$$

See Appendix for examples of calculations.

CHAPTER 6…RESULTS & DISCUSSION

The findings obtained for the analyses carried out on ten indigenous fruits are presented in this chapter. Firstly, we present the results obtained for the phenolic profiles of a sample of ten indigenous berries plus two Northern Hemisphere berries (Blueberry and Cranberry) by the following methods:

1. Total Phenolic Content by the Mazza method (mg GAE/100g FW)
2. Total Flavonol Content (mg Quercetin equiv/100g FW)
3. Total Monomeric Anthocyanin Content (mg cyanidin-3-glucoside equiv/ 100g FW).

Secondly, we present the results obtained for the Antioxidant Capacity determined by the following methods:

4. TEAC Trolox Equivalent Antioxidant Capacity assay (μmol Trolox equiv/g FW)
5. DPPH assay (μmol Ascorbic Acid equiv/g FW)
6. Molybdenum Reduction assay (μmol Gallic Acid equiv/g FW or μmol Ascorbic Acid equiv/g FW)
7. $ORAC_{FL}$ Oxygen Radical Absorbance Capacity assay (Hydrophilic and Lipophilic) (μmol Trolox equiv/g FW)
8. Total Phenolic Content (FCR) (mg GAE/100g FW)
9. Iron Chelating Activity (ICA) (% CA for 15.6 mg/ml fruit extract)

Finally, we present the following:

- Statistical Analysis in the form of Correlation Coefficients calculated for pairings of data of the various assays
- Comparative ranges of results for Blueberry and Cranberry found in the literature
- Antioxidant Potency Composite Index calculated for ten indigenous fruits and two controls.

Results: Phenolic Profiles

Table 6.1 shows the Total Phenolic Content as well as the contents of phenolic compound subgroups (Flavonols, Anthocyanins) found for 10 indigenous fruits and 2 controls.

Table 6.1 Results: Total Phenolic Content, Total Flavonols, and Total Monomeric Anthocyanins

	Total Phenolic Content[a]	Total Flavonols[b]	Total Monomeric Anthocyanins[c]
Blue berry	205.3 ± 5	34.8 ± 2.3	81.4 ± 8.5
Cranberry	340.2 ± 76.0	130.1 ± 33.4	9.7 ± 9.5
Waterberry	193.9 ± 21.8	64.9 ± 6.6	53.2 ± 7.0
Colpoon	470.2 ± 9.7	274.5 ± 9.2	139.8 ± 5.3
Wild Plum	595.7 ± 41.1	105.5 ± 12.8	nd
Tortoise berry	398.8 ± 32.8	234.2 ± 17.1	nd
Num-Num	505.5 ± 141.1	241.8 ± 90.1	nd
Christmas berry	1850.1 ± 75.7	758.1 ± 53.5	nd
Bietou	894 ± 242	419.4 ± 128	nd
Cross berry	179.3 ± 39.0	19.8 ± 12	nd
Sour fig	283.6 ± 12.7	101.9 ± 4.7	nd
Wild olive	627.2 ± 110.4	203.7 ± 12.6	193.5 ± 175.6

[a] Total Phenolic Content by Mazza method expressed as mg of Gallic acid equiv/100g FW. [b] Total Flavonol Content expressed as mg of Quercetin equiv/100g FW. [c] Total Monomeric Anthocyanin Content expressed as mg of cyanidin-3-glucoside equiv/ 100g FW.

Nd: Not detected

The Total Phenolic Content of the ten indigenous fruits was estimated using the Mazza method as it allowed for the absorbance to be measured without turbidity. Total Flavonols were also assessed by the Mazza method and Total Monomeric Anthocyanins by the pH Differential Method which allows for quantification of important contributors to the bioactivity of the fruit.

As shown in Table 6.1 results obtained for the Total Phenolic Content by the Mazza method show a ten-fold difference across the various species, with Christmas berry returning the highest results (1850.1 mg GAE/100g FW) and Crossberry the lowest (179.3 mg). A mean value of 600 mg GAE/100 g FW for the ten indigenous fruits was obtained. Christmas berry (1850.1 ± 75.7 mg GAE/100 g FW) had the highest Total Phenolic Content, followed by Bietou (894 ± 242 mg GAE/100 g FW), Wild Olive (627.2 ± 110.4 mg GAE/100 g FW), Wild Plum (595.7 ± 41.1 mg GAE/100 g FW), Num-num (505.5 ± 141.1 mg GAE/100 g FW), Colpoon (470.2 ± 9.7 mg GAE/100 g FW), Tortoise berry (398.8 ± 32.8 mg GAE/100 g FW), Sour fig (283.6 ± 12.7 mg GAE/100 g FW), Water berry (193.9 ± 21.8 mg GAE/100 g FW) and Cross berry (179.3 ± 39.0 mg GAE/100 g FW) showed the lowest Total Phenolic Content. The results for the control Blueberry (205.3 mg) lay below eight of the indigenous berries; while the results for Cranberry (340.2 mg) lay below seven of the indigenous berries. This is illustrated by Graph 6.1.

The results for the Total Flavonols by the Mazza method (Table 6.1) showed a range of values from 19.8 ± 12 mg QE/100 g FW to 758.1 ± 53.5 mg QE/100 g FW showing a difference of 38-fold and a mean value of 242.5 mg QE/100 g FW. Christmas berry (758.1 ± 53.5 mg QE/100 g FW) had the highest Total Flavonol content, followed by Bietou (419.4 ± 128 mg QE/100 g FW), Colpoon (274.5 ± 9.2 mg QE/100 g FW), Num-num (241.8 ± 90.1 mg QE/100 g FW), Tortoise berry (234.2 ± 17.1 mg QE/100 g FW), Wild Olive (203.7 ± 12.6 mg QE/100 g FW), Wild Plum (105.5 ± 12.8 mg QE/100 g FW), Sour Fig (101.9 ± 4.7 mg QE/100 g FW), Waterberry (64.9 ± 6.6 mg QE/100 g FW) and finally Cross berry (19.8 ± 12 mg QE/100 g FW). These values obtained for Blueberry and Cranberry were both well below the mean value of the indigenous fruits.

As shown in Table 6.1, Total Monomeric Anthocyanins were detected in only three indigenous fruits and the two controls. The units in which they are reported are mg Cyanidin-3-Glucoside Equivalents /100 g FW. The results for the fruits are as follows: Wild Olive (193.5 ± 175.6) with the highest level of Anthocyanins, Colpoon (139.8 ± 5.3) and the lowest Waterberry (53.2 ± 7.0). These results varied from 53.2 to 193.5, a 3.7-fold difference, with a mean value of 128.8 mg Cyanidin-3-Glucoside Equivalents / 100 g FW. Blueberry (81.4 ± 8.5) demonstrated a value within the range of the indigenous fruits; however Cranberry (9.7 ± 9.5) showed a value below the range.

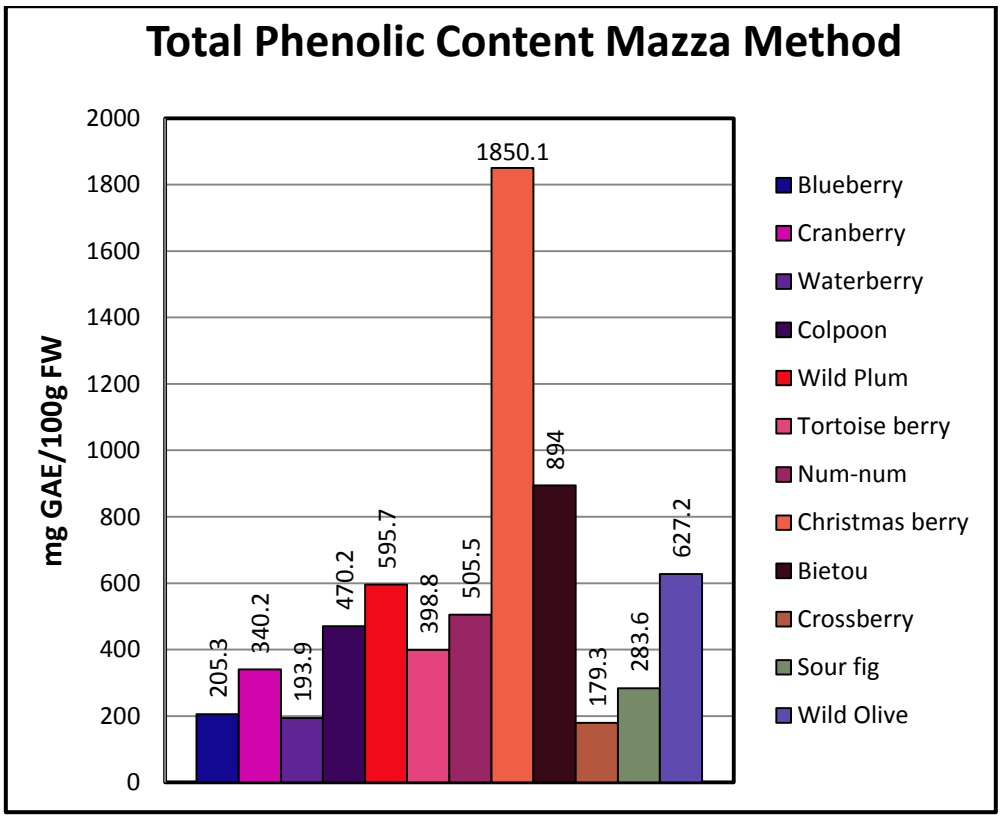
As shown in Graph 6.1, Christmas berry (1,850.1 mg GAE/100g FW) had the highest concentration of Total Phenolic Content, followed by Bietou (894.0 mg), Wild Olive (627.2 mg), and Wild Plum (595.7 mg). Among the indigenous berries with moderate levels of Total Phenolic Content were Num-num (505.5mg), Colpoon (470.2 mg), Tortoise berry (398.8 mg),

Sour Fig (283.6 mg), Waterberry (193.9 mg) and finally Crossberry (179,3 mg). These results compared well to the Northern Hemisphere control berries, with Cranberry (340.2 mg) and Blueberry (205.3 mg).

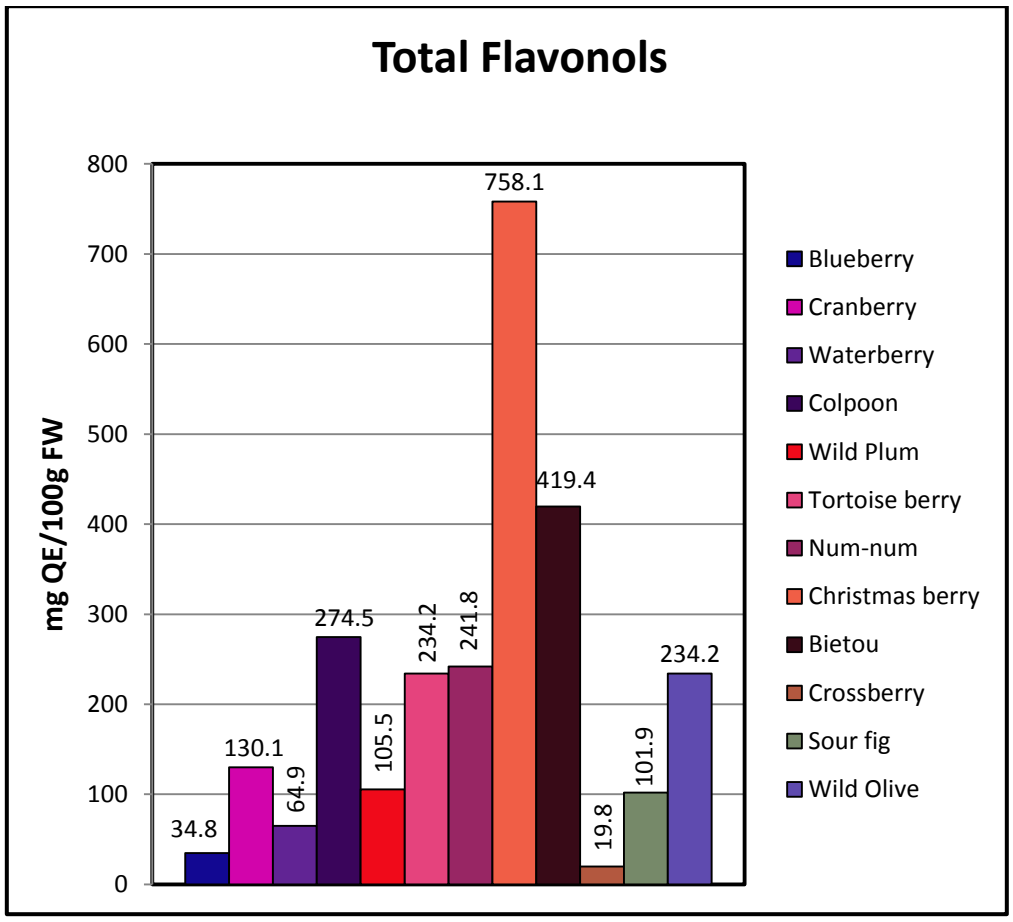
As shown in Graph 6.2, the highest flavonol (ie quercetin) content was found in Christmas berry (758.1 mg QE/100g FW), followed by Bietou (419.4 mg) and Colpoon (274.5mg). Indigenous berries with moderate levels of flavonols included Num-num (241.8 mg), Tortoise berry (234.2 mg), Wild Olive (203.7 mg). Those with the lowest levels were Wild Plum (105.5 mg), Sour Fig (101.9 mg), Waterberry (64.9 mg) and Cross berry (19.8 mg). By comparison, the concentration of Cranberry was (130.1 mg) and that of Blueberry (34.8 mg).

As shown in Graph 6.3, anthocyanins were detected in only 3 out of 10 indigenous fruits. This subgroup of phenolic compounds (ie cyanidin) was found only in Wild Olive (193.5 mg CE/100g FW), followed by Colpoon (139.8 mg) and Waterberry (53.2 mg). By comparison, the concentration in Blueberry was (81.4 mg) and Cranberry (9.7 mg).

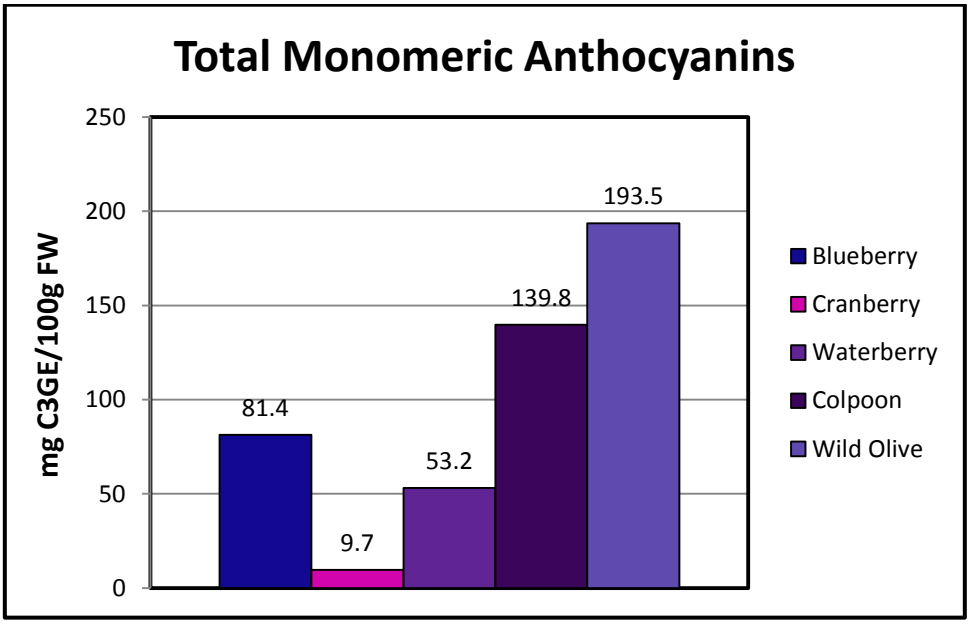
The ratio of Total Anthocyanins to Total Phenolic Content (in the five fruits that yielded positive results for Anthocyanins) were all in the range 0.27 – 0.30, with Blueberry at 0.40. However, Cranberry had a very low ratio of 0.03. This is illustrated by Graph 6.4.



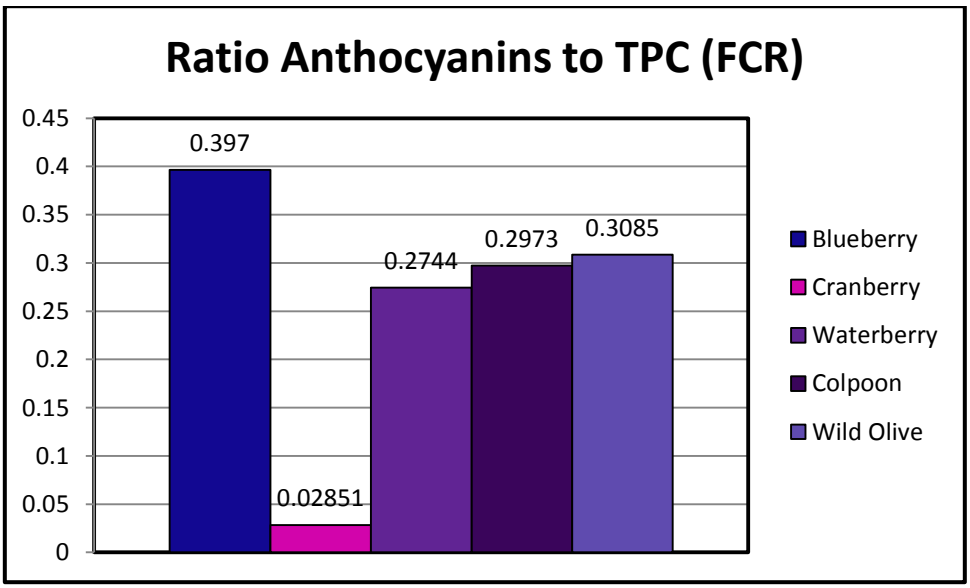
Graph 6.1 Bar graph illustrating Total Phenolic Content by Mazza method for all samples



Graph 6.2 Bar graph illustrating Total Flavonols for all samples



Graph 6.3 Bar graph illustrating Total Monomeric Anthocyanins for samples with positive results



Graph 6.4 Bar graph illustrating the ratio of Anthocyanins: TPC (FCR) for samples with positive results

Anthocyanin content is indicated by the dark purple colouration of the ripe fruits. As anthocyanins are considered to be major contributors to the bioactivity of the fruit, their ratio to Total Phenolic Content compared to the ratio obtained for the control berries may serve as an indicator of their healthful properties. As shown in Graph 6.4, in this case their ratios were slightly lower than that of Blueberry, but considerably higher than that of Cranberry.

Results: Antioxidant capacity as TEAC, DPPH & Molybdenum Reduction assays

Table 6.2 shows the results obtained for the Antioxidant Capacity assays ie the TEAC, DPPH and Molybdenum Reduction assays:

Table 6.2 Results of Antioxidant Capacity assays TEAC, DPPH and Molybdenum Reduction

	TEAC Assay[a]	DPPH assay[b]	Molybdenum Reduction assay	
			Gallic Acid Equivalents[c]	Ascorbic Acid Equivalents[d]
Blue berry	8.3 ± 0.4	5.4	-	-
Cranberry	9.5 ± 0.1	6.8	13.6 ± 0.4	25.9 ± 1.2
Waterberry	3.2 ± 0.1	3.3	26.6 ± 5.3	62.3 ± 11.1
Colpoon	8.5 ± 0.2	12.9	-	-
Wild Plum	55.6 ± 1.8	76.2	71.9 ± 2.0	160.6 ± 4.3
Tortoise berry	12.2 ± 0.2	7.9	38.7 ± 5.0	73.7 ± 11.3
Num-Num	7.6 ± 0.1	6.4	-	-
Christmas berry	8.7 ± 0.3	10.1	60.5 ± 7.1	141 ± 20.6
Bietou	7.6 ± 0.2	4.3	-	-
Crossberry	2.6 ± 0.3	3.8	26.7 ± 2.7	58.8 ± 5.8
Sour fig	0.6 ± 0.3	2.1	-	-
Wild olive	34.2 ± 0.6	12.2	120.6 ± 6.2	231.0 ± 12.2

[a] TEAC assay results expressed as μmol Trolox Equiv/g FW. [b] DPPH assay results expressed as μmol Ascorbic Acid Equiv/g FW. [c] Molybdenum Reduction assay results expressed as μmol Gallic Acid Equiv/g FW. [d] Molybdenum Reduction assay results expressed as μmol Ascorbic Acid Equiv/g FW.

In this case only six indigenous berries were assayed due to seasonal availability, with the use of one comparison berry, Cranberry.

In terms of the results for Antioxidant Capacity, see Table 6.2 for the full set of values. The methods used for this investigation were TEAC, DPPH, and the Molybdenum Reduction assay.

The TEAC (Trolox Equivalent Antioxidant Capacity) values ranged from 0.6 ± 0.3 to 55.6 ± 1.8 µmol Trolox Equivalents/g FW with a mean value of 14.08 and a 93-fold variation. Wild Plum (55.6 ± 1.8 µmol TE/g FW) gave the best result, with Sour fig (0.6 ± 0.3 µmol TE/g FW) the poorest. The rank order is as follows: Wild plum (55.6 ± 1.8 µmol TE/g FW), Wild Olive (34.2 ± 0.6 µmol TE/g FW), Tortoise berry (12.2 ± 0.2 µmol TE/g FW), Christmas berry (8.7 ± 0.3 µmol TE/g FW), Colpoon (8.5 ± 0.2 µmol TE/g FW), Num-num (7.6 ± 0.1 µmol TE/g FW), Bietou (7.6 ± 0.2 µmol TE/g FW), Waterberry (3.2 ± 0.1 µmol TE/g FW), Crossberry (2.6 ± 0. µmol TE/g FW) and Sour fig (0.6 ± 0.3 µmol TE/g FW). Controls Blueberry (8.3 ± 0.4 µmol TE/g FW) and Cranberry (9.5 ± 0.1 µmol TE/g FW) had values falling in the middle of the range of indigenous fruits.

From Table 6.2, the DPPH assay results ranged from 2.1 µmol Ascorbic Acid Equivalents /g FW to 76.2 µmol AAE/g FW with a mean of 13.92 µmol AAE/g FW and a 36-fold difference across the ten tested indigenous fruits. Wild Plum (76.2 µmol AAE/g FW) gave the highest result, followed by Colpoon (12.9 µmol AAE/g FW), Wild Olive (12.2 µmol AAE/g FW), Christmas berry (10.1 µmol AAE/g FW), Tortoise berry (7.9 µmol AAE/g FW), Num-num (6.4 µmol AAE/g FW), Bietou (4.3 µmol AAE/g FW), Crossberry (3.8 µmol AAE/g FW), Waterberry (3.3 µmol AAE/g FW) and Sour fig (2.1 µmol AAE/g FW). The controls, Blueberry and Cranberry, yielded 5.4 µmol AAE/g FW and 6.8 µmol AAE/g FW respectively.

The Molybdenum Reduction assay (Table 6.2) was carried out on a more limited number of fruits using two different comparative standard compounds, Gallic Acid and Ascorbic Acid. Six indigenous berries and one control (Cranberry) were used for this determination.

In the first procedure the values ranged from 26.6 ± 5.3 to 120.6 ± 6.2 µmol GAE/g FW with a mean value of 57.5 µmol GAE/g FW and a 4.4-fold difference. The fruit with the highest value was Wild Olive (120.6 ± 6.2 µmol GAE/g FW), followed by Wild Plum (71.9 ± 2.0 µmol GAE/g FW), Christmas berry (60.5 ± 7.1 µmol GAE/g FW), Tortoise berry (38.7 ± 5.0 µmol GAE/g FW), Crossberry (26.7 ± 2.7 µmol GAE/g FW) and Waterberry (26.6 ± 5.3 µmol GAE/g FW).

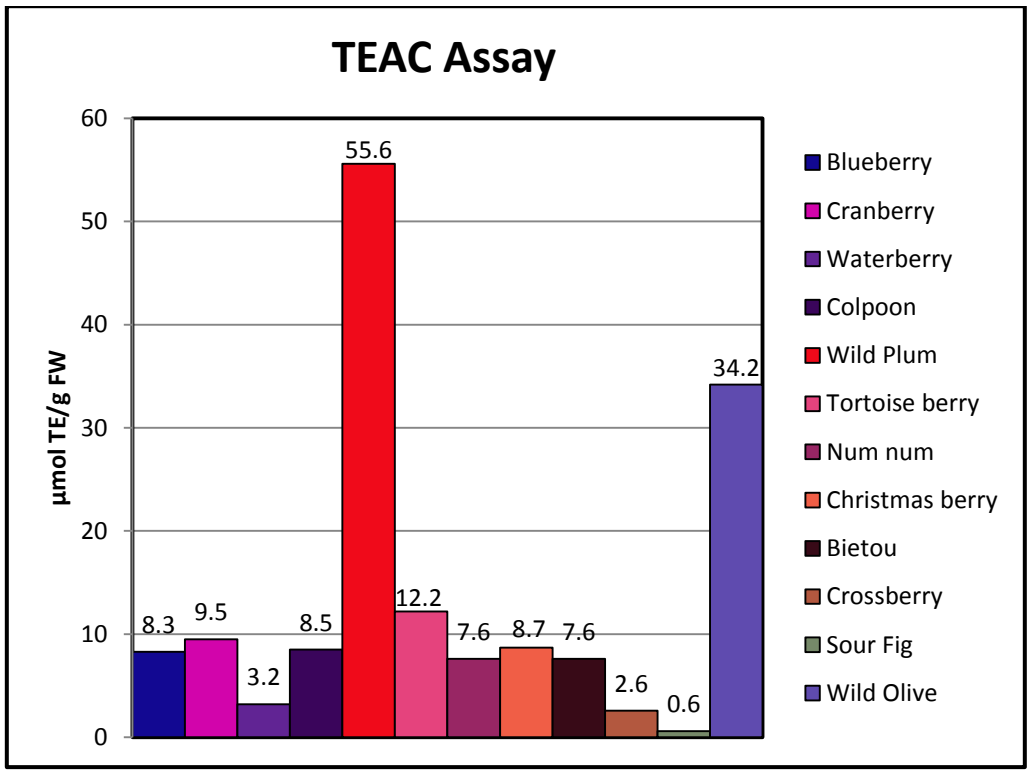
The same rank order was found with the second comparative standard compound, that is Wild Olive (231.0 ± 12.2 µmol AAE/g FW), followed by Wild Plum (160.6 ± 4.3 µmol AAE/g FW), Christmas berry (141 ± 20.6 µmol AAE/g FW), Tortoise berry (73.7 ± 11.3 µmol AAE/g FW), Waterberry (62.3 ± 11.1 µmol AAE/g FW) and Crossberry (58.8 ± 5.8 µmol AAE/g FW). In this case the range lay between 58.8 ± 5.8 and 231.0 ± 12.2 µmol AAE/g FW with a mean of 121.2 µmol AAE/g FW and a 4.9-fold difference.

In both Molybdenum Reduction assays, the result for the control Cranberry lay below the indigenous fruit values at (13.6 ± 0.4 µmol GAE/g FW) and (25.9 ± 1.2 µmol AAE/g FW) respectively.

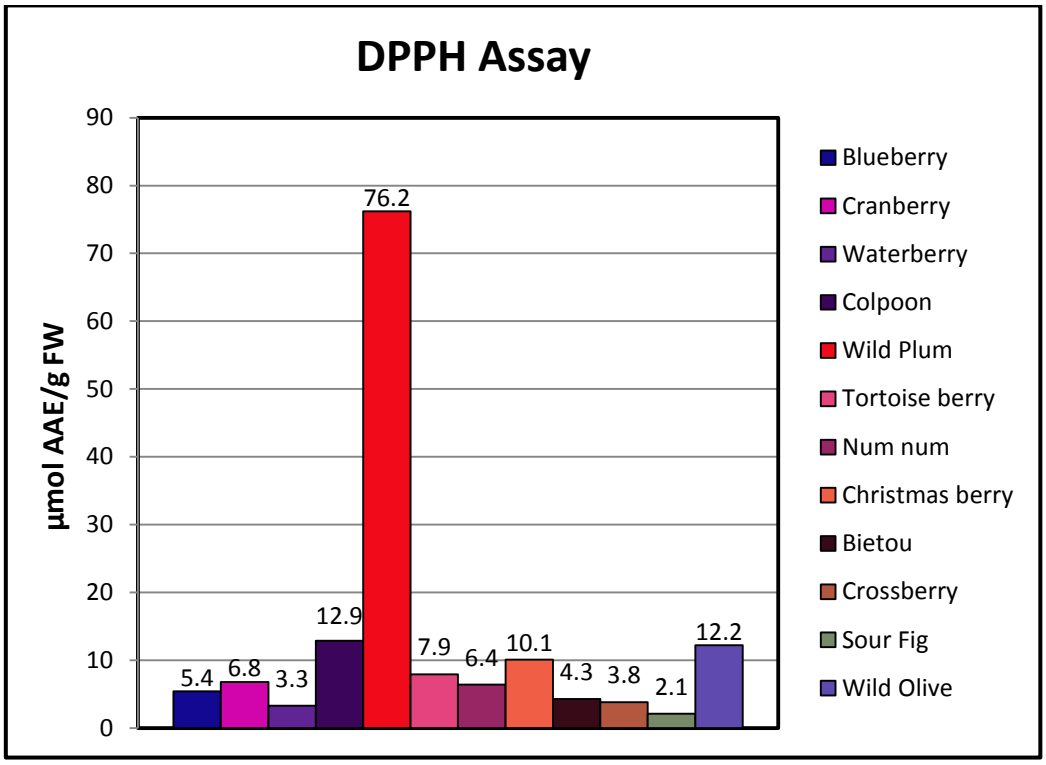
As shown in Graph 6.5, in the TEAC assay, the berries yielding the highest antioxidant capacity results were Wild Plum (55.6 µmol TE/g FW), Wild Olive (34.2 µmol), and Tortoise Berry (12.2 µmol). This was followed by Christmas berry (8.7 µmol), Colpoon (8.5 µmol), Bietou (7.6 µmol), and Num-num (7.6 µmol). These results compared well against the results for Blueberry (8.3 µmol) and Cranberry (9.5 µmol). At the lower end of the results, Waterberry yielded (3.2 µmol), Crossberry (2.6 µmol) and Sour Fig (0.6 µmol).

As shown in Graph 6.6, in the DPPH assay, the results for Wild Plum were once again the highest at (76.2 µmol AAE/g FW), followed by Colpoon at (12.9 µmol), Wild Olive at (12.2 µmol) and Christmas berry at (10.1 µmol). The results for Tortoise berry (7.9 µmol), Num-num (6.4 µmol), Bietou (4.3 µmol) fell in the middle of the range. The comparative results for Cranberry were (6.8 µmol) and Blueberry (5.4 µmol). At the lower end of the results scale, Crossberry featured once again (3.8 µmol), along with Waterberry (3.3 µmol) and Sour fig (2.1 µmol).

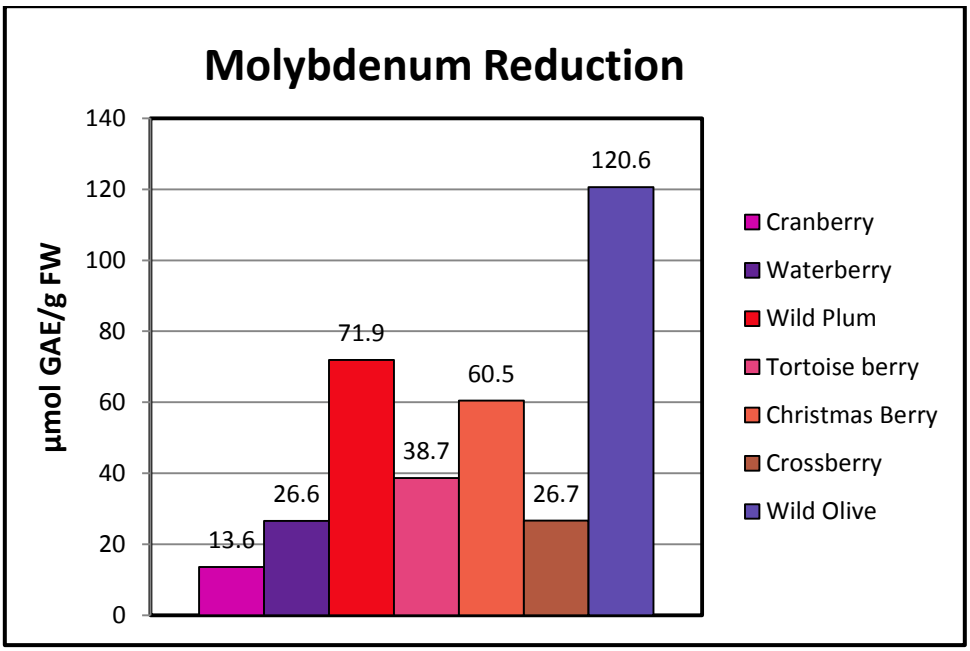
The Molybdenum Reduction assay was carried out on a more limited range of six indigenous fruits due to seasonal availability problems, along with one comparison berry, Cranberry. From the Gallic Acid calibration values the highest results (as shown in Graph 6.7) yielded were for Wild Olive (120.6 µmol GAE/g FW), followed by Wild Plum (71.9 µmol), Christmas Berry (60.5 µmol), Tortoise Berry (38.7 µmol), Crossberry (26.7 µmol), Waterberry (26.6 µmol). Cranberry gave results of (13.6 µmol). From the Ascorbic Acid calibration curve, the highest results yielded (as shown in graph 6.8) were for Wild Olive (231.0 µmol AAE/g FW), followed by Wild Plum at (160.6 µmol), Christmas berry at (141.0 µmol) and Tortoise berry at (73.7 µmol), Waterberry (62.3 µmol) and Crossberry (58.8 µmol). By comparison, Cranberry yielded (25.9 µmol).



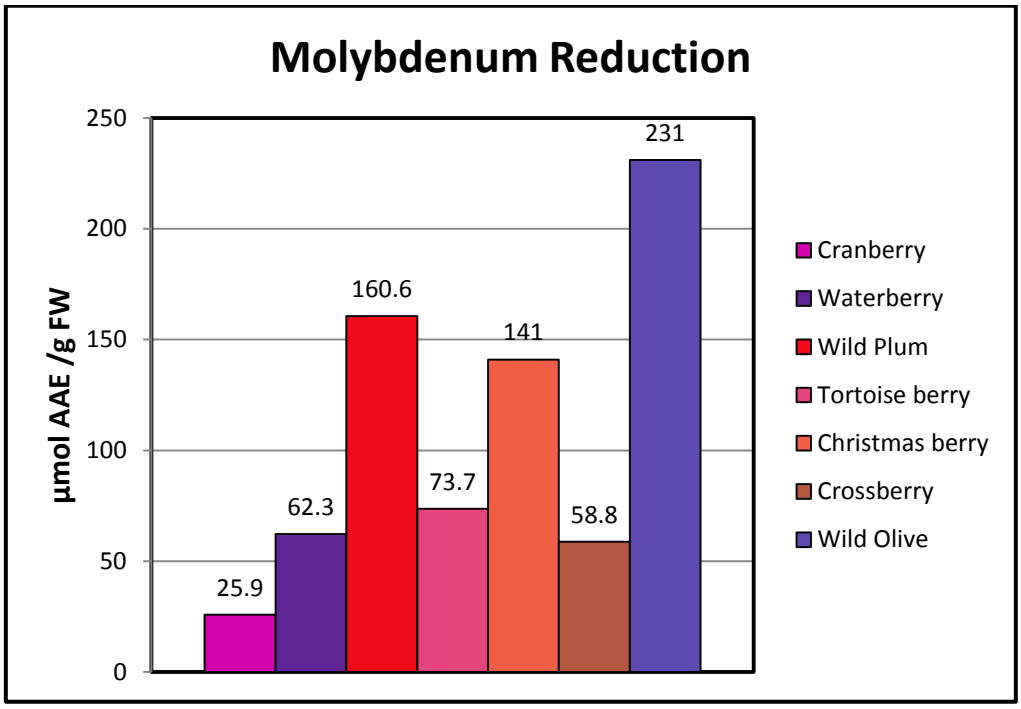
Graph 6.5 Bar graph illustrating TEAC results for all samples



Graph 6.6 Bar graph illustrating results of DPPH Assay for all samples



Graph 6.7 Bar graph illustrating results of Molybdenum Reduction assay using GAE



Graph 6.8 Bar graph illustrating results of Molybdenum Reduction assay using AAE

Results: Antioxidant Capacity as ORAC and TPC (FCR)

The results of the following two assays $ORAC_{FL}$ and Total Phenolic Content (FCR) are reported in Table 6.3 as the averages of three experimental readings and are represented as the mean ± standard deviation.

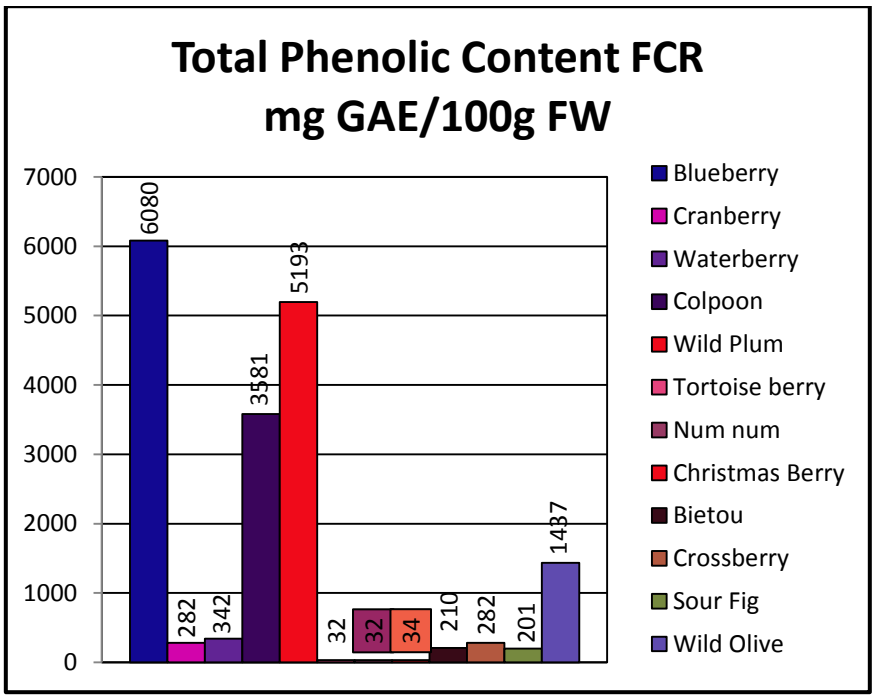
Table 6.3 Results ORAC values (±standard deviation) and Total Phenolic Content (FCR)

	H-$ORAC_{FL}$ (µmole TE/g FW)	L-$ORAC_{FL}$ (µmole TE/g FW)	TAC (µmole TE/g FW)	TPC (FCR) (mg GAE/100g FW)
Blueberry	84.30 ± 5.36	34.94 ± 2.65	119.24	6080 ± 2.35
Cranberry	34.23 ± 1.76	30.22 ± 4.45	64.45	282 ± 4.69
Water berry	77.04 ± 1.45	48.32 ± 2.44	125.36	342 ± 0.75
Colpoon	323.39 ± 2.23	55.60 ± 1.94	378.99	3581 ± 5.14
Wild Plum	125.90 ± 4.13	27.10 ± 2.51	153	5193 ± 1.12
Tortoise berry	44.51 ± 0.42	3.46 ± 0.33	47.97	182.40 ± 1.18
Num-num	29.93 ± 6.49	20.02 ± 6.72	49.95	32 ± 2.07
Christmas berry	261.37 ± 1.98	80.98 ± 7.86	342.35	34 ± 2.80
Bietou	48.34 ± 8.24	0.63 ± 6.19	48.97	210 ± 0.48
Crossberry	238.37 ± 5.01	12.98 ± 4.16	251.35	282 ± 6.71
Sour fig	63.25 ± 5.33	0.22 ± 2.54	63.47	201 ± 0.75
Wild olive	267.38 ± 1.34	69.14 ± 1.78	336.52	1437 ± 0.59

From Table 6.3 the ORAC assay yielded the following results: For Total Antioxidant Capacity (the sum of H-$ORAC_{FL}$ and L-$ORAC_{FL}$) the highest ranking fruit was Colpoon (378.99 µmol TE/g FW), followed by Christmas berry (342.35 µmol TE/g FW), then Wild Olive (336.52 µmol TE/g FW), Crossberry (251.35 µmol TE/g FW), Wild Plum (153.00 µmol TE/g FW), Waterberry (125.36 µmol TE/g FW), Sour fig (63.47 µmol TE/g FW), Num-num (49.95 µmol TE/g FW) µmol TE/g FW) and Bietou (48.97 µmol TE/g FW). The range lay between 378.99 and 48.97 µmol TE/g FW with a mean of 175.79 µmol TE/g FW and a 7-fold difference. Blueberry ranked 7th in this assay with119.24 µmol TE/g FW, while Cranberry ranked 8th with 64.45 µmol TE/g FW. This was well below the mean for the indigenous fruits.

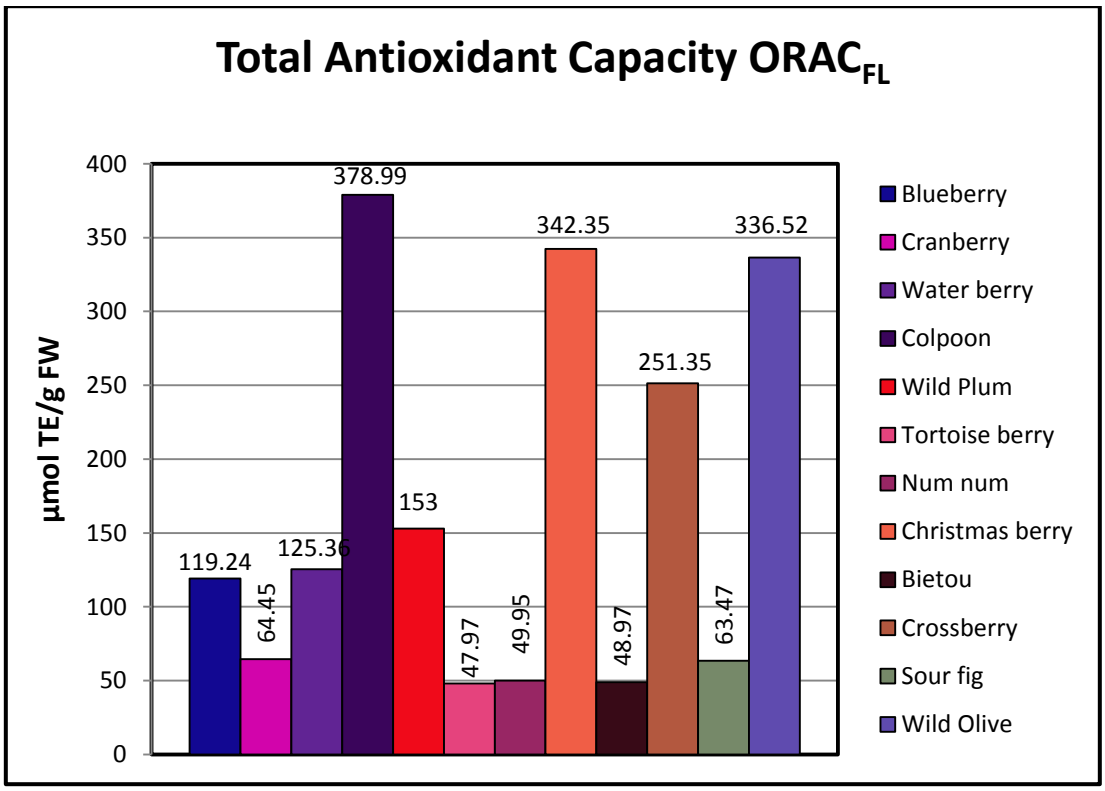
In the Total Phenolic Content (FCR) assay (Table 6.3) the results were as follows: Wild Plum (5193 ± 1.12 mg GAE/100g FW), Colpoon (3581 ± 5.14 mg GAE/100g FW), Wild Olive (1437 ± 0.59 mg GAE/100g FW), Waterberry (342 ± 0.75 mg GAE/100g FW), Crossberry (282 ± 6.71 mg GAE/100g FW), Bietou (210 ± 0.48 mg GAE/100g FW), Sour fig (201 ± 0.75 mg GAE/100g FW), Christmas berry (43 ± 2.80 mg GAE/100g FW), and Num-num (32 ± 2.07 mg GAE/100g FW). The mean value obtained was 1257 mg GAE/g FW and the range lay between 5193 and 32 mg GAE/100g FW, with a 162-fold difference between the highest and lowest values. The highest ranking of Blueberry (6080 ± 2.35 mg GAE/100g FW) is shown in Graph 6.10, with Cranberry tying with Crossberry ranking 6th (282 ± 4.69 mg GAE/100g FW).

As shown in Graph 6.10, for Total Antioxidant Capacity (the sum of H-$ORAC_{FL}$ and L-$ORAC_{FL}$) the highest ranking fruit was Colpoon (378.99 µmol TE/g FW), followed by Christmas berry (342.35 µmol TE/g FW), then Wild Olive (336.52 µmol TE/g FW), Crossberry (251.35 µmol TE/g FW), Wild Plum (153.00 µmol TE/g FW), Waterberry (125.36 µmol TE/g FW), Sour fig (63.47 µmol TE/g FW), Num-num (49.95 µmol TE/g FW) µmol TE/g FW) and Bietou (48.97 µmol TE/g FW). The range lay between 378.99 and 48.97 µmol TE/g FW with a mean of 175.79 µmol TE/g FW and a 7-fold difference. Blueberry ranked 7th in this assay (119.24 µmol TE/g FW), while Cranberry ranked 8th (64.45 µmol TE/g FW); this was well below the mean for the indigenous fruits.



Graph 6.9 Bar graph Illustrating Total Phenolic Content (FCR) for all samples

The results for the Total Phenolic Content by the Mazza method (Graph 6.1) differ substantially from the results from the Folin Ciocalteu method (Graph 6.9). For example, the result obtained for Christmas berry by the Mazza method was 1850.1 mg GAE/100g FW compared to the FCR method 34 mg GAE/100g FW. Similarly, Blueberry yielded 205.3 mg GAE/100g FW by the Mazza method compared to 6080 mg GAE /100g FW by the FCR method. The FCR method was carried out semi-automatically using a 96 well micro-plate reader, whereas in the Mazza method each cuvette was read separately. When carried out individually, the formation of precipitates resulted in disparate readings for the FCR method.



Graph 6.10 Bar graph illustrating TAC ORAC$_{FL}$ for all samples

As illustrated in Graph 6.10, the results obtained for the Total Antioxidant Capacity of Colpoon, Christmas berry, Crossberry and Wild Olive were more than double that of all the other fruits, including Blueberry and Cranberry. The results for Blueberry compared with both Waterberry and Wild Plum; whereas Cranberry, Num-num, Bietou and Sour fig yielded lower but similar results.

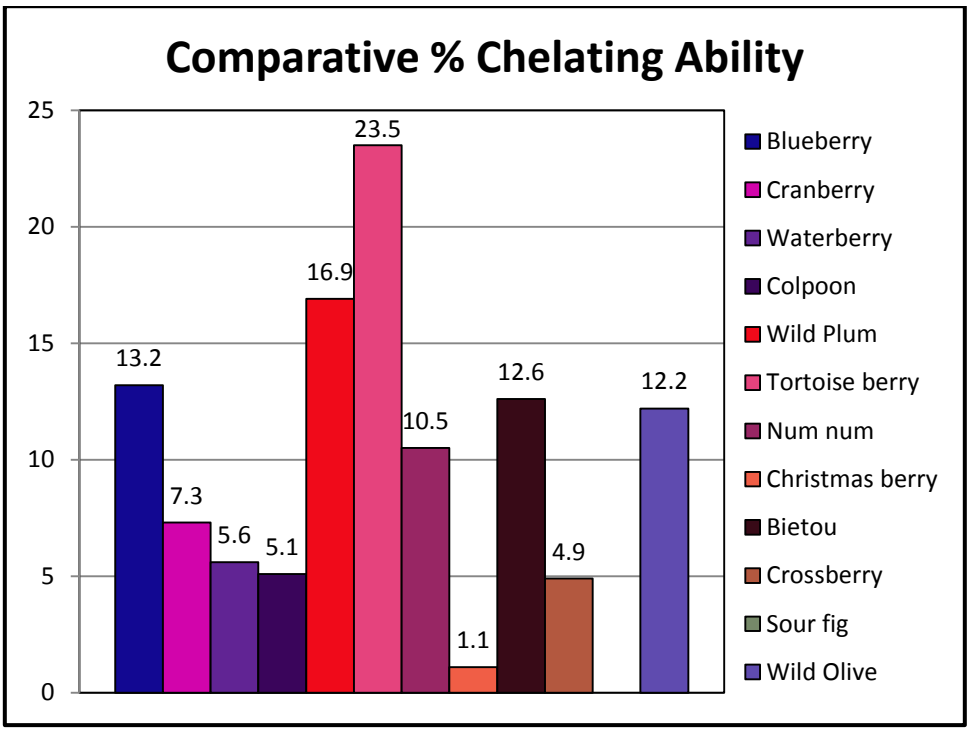
Results: Iron Chelating Activity

The results for ten indigenous fruits and two controls assayed for % Iron Chelating Activity are included in Table 6.4.

Table 6.4 Percentage Chelating Activity

Sample Extracts (15.6 mg/ml)	% Chelating Activity (Mean value)
Blueberry	**13.2**
Cranberry	**7.3**
Waterberry	**5.6**
Colpoon	**5.1**
Wild Plum	**16.9**
Tortoise berry	**23.5**
Num-num	**10.5**
Christmas Berry	**1.1**
Bietou	**12.6**
Crossberry	**4.9**
Sour fig	**0**
Wild Olive	**12.2**

The results in the case of the % Iron Chelating Activity assay (Table 6.4) were as follows: 23.5 % Tortoise berry, Wild Plum 16.9 %, Bietou 12.6 %, Wild Olive 12.2 %, Num-num 10.5 %, Waterberry 5.6 %, Colpoon 5.1 %, Crossberry 4.9 %, Christmas berry 1.1 % and Sour fig showed no activity. Blueberry gave a value of 13.2 % and Cranberry 7.3 %.



Graph 6.11 Bar graph comparing % Chelating Activity for 15.6 mg/ml fruit extracts.

As shown in Graph 6.11, the results from the Iron Chelating Activity assay revealed Tortoise berry and Wild Plum at above 15%; followed by Blueberry, Bietou, Wild Olive, Num-num at between 10-15%. Cranberry, Waterberry, Colpoon, Crossberry, and Christmas berry follow at below 10%. Sour fig showed no activity.

Statistical analysis

Similar studies carried out to explore the relationship between phenolic composition and antioxidant activity of certain fruits made use of Pearson Product Moment correlation and Multiple Regression analysis to analyse the data [164], [165], [166]. However, the relationship existing between the phenolic composition of berry extracts and their antioxidant activity is complex and cannot be fully elucidated even when analysed by means of statistical tools [165]. For example, an examination of whether the results correlate, or not, only indicates the presence or absence of a relationship, it does not reveal the nature of the relationship. And it should be remembered that 'correlation is not causation' – the possibility exists that the results may have been influenced by a 3rd variable [167].

The Pearson Correlation Coefficient analysis for association between paired samples (at $P<0.05$) from Microsoft Excel was used to test whether any relationships exist between the Total

Phenolic Content, Total Flavonols and Total Anthocyanins obtained for the samples and the various Antioxidant Activity results are summarized in Table 6.5 that follows.

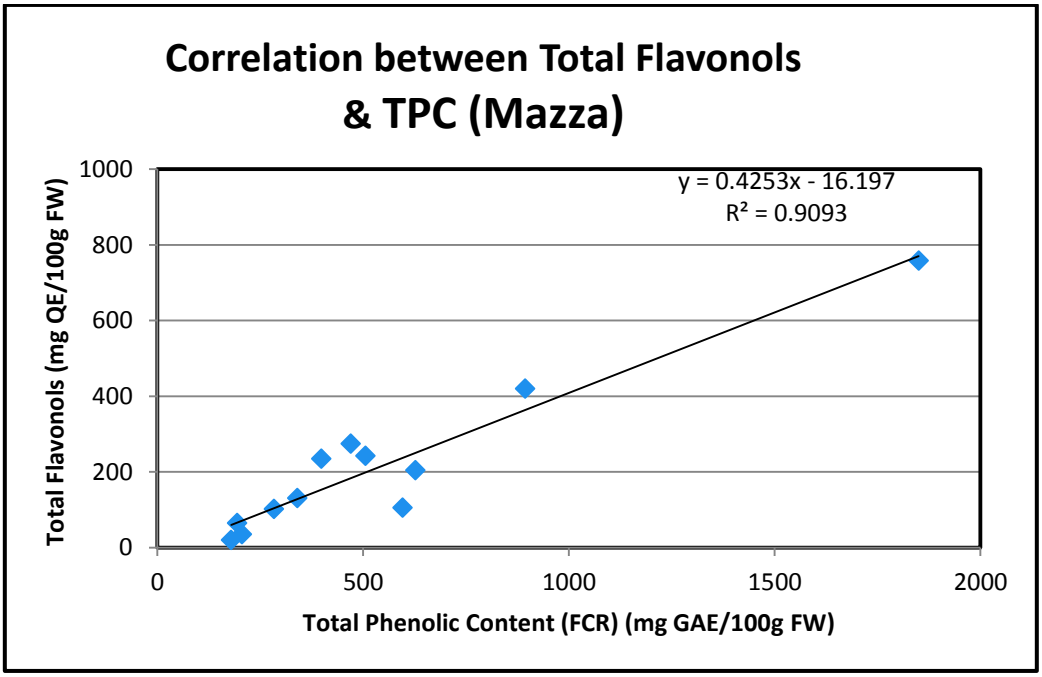
Table 6.5 Correlation coefficients (R) found for pairings of data of various assays

Correlation Coefficients (R) (Pearson) between Total Phenolic Content, Total Flavonols, Total Monomeric Anthocyanins and TEAC, DPPH, ORAC in fruit samples (Significance at P≤ 0.05).							
	TPC (Mazza) (mg GAE/100g FW)	**TF** (mg QE/100g FW)	**TA** (mg C3G/100g FW)	**TEAC** (µmol TE/g FW)	**DPPH** (µmol AAE/g FW)	**TAC $ORAC_{FL}$** (µmol TE/g FW)	**TPC (FCR)** (mg GAE/100g FW)
TPC (Mazza) (mg GAE/100g FW)	*	0.9536 (n=12)	0.7894 (n=5)	0.1228 (n=12)	0.1047 (n=12)	0.3833 (n=11)	-0.2219 (n=11)
TF (mg QE/100g FW)		*	Not applicable.	-0.0760 (n=12)	-0.0904 (n=12)	0.3587 (n=11)	-0.3168 (n=11)
TA (mg C3G/100g FW)			*	0.6337 (n=5)	0.4986 (n=5)	0.9044 (n=5)	0.2439 (n=5)
TEAC (µmol TE/g FW)				*	0.8935 (n=12)	0.1850 (n=11)	0.5090 (n=11)
DPPH (µmol AAE/g FW)					*	0.0754 (n=11)	0.5589 (n=11)
TAC $ORAC_{FL}$ (µmol TE/g FW)						*	0.1462 (n=11)
TPC (FCR) (mg GAE/100g FW)							*

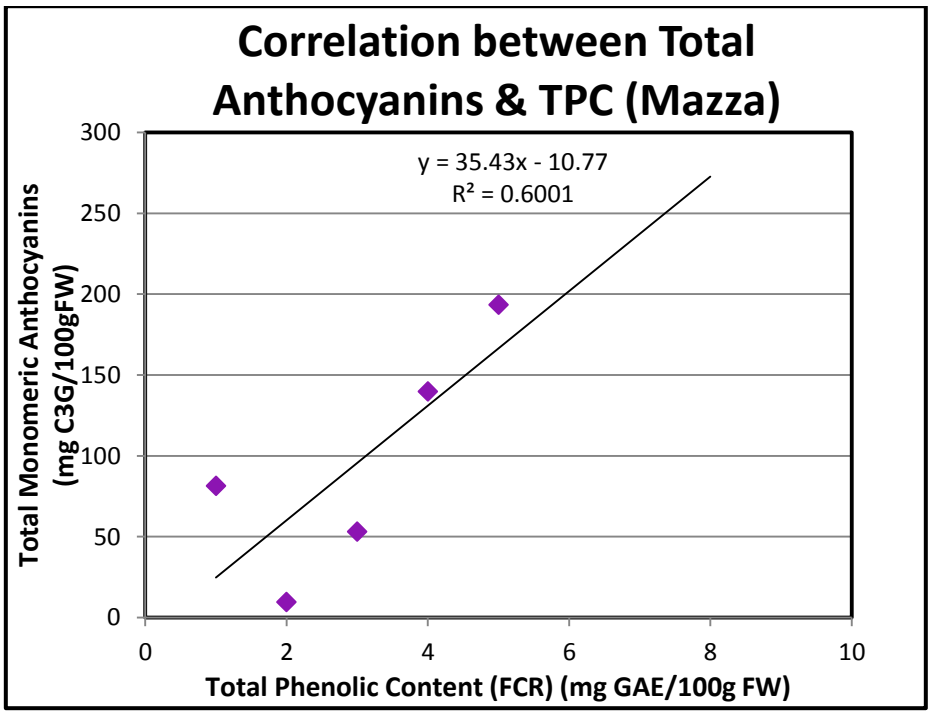
From the above Table 6.5, the Total Phenolic Content (Mazza) data correlated highly with that of Total Flavonols (Pearson: 0.9536, n=12) as illustrated in Graph 6.12. Anthocyanins were detected in only three of the indigenous fruits (Waterberry, Colpoon and Wild Olive) and two Northern Hemisphere berries; however Total Phenolic Content (Mazza) correlated well with Total Anthocyanins for these fruits (Pearson: 0.7894, n=5) as illustrated in Graph 6.14. The

correlation between TEAC and DPPH was significant (Pearson: 0.8935, n=12) as illustrated in Graph 6.15. The Total Phenolic Content (FCR) correlated weakly with Total Antioxidant Capacity (Pearson: 0.1462, n=11). This is in contrast to the result obtained by Prior (r_{xy} = 0.77) [42]. The Total Phenolic Content (Mazza) also correlated weakly with Total Antioxidant Activity TAC (Pearson: 0.3833, n=11).

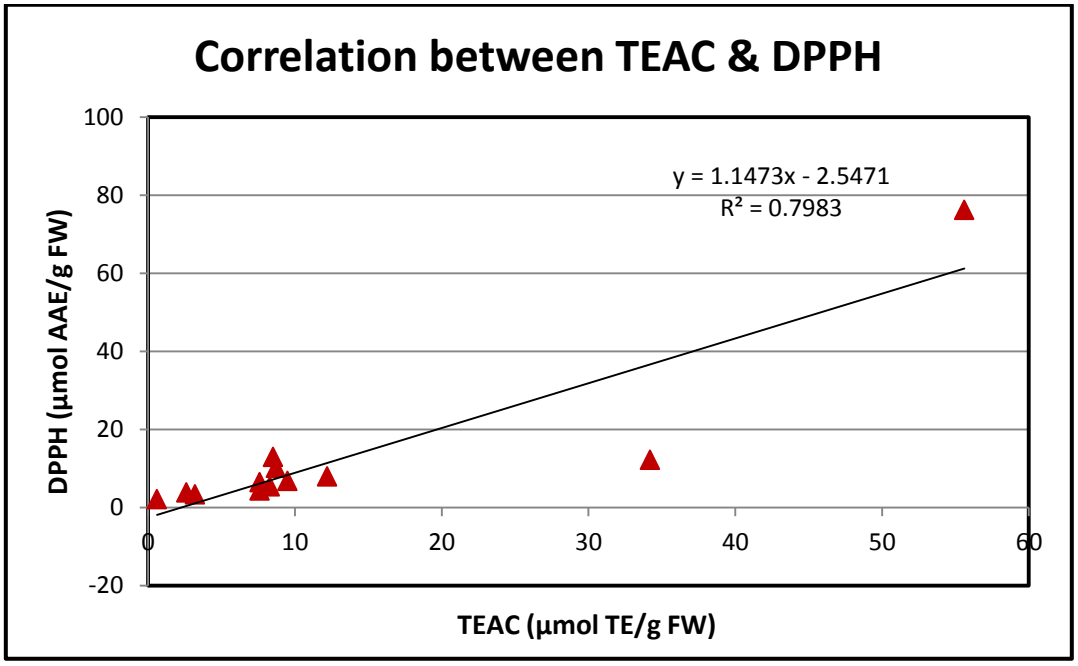
However, the correlation between Total Monomeric Anthocyanins and TAC was significant (Pearson: 0.9044, n=5) as illustrated in Graph 6.15. A similar result has previously been found by other research groups (r_{xy} = 0.92) (42). Total Monomeric Anthocyanins also correlated well with TEAC (Pearson: 0.6337, n=5). DPPH correlated moderately well with TPC (FCR) (Pearson: 0.5589, n=11); also TEAC correlated moderately well with TPC (FCR) (Pearson: 0.5090, n=11). Similarly, Total Monomeric Anthocyanins correlated moderately well with DPPH (Pearson: 0.4986, n=5).



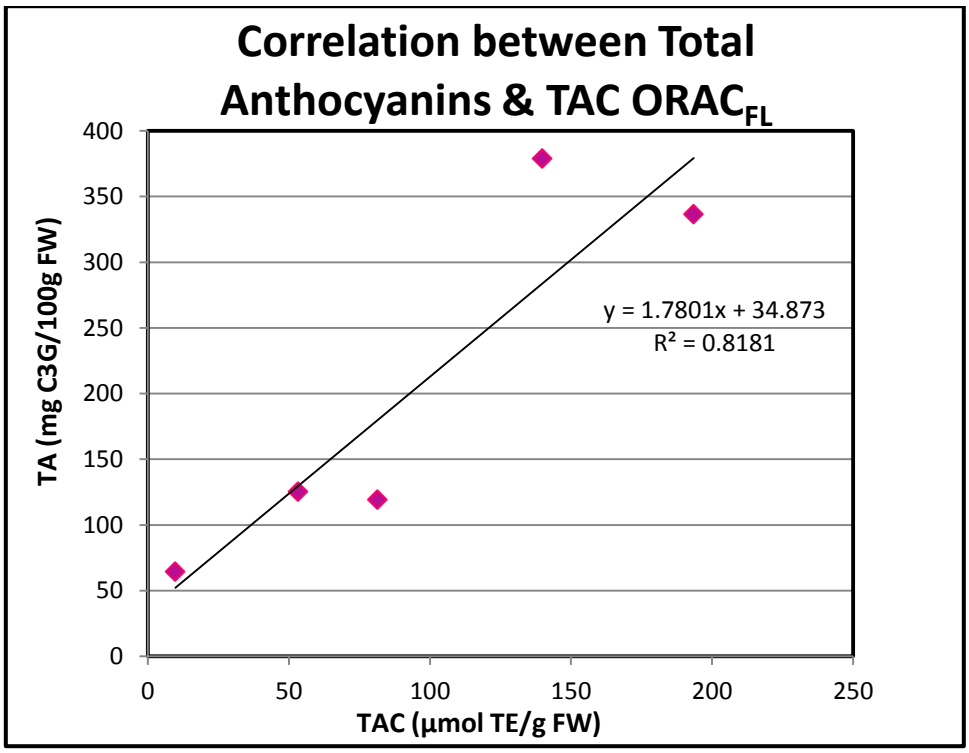
Graph 6.12 Correlation between Total Flavonols and Total Phenolic Content (Mazza)



Graph 6.13 Correlation between Total Monomeric Anthocyanins & Total Phenolic Content (Mazza)



Graph 6.14 Correlation between TEAC & DPPH



Graph 6.15 Correlation between Total Anthocyanins and TAC ORAC$_{FL}$

To summarize, the statistical analysis by means of Pearson correlation coefficients (R) as shown in Table 6.5, revealed strong positive correlations in five cases:

1. Total Phenolic Content (Mazza) versus Total Flavonols [R= 0.9536 (n=12)]. See Graph 6.13.
2. Total Phenolic Content (Mazza) versus Total Monomeric Anthocyanins [R= 0.7894 (n=5)]. See Graph 6.14.
3. DPPH versus Trolox Equivalent Antioxidant Capacity TEAC [R= 0.8935 (n=12)]. See Graph 6.15.
4. Total Antioxidant Capacity (ORAC$_{FL}$) versus Total Monomeric Anthocyanins [R= 0.9044 (n=5)]. See Graph 6.16.

From the above positive correlations it appears that the Flavonol content as well as the Total Anthocyanins present contributed to the Total Phenolic Content; the Total Anthocyanins present also contributed to the ORAC$_{FL}$ as well as TEAC, which indicates that anthocyanins are important players in combatting peroxyl ROO$^{\bullet}$ as well as ABTS $^{\bullet+}$ radicals.

The strong correlation between the DPPH and TEAC results in measuring antiradical activity showed that these assays, although using different radicals, gave similar results.

The weak correlation between TEAC and TPC (Mazza) [R= 0.1228 (n=12)] suggests that phenolic compounds are not the main compounds in these fruits responsible for their free radical scavenging ability. However, the stronger relationship between TEAC and TPC (FCR) [R=0.5090 (n=11)] seems to contradict the above statement.

A weaker relationship exists between TPC (FCR) and TAC $ORAC_{FL}$ [R=0.1462 (n=11)] compared to TPC (Mazza) and TAC $ORAC_{FL}$ [R=0.3833 (n=11)].

Comparative ranges of results for Blueberry and Cranberry

Results obtained in various studies of Blueberry and Cranberry fruits for TPC (FCR) and ORAC assays have also spanned a considerable range as demonstrated in the following Table 6.6:

Table 6.6 Table of comparative ranges of results for Blueberry and Cranberry assays, 2001-2012

Northern Hemisphere Berry Type	TPC (FCR) mg GAE/100g FW	TEAC µmol TE/g FW at 6 min	ORAC µmol TE/g FW	Reference
Blueberry varietals	262 – 930	8.11 – 38.29		Sellapan et al (2002) [140]
	262 - 497		54.81 – 87.56	Speisky et al (2012) [168]
	77 - 820			Koca et al (2009) [169]
	46.24			Fu et al (2011) [170]
	412			Zheng et al (2003) [53]
	316 - 489		48.9 - 52.7	Qi et al (2011) [171]
	450 - 800		31.1 - 35.8	Prior et al (2001) [164]
	315			Medina et al (2011) [172]
	46	1.26		Fu et al (2011) [170]
Blueberry Juice	230			Seeram et al (2008) [155]
Cranberry	315			Zheng et al (2003) [53]
	1722		37.4	Prior et al (2001) [164]
Cranberry juice	170			Seeram et al (2008) [155]

Across the various studies the TPC (FCR) results for Blueberry ranged from 46 – 930 mg GAE/100g FW, whereas the result from our analysis was very high at 6080 mg GAE/100g FW. The results for TEAC spanned 1.26 – 38.29 µmol TE/g FW, our result being 8.3 µmol TE/g FW. The results for ORAC likewise ranged from 31.1 – 87.6 µmol TE/g FW, the result for H-ORAC in our analysis was 84.3 µmol TE/g FW.

The results for Cranberry TPC (FCR) lay between 170 – 1722 mg GAE/100g FW. Our results were 282 mg GAE/100g FW. There were no TEAC findings reported for Cranberry in the

literature, our result being 9.5 μmol TE/g FW. There was one result found for ORAC: 37.4 μmol TE/g FW, whereas our H-ORAC compared yielding 34.2 μmol TE /g FW.

Antioxidant Potency Composite Index

To arrive at an antioxidant potency ranking of the samples, the results from eight assays were combined into a single Antioxidant Potency Composite Index [155] by the following procedure:

- All eight assays were equally weighted (TPC, TF, TA, TEAC, DPPH, TAC, TPC (FCR), ICA)
- An index value of 100 was assigned to the best score for each test
- The index score of the other samples in each assay was calculated as follows:

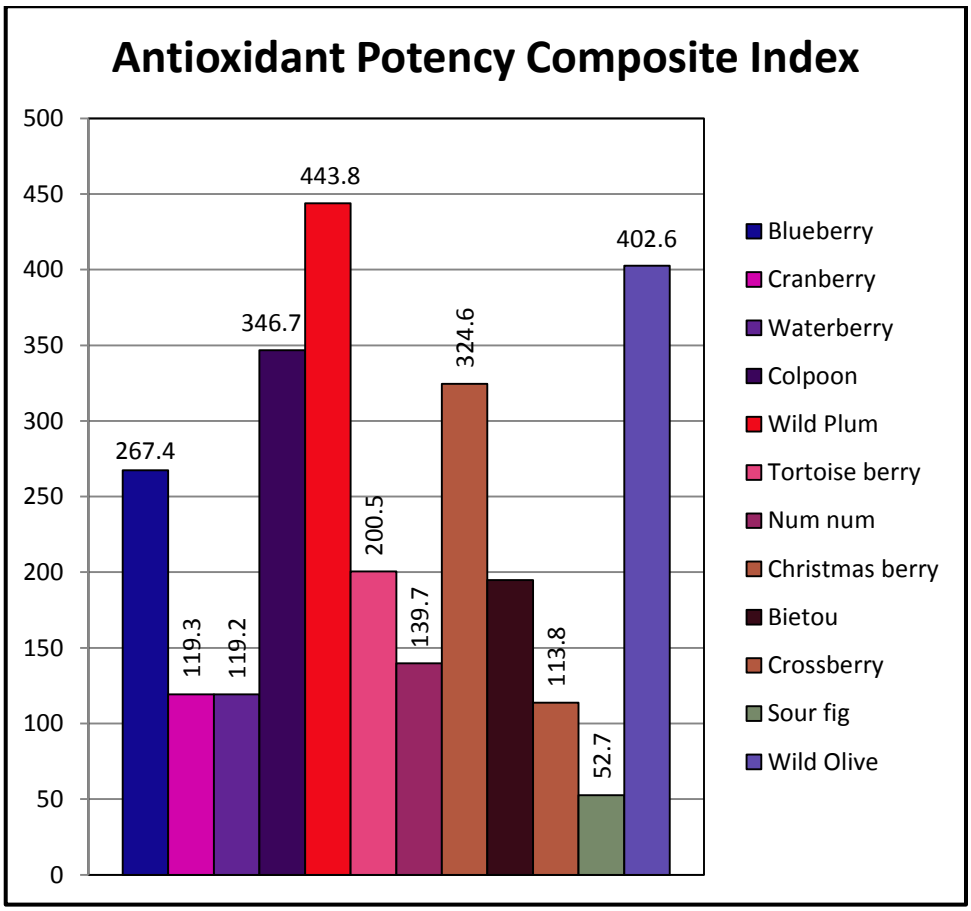
$$Antioxidant\ Index\ score\ = \left[\left(\frac{Sample\ score}{Best\ score}\right) \times 100\right]$$

- An average of all eight assays for each sample was calculated giving an overall mean index value
- A simple rank order was reported

The following Table 6.7 lists the Sample Rank Order obtained for ten indigenous fruits along with the controls, Blueberry and Cranberry.

Table 6.7 Antioxidant Potency Composite Index for eight assays and resultant Sample Rank Order

Samples	TPC (Mazza)	TF (Mazza)	TA	TEAC	DPPH	TAC	TPC (FCR)	ICA	APC Index	Sample Rank Order
Blueberry	11	4.6	42.1	14.9	7.1	31.5	100	56.2	267.4	5
Cranberry	18.4	17.2	5.0	17.1	8.9	17.0	4.6	31.1	119.3	9
Waterberry	10.5	8.6	27.5	5.8	4.3	33.1	5.6	23.8	119.2	10
Colpoon	25.4	36.2	72.3	15.3	16.9	100	58.9	21.7	346.7	3
Wild Plum	32.2	13.9	0	100	100	40.4	85.4	71.9	443.8	1
Tortoise berry	21.6	30.9	0	21.9	10.4	12.7	3.0	100	200.5	6
Num-num	27.3	31.9	0	13.7	8.4	13.2	0.5	44.7	139.7	8
Christmas berry	100	100	0	15.7	13.3	90.3	0.6	4.7	324.6	4
Bietou	48.3	55.3	0	15.7	5.6	12.9	3.5	53.6	194.9	7
Crossberry	9.7	2.6	0	4.7	5.0	66.3	4.6	20.9	113.8	11
Sour fig	15.3	13.4	0	1.1	2.8	16.8	3.3	0	52.7	12
Wild Olive	33.9	26.9	100	61.5	16.0	88.8	23.6	51.9	402.6	2



Graph 6.16 Antioxidant Potency Composite Index

In order to attempt to achieve a fair ranking, the results for Total Phenolic Content by the Mazza method as well as by the Folin Ciocalteu method were included in the composite index, although disparate.

The 1st ranking of Wild Plum is a reflection of attaining the highest TEAC and DPPH values along with a high Total Phenolic Content (FCR). It scored zero for Total Monomeric Anthocyanins yet achieved a composite index value of 443.8.

Wild Olive achieved the 2nd highest ranking due to having the highest Total Monomeric Anthocyanins as well as a high Total Antioxidant Capacity (L-$ORAC_{FL}$ + H-$ORAC_{FL}$). It achieved a composite index value of 402.6.

Colpoon yielded 3rd place with the highest Total Antioxidant Capacity (L-$ORAC_{FL}$ + H-$ORAC_{FL}$) result and a high Total Monomeric Anthocyanin content. It achieved a composite index value of 346.7.

Christmas berry yielded the 4th place due to the highest Total Phenolic Content (Mazza method) as well as the highest Total Flavonol Content, this despite scoring zero for Total Monomeric Anthocyanins. It achieved a composite index value of 324.6.

Blueberry came in at 5th place with the highest score for Total Phenolic Content (FCR). It achieved a composite index value of 267.4.

Tortoise berry held 6th place with the highest Iron Chelating Activity (despite a zero score for Total Monomeric Anthocyanins) and attained a composite index value of 200.5.

Bietou held 7th place, having scored zero for Total Monomeric Anthocyanins, with a composite index value of 194.9.

Num-num, Cranberry and Waterberry held places 8-10 with composite index values of 139.7, 119.3 and 119.2. Num-num obtained a zero score for Total Monomeric anthocyanins.

Crossberry ranked 11th with zero score for Total Monomeric Anthocyanins and composite index of 113.8.

Finally, Sour fig ranked 12th with a composite index value of 52.7, having scored zero for Total Monomeric Anthocyanins.

Comparatively speaking, Blueberry's 'overall antioxidant potency' is 56.8% of that of Wild Plum, 60.2% that of Wild Olive, 65% that of Colpoon and 66% that of Christmas berry. Cranberry (with 41.8% of the overall antioxidant potency of Blueberry) is 23.7% that of Wild Plum, 25% that of Wild Olive, 27% that of Colpoon, and 27.6% that of Christmas berry.

CHAPTER 7…CONCLUSION

"Please, test your servants for ten days, and let us be given some vegetables to eat and water to drink; then compare our appearance with the appearance of the youths who are eating the king's delicacies, and deal with your servants according to what you see." Daniel 1:12 circa 536 BC

In conclusion, determination of the Antioxidant Potency Composite Index (for eight assays) resulted in the following rank order for the indigenous fruits and control berries:

1. *Harpephyllum caffrum (*Wild Plum)
2. *Olea europaea* subsp. *Africana* (Wild Olive)
3. *Osyris* compressa (Colpoon)
4. *Chironia baccifera* (Christmas berry)
5. *Vaccinium corymbosum* (Blueberry)
6. *Nylandtia spinosa* (Tortoise berry)
7. *Chrysanthemoides monilifera* (Bietou)
8. *Carissa macrocarpa* (Num-num)
9. *Vaccinium macrocarpon* (Cranberry)
10. *Syzygium cordatum* (Waterberry)
11. *Grewia occidentalis* (Crossberry)
12. *Carpobrotus edulis* (Sour fig)

The North American 'gold standards' Blueberry ranked 5th and Cranberry tied in 9th place with Waterberry. Comparison of the results of Wild Plum to Blueberry gave a value 176% that of Blueberry. Likewise, Wild Olive gave 166%, Colpoon 154% and Christmas berry 152%.

The reasons for the attainment of the four highest rankings by Wild Plum, Wild Olive, Colpoon and Christmas berry were the following: Wild Plum attained this ranking due to having the highest TEAC and DPPH values and the second highest TPC (FCR), yet despite having zero Total Monomeric Anthocyanins. Wild Olive, a purple berry, yielded the highest Total Monomeric Anthocyanins and the 3rd highest TAC. Colpoon had the highest TAC and 2nd highest Total Monomeric Anthocyanins; whereas Christmas berry had the highest TPC

(Mazza) and TF (Mazza) and the 2nd highest TAC but zero Total Monomeric Anthocyanins. Blueberry, ranking 5th, had the highest TPC (FCR).

The levels of anthocyanins and total phenolics found in a fruit have a direct bearing on the antioxidant activity delivered by the fruit. Anthocyanins are considered to be among the most potent flavonoids in scavenging free radicals in vitro, possibly accounting for their anti-tumour effects [173]. A 2003 English study showed that an inverse relationship exists between increased consumption of fruit in childhood and cancer incidence in adulthood [174]. This is consistent with the findings of an inverse relationship for adult fruit intake and cancer risk [175].

It has been suggested that a plant-centred diet be more strongly encouraged to counteract the effects of eating refined grains, which have been processed to the extent of being devoid of nutrient-rich bran and germ [176]. Maize porridge is the most frequently consumed food by 1-5 year olds (355g average per capita per day), yet of all the common starches consumed in South Africa, maize meal porridge has the lowest Total Antioxidant Capacity [41]. The post-prandial surge associated with such a high Glycaemic Index meal has negative health implications. However, the inflammatory responses triggered following digestion can be offset by the inclusion of high antioxidant fruits along with the meal [22], [115]. This protection could be provided by following the Zulu tradition of adding Bietou berries to the porridge [66] or taking the juice in water or tea. These fruits have also traditionally been made into a nourishing syrup or jam.

The contribution to ORAC from fruits in the diet may come from a high consumption of low ranking fruits or from high ranking fruits that are consumed in small amounts [166]. By adding 25g of certain indigenous fruits to the average diet consumed in South Africa the following increases in Trolox Equivalents per day could be achieved:

1. 25g Wild Plum (weight excluding seeds) 4,000 µmol TE
2. 25g Waterberry (weight excluding seeds) 3,000 µmol TE
3. 25g Num-num (seeds consumed along with pulp) 1,249 µmol TE
4. 25g Sour fig (seeds consumed along with pulp) 1,587 µmol TE

All of these fruits are known and have been traditionally used by rural communities in South Africa.

The potential for utilisation of various South African indigenous species for food needs to be investigated. A number of underutilized native fruits have been developed successfully for commercial growing [177] in Australia and Malaysia, both for local consumption and inclusion in health promotion campaigns [178].

Due to their bitter taste, among the indigenous fruits examined, Christmas berry, Colpoon and Wild Olive would not be consumed as food but rather could be utilized as medicine. The tradition of consuming bitter alcoholic digestive tonics after a heavy meal is a long-standing tradition in Europe. Consider Fernet-Branca, the herbal bitters used in Italy as a digestive for its health giving properties [179]. The Khoe San are reported to have used the fruit and leaves of Christmas berry as a bitter tonic to treat stomach ulcers as well as diarrhoea. The very high ranking of Christmas berry as well as Wild Olive in Total Phenolic Content (Mazza) (Chapter 6 Table 6.1) reflects the traditional use of these herbs/fruits for treating diarrhoea.

Seemingly unpromising thin-fleshed fruit with large seeds like blackcurrants and pomegranate have been successfully commercialized and promoted as health giving, being used for increasing the antioxidant capacity of apple juice [155]. Blackcurrants are similar to the Bietou whose fruits have traditionally been made into a nourishing syrup and jam. The French Crème de Cassis manufactured from blackcurrant juice, has been described as 'a cocktail of anthocyanins' [180] and has become known world-wide.

The Israeli Negev trials [15] highlighted the problems besetting the commercial cultivation of Wild Plum, such as the very small fruits (labour intensive), and the flesh being only 10% of the total weight. It was suggested that the tree be used for home gardens and city and park landscaping so that the fruits may still be picked and enjoyed by 'children and others'. The highest ranking of Wild Plum in the Antioxidant Potency Composite Index justifies this recommendation. However, a lemonade-type fruit juice and rosé wine have been made from the pulp, as well as jam and jelly. Waterberry has also been made into jellies and fermented to give beverages. The high anthocyanin content in this fruit lends itself to making a so-called anthocyanin cocktail. In the case of Num-num, the potential for commercializing this fruit is considered to be favourable. It flowers and fruits almost continuously throughout the year; it can be processed into jam and can also be sold as fresh fruit as it has a long shelf life. The Sour fig has already been successfully commercialized, being sold in open markets in dried form and made into jam.

Freely available indigenous fruits with relatively high levels of antioxidant capacity constitute an untapped resource that deserves to be promoted more extensively by community health educators. As freely available, yet nutritionally superior alternatives to the relatively expensive 'exotic' fruits, these could help in diversifying monotonous diets [181], [182]. As dietary diversity correlates strongly with longevity and a decreased risk of mortality [183], indigent groups need to be empowered to increase their intake of essential nutrients [184], as well as those substances like polyphenols that have been implicated in chronic disease prevention.

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APPENDIX

Examples of Calibration Curves and Calculations

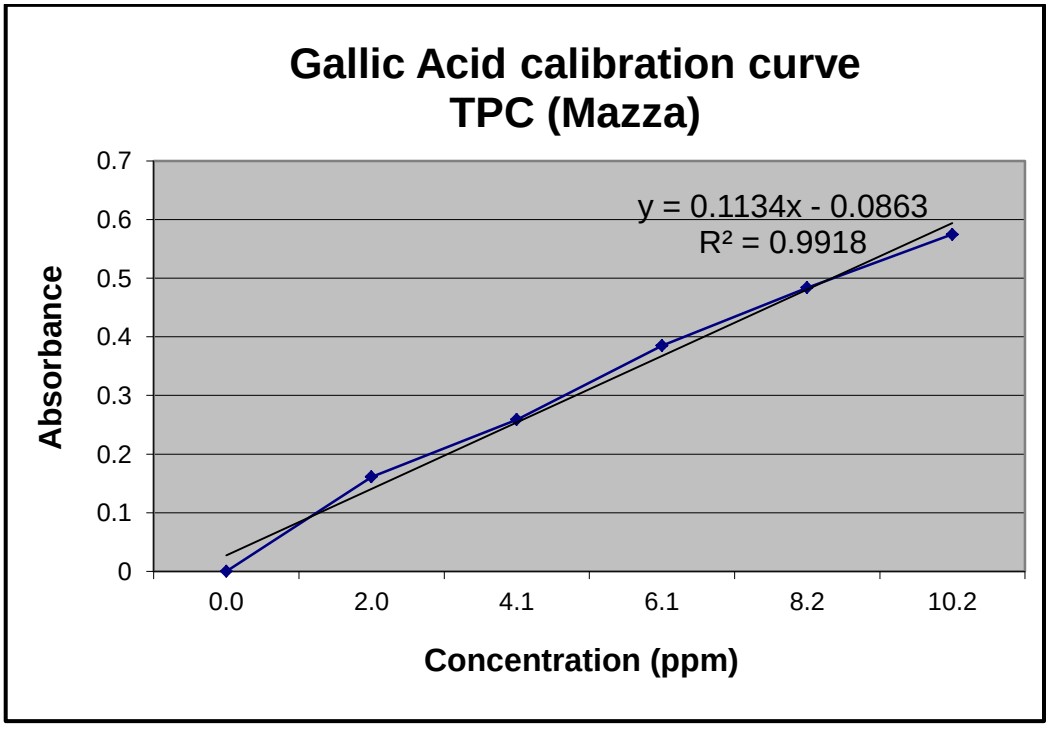
1. Total Phenolic Content (Mazza)

Table A.1 Standards prepared for Gallic Acid calibration curve (Mazza)

ml Stock Standard in 5ml	Gallic Acid after dilution (ppm)	Concentration for spectroscopy (ppm)
0	0	0
0.2	40	2
0.4	80	4
0.6	120	6
0.8	160	8
1	200	10

Table A.2 Absorbance readings of Gallic Acid calibration curve (Mazza)

Concentration (ppm)	Absorbance 1	Absorbance 2	Absorbance 3	Average Absorbance
0.000	0	0	0	0.000
2.046	0.148	0.178	0.156	0.161
4.091	0.264	0.272	0.242	0.259
6.137	0.386	0.378	0.392	0.385
8.182	0.486	0.474	0.492	0.484
10.228	0.575	0.567	0.583	0.575



Graph A.1 Gallic Acid calibration curve for TPC (Mazza)

The concentration of the sample in milligrams Antioxidant Equivalents per litre was calculated from the absorbance reading using the calibration curve for the standard. This concentration was then converted to milligrams Antioxidant Equivalents per 100g Fresh Weight by the equation:

Conc. (mg AE/100gFW) = Conc. (mg AE/L) $\times \frac{Dilution\ \ factor \times 100}{40 \times Mass\ of\ \ sample}$

Calculation Total Phenolic Content (Mazza) (Blueberry 1)

Absorbance ÷ slope

= 0.303 ÷ 0.064

= 4.73 mg Gallic Acid Equivalents/L

4.73 × 20 (dilution during preparation for spectroscopy) ÷ 40

(25ml extract) × $^{100}/_{\text{mass of sample}}$

= 4.73 × 20 (dilution during preparation for spectroscopy) ÷ 40

(25ml extract) × $^{100}/_{1.1856}$

= 199.5 mg Gallic Acid Equivalents/100g FW

= 4.73 mg Gallic Acid Equivalents/L

4.73 × 20 (dilution during preparation for spectroscopy) ÷ 40

(25ml extract) × $^{100}/_{\text{mass of sample}}$

= 4.73 × 20 (dilution during preparation for spectroscopy) ÷ 40

(25ml extract) × $^{100}/_{1.1856g}$

= 199.5 mg Gallic Acid Equivalents/100g FW

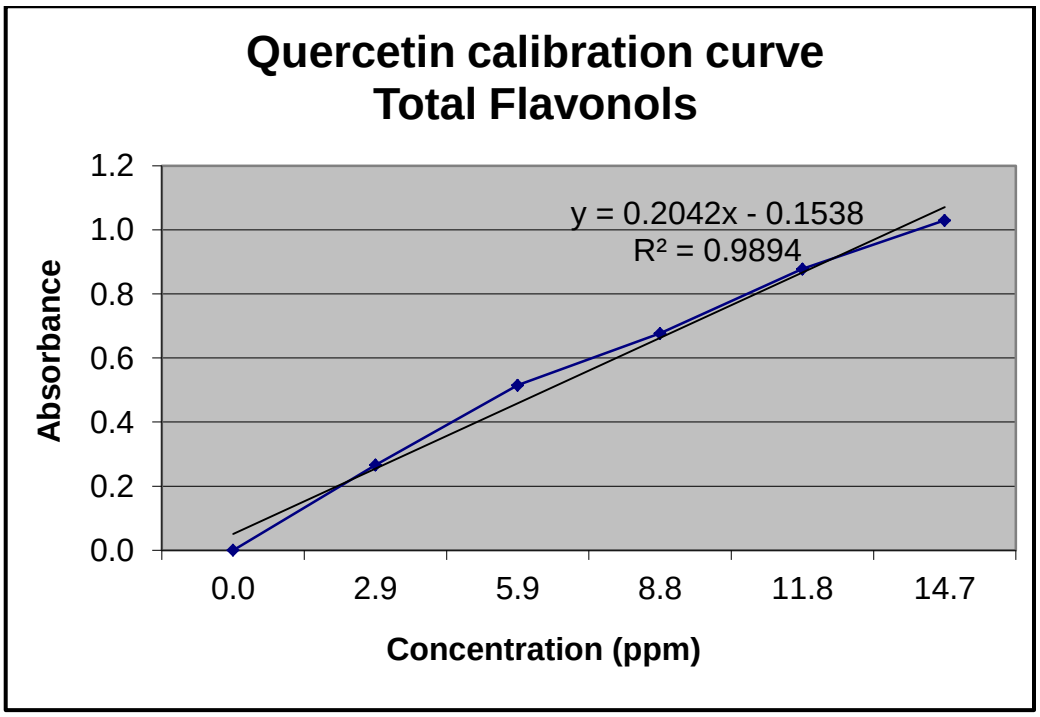
2. Total Flavonols

Table A.3 Standards prepared for Quercetin calibration curve

Stock Standard ml in 5ml	Dilution Range for Quercetin (ppm)	Concentration for spectroscopy (ppm)
0	0	0
0.3	60	3
0.6	120	6
0.9	180	9
1.2	240	12
1.5	300	15

Table A.4 Absorbance readings of standards for Quercetin calibration curve

Concentration (ppm)	Absorbance 1	Absorbance 2	Absorbance 3	Average Absorbance
0	0	0	0	0
2.940	0.260	0.264	0.275	0.266
5.886	0.523	0.510	0.513	0.515
8.829	0.693	0.666	0.671	0.677
11.770	0.926	0.834	0.875	0.878
14.700	1.086	0.857	1.148	1.030



Graph A.2 Quercetin calibration curve for Total Flavonols

The concentration of the sample in milligrams Antioxidant Equivalents per litre was calculated using its absorbance and the slope and absorbance for the standard. This concentration was then converted to milligrams Antioxidant Equivalents per 100g Fresh Weight by the equation:

$$\text{Conc. (mg AE/100gFW)} = \text{Conc. (mg AE/L)} \times \frac{Dilution\ \ factor \times 100}{40 \times Mass\ of\ \ sample}$$

Calculation Total flavonols (Tortoise berry 1)

Absorbance ÷ slope

= 0.217 ÷ 0.053

= 4.094 mg Quercetin Equivalents/L

4.094 × dilution factor (if extract is initially diluted) × 20 (dilution during preparation for spectroscopy) ÷ 40 (25ml extract) × 100/mass of sample

$4.094 \times {}^{50}/_{0.9503g}$

= 215.4 mg Quercetin Equivalents/100g FW

3. Total Monomeric Anthocyanins

Calculations (Blueberry 1)

Table A.5 Sample results for Total Monomeric Anthocyanins

Sample	Absorbance Readings				Absorbance	Concentration (mg Cyanidin-3-Glucoside/100g FW)
	pH 1.0 (510nm)	pH 1.0 (700nm)	pH 4.5 (510nm)	pH 4.5 (700nm)		
1	0.358	0.035	0.073	0.032	0.282	79.7
2	0.393	0.054	0.116	0.054	0.277	73.8
3	0.204	0.000	0.027	0.008	0.185	90.6
					Average	81.4
					Std Dev.	8.5

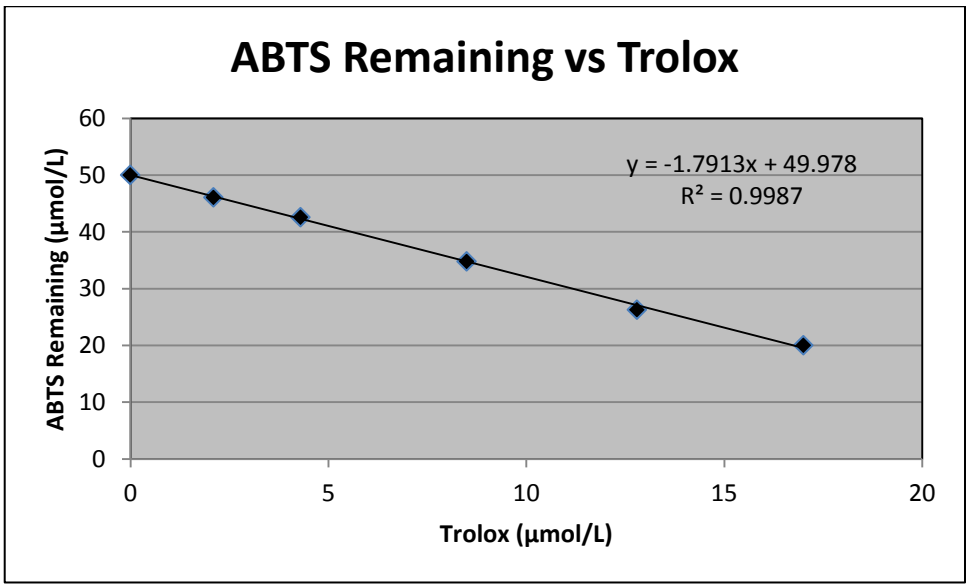
4. TEAC

Table A.6 Trolox standards for calibration curve

Trolox Stock 2 mM (ml)	EtOH (ml)	Concentration (mM)
0	5	Blank
0.25	4.75	0.1
0.5	4.5	0.2
1	4	0.4
1.5	3.5	0.6
2	3	0.8

Table A.7 Absorbance readings of Trolox standards and ABTS Remaining

Concentration Trolox (μM)	Absorbance 1	Absorbance 2	Absorbance 3	Average Absorbance	ABTS Remaining (μM)
0.0	0.800	0.800	0.800	0.800	50.0
2.1	0.740	0.736	0.739	0.738	46.1
4.3	0.682	0.685	0.678	0.682	42.6
8.5	0.559	0.555	0.558	0.557	34.8
12.8	0.410	0.421	0.429	0.420	26.3
17.0	0.319	0.323	0.316	0.319	20.0



Graph A.3 ABTS Remaining versus Trolox

A table was drawn up of the Trolox concentrations and corresponding absorbencies. The ABTS radical concentration remaining, in µM, was calculated by dividing the absorbance with the molar extinction coefficient of ABTS (ε, 0.016 dm^3 $\mu M^{-1}cm^{-1}$). Then the slope and intercept of the regression line from the Trolox concentrations and the calculated ABTS Remaining values was calculated using Excel. The ABTS Remaining in the samples was also calculated by dividing the absorbance with the Molar Extinction Coefficient. The TEAC values were then determined using the slope and intercept values of the regression line. Finally, the TEAC values were converted to the final values by taking the dilutions and the masses of the berries into account.

Calculation TEAC (Colpoon 1)

Table A.8 Calibration curve for Trolox

Trolox (µmol/L)	Absorbance	Conc ABTS remaining (µmol/L)
0.00	0.661	41.313
1.99	0.594	37.125
3.98	0.54	33.750
7.96	0.43	26.875
11.94	0.288	18.000
15.92	0.198	12.375

Using the average set of values for Colpoon calculation

Slope = -1.840

Intercept = 41.054

Absorbance ÷ ε = ABTS radical concentration remaining

0.466 ÷ 0.016 = 29.125 µmol/L ABTS remaining

(Conc. ABTS remaining (µmol/L) – intercept) ÷ slope = TEAC

(29.125 – 41.054)/-1.840 = 6.483 µmol/L Trolox Equivalent Antioxidant Capacity

TEAC × dilution factor (if extract initially diluted) × 50 (sample dilution 20 µL in 1000 µL) = TEAC of original berry extract

6.478 × 50 = 324.158 µmol Trolox Equivalent Antioxidant Capacity (per litre)

TEAC ÷ 40 (25ml extract) ÷ mass of sample = µmol TEAC /g FW

324.158 ÷ 40 ÷ 0.9499

=8.5 µmol TEAC/g FW

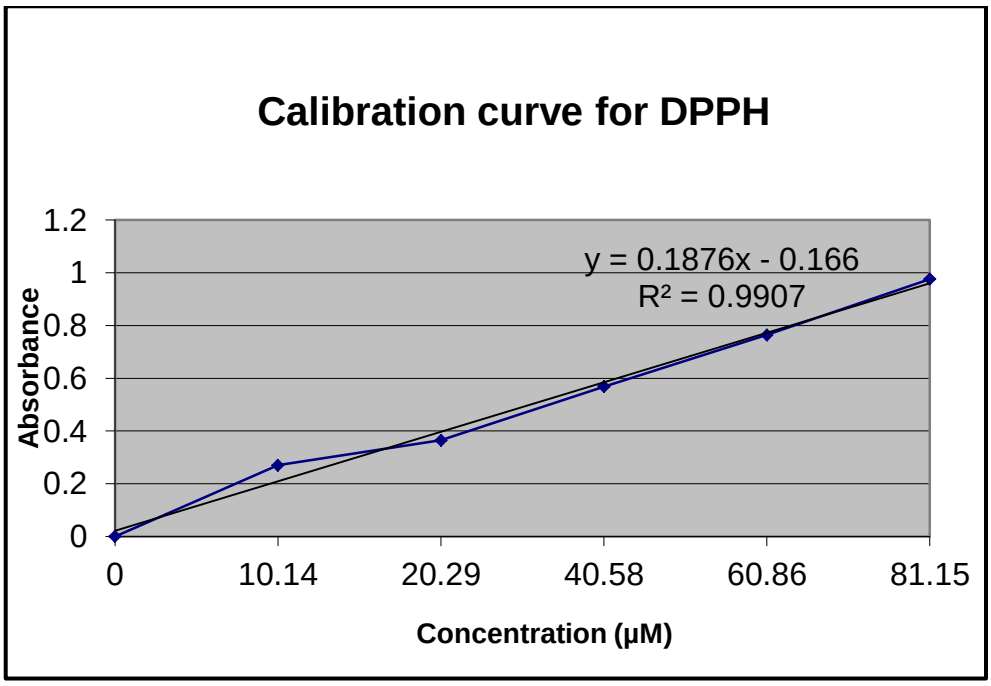
5. DPPH

Table A.9 Standards for DPPH calibration curve

Stock Solution DPPH ml in 10 ml	Concentration (µM)
0	0
0.2	20
0.4	40
0.6	60
0.8	80

Table A.10 Absorbance readings of DPPH standards for calibration curve

Concentration DPPH (µM)	Absorbance 1	Absorbance 2	Absorbance 3	Average Absorbance
0	0.000	0.000	0.000	0.000
10.14	0.270	0.270	0.270	0.270
20.29	0.365	0.365	0.365	0.365
40.58	0.568	0.568	0.568	0.568
60.86	0.764	0.763	0.764	0.764
81.15	0.976	0.975	0.975	0.976



Graph A.4 DPPH calibration curve

The slope and intercept from the DPPH calibration curve was used to determine the DPPH remaining from the absorbance after the samples and standards reacted with the DPPH. The slope and intercept was then determined for the Ascorbic Acid/DPPH Remaining calibration curve each time. This information was then used to determine the Ascorbic Acid Equivalents from the DPPH Remaining of the sample. Finally the concentrations were converted to µmol Ascorbic Acid Equivalents/g FW.

Regression line for DPPH calibration curve: $y = 0.011126x + 0.095313$

Regression line for Ascorbic Acid calibration curve: $y = -11.001x + 52.372$

Calculation DPPH (Crossberry 1)

Regression line for DPPH calibration curve:

$y = 0.011126x + 0.095313$

DPPH remaining = (Absorbance – 0.095313)/0.011126

= (0.558 – 0.095313)/0.011126

= 41.586

Table A.11 Absorbance of Crossberry sample

Absorbance 1	Absorbance 2	Mean Absorbance	DPPH Remaining
0.556	0.56	0.558	41.586

Table A.12 Ascorbic Acid calibration curve

Ascorbic Acid concentration	Absorbance	DPPH Remaining
0.00	0.650	49.855
0.54	0.542	40.148
1.07	0.485	34.980
1.61	0.444	31.340
2.14	0.349	22.756
2.68	0.280	16.600

Regression line for Ascorbic Acid calibration curve:

$y = -11.84x + 48.469$

mg Ascorbic Acid Equivalents/L = [DPPH Remaining – 48.469]/-11.84

= (5.185 – 48.469)/-11.84

= 41.586 mg Ascorbic Acid Equivalents/L

µmol AAE/g FW = mg AAE/L × 40 (0.1 in 4ml) ÷ 40 (25ml extract) ÷ mass of sample ÷ 176.13 (MW of Ascorbic Acid)

= 41.586 × 40 ÷ 40 ÷ 0.8655 ÷ 176.13

= 3.813 µmol AAE/g FW

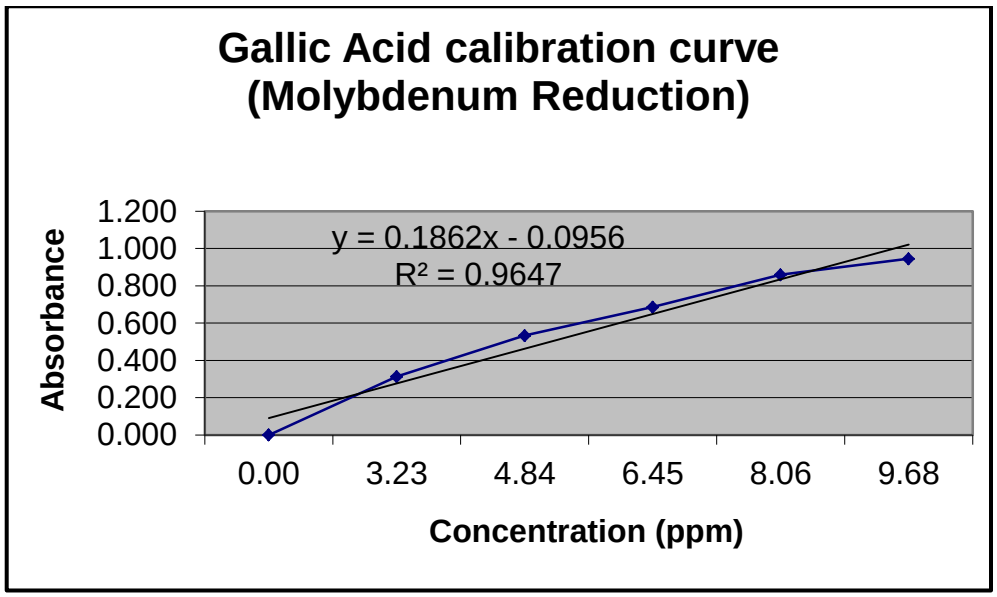
6. Molybdenum Reduction

Table A.13 Ascorbic Acid and Gallic Acid calibration

Stock Standard solution (1000ppm) ml in 10 ml	Concentration (ppm)
1	100
2	200
3	300
4	400
5	500

Table A.14 Absorbance readings for Gallic Acid standards

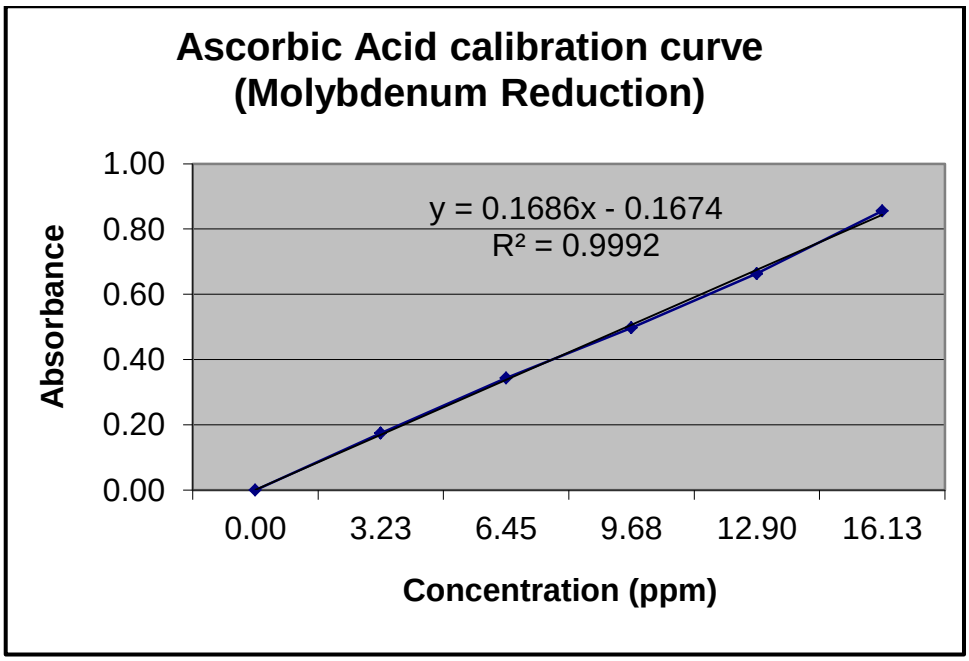
	Concentration of Gallic Acid (ppm)	Absorbance 1	Absorbance 2	Absorbance 3	Average Absorbance
Blank	0.00	0.000	0.000	0.000	0.000
Std 1	3.23	0.263	0.296	0.380	0.313
Std 2	4.84	0.440	0.602	0.556	0.533
Std 3	6.45	0.526	0.740	0.791	0.686
Std 4	8.06	0.648	0.959	0.971	0.859
Std 5	9.68	0.776	1.063	0.995	0.945



Graph A.4 Gallic Acid calibration curve for Molybdenum Reduction

Table A.15 Absorbance readings for Ascorbic Acid standards for Molybdenum Reduction

	Concentration of Ascorbic Acid (ppm)	Absorbance 1	Absorbance 2	Absorbance 3	Average Absorbance
Blank	0.00	0.000	0.000	0.000	0.000
Std 1	3.23	0.176	0.188	0.160	0.175
Std2	6.45	0.291	0.397	0.343	0.344
Std3	9.68	0.432	0.549	0.511	0.497
Std4	12.90	0.594	0.769	0.629	0.664
Std5	16.13	0.875	0.890	0.802	0.856



Graph A.5 Ascorbic Acid calibration curve for Molybdenum Reduction

The concentration of the sample in milligrams Antioxidant Equivalents per litre was calculated using its absorbance and the slope and intercept of the calibration curve for the Molybdenum assay. This concentration was then converted to milligrams Antioxidant Equivalents per gram of Fresh Weight by the equation:

$$\text{Conc. (mg AE/g FW)} = \text{Conc. (mg AE/L)} \times \frac{Dilution\ \ factor \times 100}{40 \times Mass\ of\ \ sample}$$

This concentration was then converted to μmol Antioxidant Equivalents per gram Fresh Weight by dividing by the Molecular Mass of the antioxidant and multiplying by a thousand.

Calculation Molybdenum Reduction Assay (Christmas berry) (Gallic acid)

(Absorbance – intercept) ÷ slope

= (0.301 – 0.0287) ÷ 0.1091

= 2.4959 mg Gallic Acid Equivalents /L (diluted sample)

2.4959 mg Gallic acid equivalents/L × dilution factor ÷ 40 (25ml extract) × 1/mass of sample

= 2.4959 × 5 (initial extract dilution) × 31 (dilution during preparation for spectroscopy) ÷ 40 (25ml extract) × 100/1.0763

= 8.9859 mg Gallic Acid Equivalents/g FW

8.9859 mg Gallic Acid Equivalents/g FW ÷ Molecular Mass × 1000

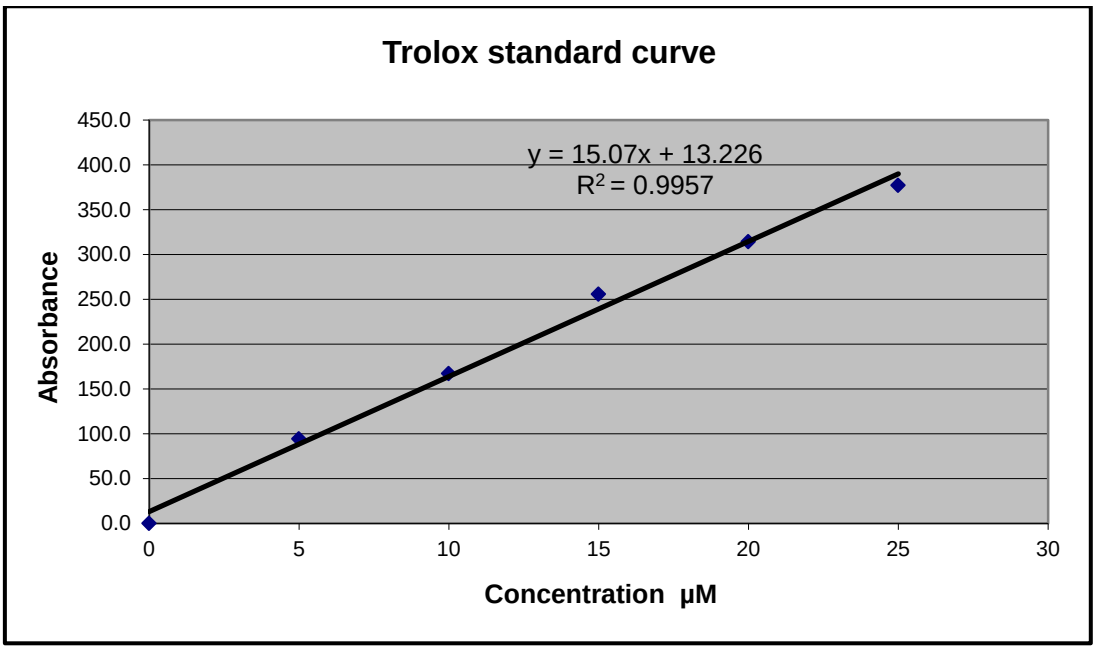
= 8.9859 mg Gallic Acid Equivalents/g FW ÷ 170.12 × 1000

= 52.8 μmol Gallic Acid Equivalents/g FW

7. ORAC

Table A.16 Absorbance readings for Trolox calibration curve

Concentration Trolox (μM)	Average Absorbance
0.0	13.226
5.0	88.576
10.0	163.926
15.0	239.276
20.0	314.626
25.0	389.976



Graph A.6 Trolox calibration curve

Calculation of ORAC value

The ORAC values were calculated using a regression equation (y = mx + c) between Trolox concentration (y) (µM) and the net area under the fluorescence decay curve (x). Data are expressed as micromoles of Trolox Equivalents / g FW for the fruits. The area under the curve (AUC) is calculated as:

$$AUC = \left(0.5 + {f_2}/{f_1} + {f_3}/{f_1} + {f_4}/{f_1} + \cdots + {f_i}/{f_1}\right) \times CT$$

Where f_1 = initial fluorescence reading at cycle *1*, f_i = fluorescence reading at cycle *i*, and CT = cycle time in minutes.

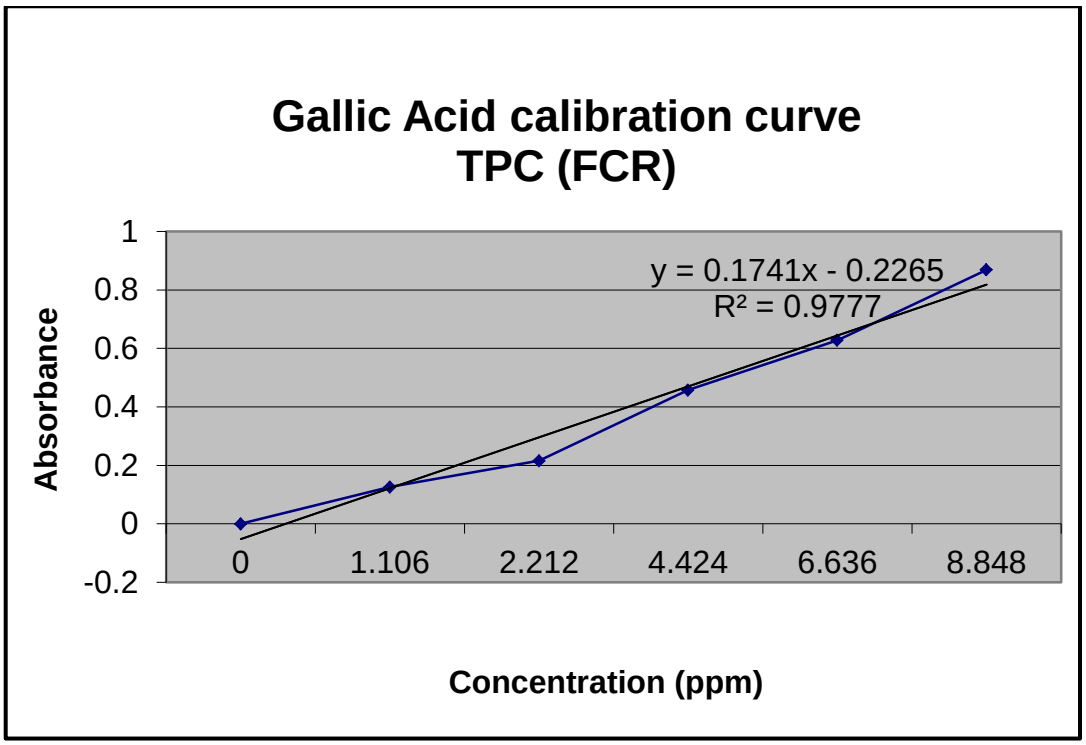
8. Total Phenolic Content (FCR)

Table A.17 Gallic Acid calibration curve for Total Phenolic Content (FCR)

Flask no.	Volume (µl)	Final concentration (ppm)
1	0	0 (Blank)
2	200	2
3	400	4
4	600	6
5	800	8

Table A.18 Absorbance readings for Gallic Acid or Total Phenolic Content (FCR)

Concentration (ppm)	Absorbance 1	Absorbance 2	Absorbance 3	Average Absorbance
0	0	0	0	0
1.106	0.129	0.126	0.124	0.126
2.212	0.217	0.219	0.213	0.216
4.424	0.452	0.473	0.448	0.458
6.636	0.628	0.632	0.623	0.628
8.848	0.879	0.857	0.872	0.869



Graph A.7 Gallic Acid calibration curve for Total Phenolic Content (FCR)

Calculation Total Phenolic Content (FCR) (Blueberry 1)

Absorbance ÷ slope

= 0.004 ÷ 0.115

= 0.035

0.035 × 100 (dilution in sample preparation)

= 3.478 mg Gallic acid equivalents/L ÷ 40 × $^{100}/_{1.1856g}$

= 7.3 mg Gallic acid equivalents/100g FW

9. Iron Chelating Activity

Table A.19 Volumes of sample and reagents for ICA assay

Volume Stock Soln (µl)	Volume Water (µl)	Volume Methanol (µl)	Volume Ferrozine (µl)	Volume Ferrous ions (µl)
500	0	900	100	100
400	100	900	100	100
300	200	900	100	100
200	300	900	100	100
100	400	900	100	100
50	450	900	100	100
25	475	900	100	100
0	500	900	100	100

Table A.24 Absorbance for Control and 15,63 mg/ml fruit samples ICA

Fruit Extract 15,63mg/ml	Abs $_{Sample}$	Abs $_{Pure\ extract}$	Abs
Control			0,818
Blueberry	0,804	0,094	0,710
Cranberry	0,798	0,04	0,758
Waterberry	0,786	0,014	0,772
Colpoon	0,793	0,017	0,776
Wild Plum	0,692	0,012	0,680
Tortoise berry	0,638	0,012	0,626
Num-num	0,752	0,020	0,732
Christmas berry	0,823	0,014	0,809
Bietou	0,729	0,014	0,715
Crossberry	0,789	0,011	0,778
Sour fig	0,845	0,014	0,831
Wild Olive	0,739	0,021	0,718

$$Absorbance = Absorbance_{Sample} - Absorbance_{Pure\ Extract}$$

Calculation Metal Chelating Activity (Christmas berry)

The % Chelating Activity for the extracts and standards was calculated using the following equation:

$$Chelating\ Effect\ (\%) = \left(1 - \frac{A_{sample}}{A_{control}}\right) \times 100$$

% Chelating Activity for 15.63 mg/L Blueberry extract = $\left[1 - \frac{0,710}{0.818}\right] \times 100$

= 13,2%