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# Routine analytical methods for use in South Pacific regional laboratories for determining naturally occurring antioxidants in food

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**Abstract:** Naturally occurring antioxidants are compounds that aid in the prevention of oxidative stress that leads to the onset of nutritionally-related chronic diseases including diabetes, cancer and coronary heart diseases. Reliable information on the antioxidant content of traditional South Pacific food is vital in reducing the level of chronic degenerative disorders amongst the local population. This paper describes, with examples from our laboratories, analytical procedures that are suitable for use in South Pacific regional laboratories for determining naturally occurring antioxidants in food and plant material. These include the trolox equivalent antioxidant capacity (TEAC) for measuring the Total Antioxidant Capacity (TAC), the Folin-Ciocalteu method for determining the total polyphenol (TPP) content and a spectrophotometric /pH difference method for determining the total anthocyanin (TAT) content. High performance liquid chromatography with ultraviolet detection (HPLC-UV) is suitable for determining individual antioxidants present in food, e.g. carotenoids and flavonols, while high performance liquid chromatography-diode array detection-tandem mass spectrometry (HPLC-DAD-MS/MS) can be used to identify and quantify low levels of naturally occurring antioxidants, e.g. catechins, present in food and plant material.

**Keyword: Food, total antioxidant capacity, total polyphenol content, total anthocyanin content, flavonols, carotenes, liquid chromatography-mass spectrometry**

## INTRODUCTION

Antioxidants are compounds that inhibit or prevent oxidation. Antioxidants neutralise free radicals by donating one of their own electrons, thus ending the electron-stealing reaction. In the human body, they act as scavengers and play a housekeeping role by mopping up free radicals and are capable of quenching or stabilising free radicals, reducing oxidative stress (Barry, 1994; Halliwell and Aruoma, 1997; Droge, 2002). The four major forms of free radicals are the hydroxyl radical ($OH^{\bullet}$), the superoxide radical ($O^{-}_{2}$), singlet oxygen ($^{1}O_{2}$) and hydrogen peroxide ($H_{2}O_{2}$). Endogenous antioxidants such as superoxide dismutase (SOD) help remove superoxides, while catalase and glutathione reductase (GHS) counteract hydrogen peroxide. Other forms of natural antioxidants are also present in foods. These include vitamin E, vitamin C and phytochemicals, e.g. carotenoids and flavonoids. An adequate intake of antioxidants, through the consumption of antioxidant rich foods, prevents the development of oxidative stress that leads to the onset of nutritionally-related chronic diseases including diabetes, cancer and coronary heart diseases (Krinsky, 1989; Hertog *et al.*, 1997a; Hertog *et al.*, 1997b; Maxwell *et al.*, 1997; Michaud *et al.*, 1998; Prior and Cao, 2000; Kaur and Kapoor, 2001; Young and Woodside, 2001; Shihabi *et al.*, 2002; Boyer and Lui, 2004; Song *et al.*, 2005).

The analytical methods used to determine the levels of antioxidants in food depend on the types of antioxidants of interest. Antioxidants can be analysed either as a 'functional group', antioxidant groups or as individual antioxidants. 'Functional group' can be measured as the total antioxidant capacity (TAC) with any of the following major assays: Trolox equivalent antioxidant capacity (TEAC) (Miller and Rice-Evans, 1997), oxygen radical absorbance capacity (ORAC) (Cao and Prior, 1998; Re *et al.*, 1999), total reactive antioxidant potential (TRAP) (Ghiselli *et al.*, 1998) and ferric reducing antioxidant power (FRAP) (Benzie and Szeto, 1999). Other methods include measuring the scavenging of stable radical species by antioxidants such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) and N,N-dimethyl-p-phenylene diamine (DMPD) (Gil *et al.*, 2000), chemiluminescent procedures (Oki *et al.*, 2002; Costin *et al.*, 2003; Anagnostopoulou *et al.*, 2006) and a cellular antioxidant assay (Wolfe and Liu, 2007).

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The major antioxidant group assays include the determination of total polyphenol (TPP) content by the Folin-Ciocalteu method (Singleton and Rossi, 1965; Singleton *et al.*, 1999) and total anthocyanin (TAT) content by spectrophotometric/ pH difference (Sellappan *et al.*, 2002). Instrumental techniques, e.g. gas chromatography (Gioti *et al.*, 2007), capillary electrophoresis (Wang and Huang, 2004; Jac *et al.*, 2006; Woraratphoka *et al.*, 2007; Tsukagoshi *et al.*, 2007) and high performance liquid chromatography with ultraviolet detection (HPLC-UV) (Lako *et al.*, 2007; Rochfort *et al.*, 2006) can be used to determine individual antioxidants in fruits, vegetables and other plant extracts. High performance liquid chromatography-diode array detector-ion trap mass spectrometry (HPLC-DAD-MS/MS) is becoming more widely available and is being used for both structure elucidation of complex antioxidants, e.g. conjugated flavonols, as well as quantification of targeted antioxidants, provided that suitable reference standards are available. Recent examples include identifying highly glycosylated acetylated flavonoids present in cauliflower, broccoli and Pak Choi (Llorach *et al.*, 2003; Vallejo *et al.*, 2004; Rochfort *et al.*, 2006) and the identification and quantification of quercetin glycosides in onions (Caridi *et al.*, 2007).

This paper describes analytical methods for determining naturally occurring antioxidants that are suitable for routine use in South Pacific regional laboratories. Data from our laboratories is used to demonstrate the range and applicability of the various procedures. These include a comparison between TEAC data generated for a variety of food grown in Fiji and Australia with literature values for the FRAP and ORAC procedures, the determination of the TPP and TAT of a selection of fresh Australian produce, the determination of individual antioxidants, e.g. β-carotene in broccoli and quercetin and kaempferol in brown onions by HPLC-UV, the quantification of gallocatechin and epigallocatechin and the tentative identification of flavonol – sugar conjugates and anthocyanidins in white clover flower by HPLC-DAD-MS/MS.

## MATERIALS AND METHODS

### *Samples and sample preparation*

#### Total antioxidant capacity (TAC): TEAC, TPP and TAT assays

Representative samples of the same variety or cultivar were purchased on the same day from at least three different supermarket chains located in Werribee and Hoppers Crossing, Victoria, Australia. Approximately 2 kg of each sample type was purchased from each site and prepared by compositing all halves of large items, 1-2 pieces of smaller produce and about 15-20 leaves picked at random. The edible portions were washed, diced or cut into smaller pieces, mixed well and assayed immediately after preparation.

#### Sample extraction

Samples were extracted according to the method of Sellappan *et al.* (2002) with some modifications. Briefly, approximately 10 g of homogenised food was mixed with 45 ml of acetonitrile containing 4% acetic acid and blended for 5 min with a hand held Bamix blender. The mixture was mechanically shaken for 30 min and then centrifuged at 5100 g for 15 min at 10°C. The supernatant (water-soluble fraction) was recovered and the pulp was further washed with 45 ml of acetonitrile containing 4% acetic acid and centrifuged. The resulting supernatants were combined and the volume adjusted to 100 ml with 95% ethanol. The solution was filtered before analysis.

#### Chemicals and reagents

β-carotene, epicatechin, epigallocatechin, gallocatechin, gallic acid, tert-butylhydroquinone (TBHQ), butylated hydroxytoluene (BHT), 2,2'-azinobis (3-ethyl-benzothiazoline-6-sulphonate) (ABTS), trolox C ((±)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), HPLC grade water/0.1% formic acid and methanol/0.1% formic acid were obtained from Sigma Chemical Co. (Castle Hill, NSW, Australia). Hydrochloric acid, triethylamine and potassium chloride were obtained from BDH Chemicals (Kilsyth, Victoria, Australia). Ammonium acetate, sodium acetate and potassium persulphate were obtained from Ajax Chemicals (NSW, Australia). All other solvents were either AR or HPLC grade and were obtained from Mallinckrodt Chemical (Australia).

#### Total antioxidant capacity (TAC)

Briefly, $ABTS^{\bullet+}$ radical cation was produced from the reaction of 7 mM 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and 2.45 mM potassium persulfate after incubation at room temperature in the dark for 16 h. The $ABTS^{\bullet+}$ solution was diluted with 95% ethanol to an absorbance of 0.70 ± 0.02 at 734 nm. For linearity, the trolox standards of different concentrations from 0.06 mg/ml to 0.12 mg/ml were prepared to give 20 – 80% inhibition of the blank absorbance. The $ABTS^{\bullet+}$ solution (2000 µl; absorbance of 0.70 ± 0.02) was read at 734 nm before 200 µl of the sample extract was added, mixed for 10 seconds and the absorbance continuously taken at 10 seconds intervals up to 1.0 min. Trolox

equivalent antioxidant capacity of the sample was calculated based on the inhibition exerted by standard Trolox solution at 1 min and expressed as milligrams of trolox equivalent antioxidant capacity (TEAC) per 100g fresh weight.

**Total polyphenol (TPP)**

Briefly, 0.5 ml of the extract and gallic acid standard solutions were mixed with Folin-Ciocalteu's Reagent (FCR – 1:10 dilution) and left to stand for 8 min at room temperature to allow the FCR to react completely. 2.0 ml of 7.5% sodium carbonate in water was added to destroy the excess reagent. The absorbances were read at 760 nm using a Shimadzu UV-vis spectrophotometer after incubating at room temperature for 2 h. Results are expressed as milligrams of gallic acid equivalents per 100 g fresh weight.

**Total anthocyanin (TAT)**

The TAT content of the food was estimated by the pH-differential method using two buffer systems: potassium chloride buffer, pH 1.0 (0.025 M) and sodium acetate buffer, pH 4.5 (0.4 M). Briefly, 0.4 ml of the extract was mixed with 3.6 ml of corresponding buffers and read against a blank at 510 nm and 700 nm. Absorbance (A) was calculated as: $A = (A_{510} - A_{700})_{pH\ 1.0} - (A_{510} - A_{700})_{pH\ 4.5}$. Monomeric anthocyanin pigment concentration in the extract was calculated as cyanidin-3-glucoside (mg/l) = A × MW × DF × 1000/(MA x 1) where A = absorbance, MW = molecular weight (449.2); DF = dilution factor, MA = molar absorptivity (26,900). The TAT content was expressed as cyanidin-3-glucoside (mg/100g).

**Quercetin and kaempferol**

Brown onions were freeze dried and crushed to a powder. One gram of the powder was mixed with 40 ml of 62.5% methanol/water containing 2 g/l TBHQ. Ten millilitres of 6 M HCl was added and the mixture refluxed for 2 h. The mixture was cooled, made to 100 ml with methanol, mixed thoroughly, sonicated for 5 min, filtered through a 0.45 µm filter disc and assayed by HPLC.

**β-carotene**

Broccoli florets were freeze dried and crushed to a powder. Then, 100 ml of hexane:acetone:ethanol (50:25:25) and 9 ml of deionised water was added to 0.5 g of the powder and the mixture shaken for 10 min using a wrist-shaker. A further 15 ml of deionised water was added and the mixture shaken for a further 5 min. The mixture was transferred to a separating funnel, the phases separated and the aqueous phase extracted with a further 20 ml of hexane:acetone:ethanol (50:25:25). The organic phases were combined, made to volume (1000 ml) with hexane and dried with anhydrous sodium sulphate. A 10 ml aliquot was removed and reduced to 1 ml with a stream of nitrogen. The extract was diluted to 5 ml with the HPLC mobile phase and filtered through a 0.45 µm nylon filter disc before analysis.

**Epigallocatechin and gallocatechin**

White clover flowers were frozen at -80°C, freeze dried and crushed to a powder. The sample (5 mg) was weighed into a 2 ml microfuge tube and 0.5 ml 80% methanol/water added. The mixture was allowed to stand at room temperature for 20 min after which time the tube was centrifuged and the supernatant removed. This extraction was repeated twice more to give a total extract volume of 1.5 ml. This volume was sufficient for flavonol analysis (method 1) but not for the quantification of epigallocatechin (EGC) and gallocatechin (GC) (method 2). For this assay, the extract was dried with gentle warming under a stream of nitrogen and reconstituted in 200 µl 80% methanol/water.

***Analyses***

**β-carotene**

The experiments were performed with a Waters Alliance 2695 HPLC equipped with Waters 626 photodiode array detector (Waters, MA). The autosampler was maintained at 4°C. The compounds were separated using a 250 mm × 2.1 mm i.d. Spherisorb 5 µm ODS2 C18 column maintained at 40°C. The mobile phase consisted of a mixture of acetonitrile: 0.05 M ammonium acetate: dichloromethane: triethylamine (75:20:50.05) containing 0.1% BHT. The flow rate was 2 ml/min and β-carotene was monitored at 448 nm. Waters Millennium software was used to process the data.

**Quercetin and kaempferol**

The experiments were performed with a Waters 600E HPLC pump (Waters, MA) equipped with an Agilent series 1100 autosampler and photodiode array detector (Agilent Technologies, Waldbronn, Germany). The compounds were separated using a 250 mm × 4.6 mm i.d. Hypersil 5 µm C18 column fitted with a C18 guard column and a mobile phase consisting of two components: A (1% formic acid/99% water) and B (1 % formic acid/49% water/50% methanol); with the following gradient: 0-4 min, 10% B, 4-21 min, 10-100% B, 21-41 min, 100% B, 41-42 min 100-10% B, 42-46 min, 10% B and a flow rate of 1 ml/min. The flavonols were detected at 370 nm. Aglient Chemstation software was used to process the data.

***Conjugated flavonols, anthocyanidins and catechins in white clover flowers***

**Method 1**

The experiments were performed with an Agilent 1100 series HPLC (Waldbronn, Germany) equipped with a quaternary gradient pump, column heater, cooled autosampler (4°C), and diode array detector, coupled to a Thermo Electron LTQ ion trap mass spectrometer (Waltham, MA). Separations were achieved using as a Thermo BDS Hypersil 150 mm × 2.1 mm i.d. 3 µm C18 column (Waltham, MA) maintained at 40°C and a mobile phase consisting of two components: A (water/0.1% formic acid) and B (acetonitrile/0.1% formic acid); with the following gradient: 0-5 min, 98 % A; 5-25 min, 98-62% A; 25-35 min, (0.3 ml/min) 62-98 % A and a flow rate of 0.2 ml/min. 5 µl of the neat extract was injected onto the column.

**Mass spectrometric acquisition parameters**

The instrument was run in polarity switching mode with MS/MS data acquired in both negative and positive mode. For the LC-MS/MS experiments, a data dependant protocol was used with a mass range of 150 to 2000 amu for the ESI negative mode and a mass range of 180 to 2000 amu for the ESI positive mode. Dynamic exclusion was engaged with a 20 second exclusion time. Data were acquired using automated MS/MS settings with a target of 30,000, a normalized collision energy of 35 and an ion max time of 200 ms. The heated capillary was maintained at 250°C and the sheath, auxiliary and sweep gases were at 13, 6 and 8 units respectively. Source voltage was set to 3.4 kV with a capillary voltage of –43 V for negative data acquisition and 4.4 kV with a capillary voltage of 26 V for positive data acquisition. Prior to data acquisition, the system was tuned using a 250 µg/ml standard of rutin. The rutin was infused via syringe pump through a T-piece at a rate of 5 µl/min with a HPLC flow rate of 0.2 ml/min and a solvent composition of 50% A and 50% B.

**Method 2**

The HPLC conditions were identical to those for method 1 except that the internal standard (epicatechin 450 ng final concentration) had been added to the concentrated extract. Ten micro-millilitres of this solution was injected onto the column.

**Mass spectrometric acquisition parameters**

For maximum sensitivity LC-MS/MS data was acquired in ESI negative mode with a mass range of 200 to 1000 amu only. The heated capillary was maintained at 250°C and the sheath, auxiliary and sweep gases were at 13, 6 and 8 units respectively. Source voltage was set to 3.4 kV with a capillary voltage of –50 V. Prior to data acquisition the system was tuned using a 20 µg/ml standard of epicatechin. The epicatechin was infused via syringe pump through a T-piece at a rate of 5 µl/min with an HPLC flow rate of 0.2 ml/min and a solvent composition of 50% A and 50% B. Standard curves for gallocatechin and epigallocatechin were prepared by serial dilution of a stock solution and analysed in conjunction with the samples. The results were processed using Xcalibur Quan with peak detection by mass and retention time. The detector response was linear ($R^2$=0.999) for epigallocatechin (8-285 ng on column) and gallocatechin (5-81 ng on column).

## RESULTS AND DISCUSSION

***Functional group assay: total antioxidant capacity (TAC)***

Fruits and vegetables contain many different phytochemicals with antioxidant potential. Although it is possible to measure all of the individual antioxidants, such procedures are time consuming and expensive. These antioxidants are often multifunctional. Their activity and mode of action in a particular test system may depend on the oxidation conditions, which may in turn affect both the kinetics of oxidation and the composition of the system. Therefore, a multi-dimensional assay protocol would be an advantage by reducing these limitations. The chemistry behind the antioxidant capacity assays has recently been reviewed by Huang *et al.* (2005).

The trolox equivalent antioxidant capacity (TEAC) is one of the most popular procedures to measure Total Antioxidant Capacity (TAC) due to its relative simplicity (Roginsky and Lissi, 2005). This technique is robust and applicable to both hydrophilic (water-soluble) and lipophilic (lipid-soluble) antioxidants, pure compounds and food extracts (Re *et al.*, 1999). A brief description of the method is described in the Materials and Methods section.

A comparison between the TEAC values of a selection of Fijian (Lako *et al.*, 2007) and Australian foods (this study) and the literature data for Ferric Reducing Antioxidant Power (FRAP) and Oxygen Radical Absorbance Capacity (ORAC) values for similar foods assayed elsewhere (Halvorsen *et al.*, 2002; Cao *et al.*, 1996; Wang *et al.*, 1996) is shown in Table 1. For comparison purposes, the literature values for FRAP and ORAC have been converted to the same units as those for TEAC, i.e. mg/100g. Most of the data are comparable despite the technical and chemical differences between the

**Table 1:** Comparison of TEAC values for a selection of Fijian and Australian produce with FRAP and ORAC literature values

| Food type | Literature values (mg/100g TEAC)[1,2,3] | | | |
|---|---|---|---|---|
| | TEAC | FRAP | ORAC/ROO$^+$ | ORAC/OH$^+$ |
| Fijian food | | | | |
| Chinese cabbage, steamed | 31 | 62 | 75 | 38 |
| Red eggplant, steamed | 110 | 26 | 98 | 28 |
| Tomato, steamed | 13 | 47 | 47 | – |
| Cucumber, raw | 2 | 8 | 13 | 8 |
| Carrot, raw | 2 | 6 | 53 | 20 |
| Hawaiian papaya, raw | 100 | 94 | – | – |
| Orange, raw | 38 | 173 | 188 | – |
| Mango, raw | 34 | 53 | – | – |
| Pineapple, raw | 25 | 158 | – | – |
| Banana, raw | 25 | 30 | 55 | – |
| Watermelon, raw | 1 | 6 | 24 | – |
| Apple (NZ), raw | 34 | 44 | 55 | – |
| Sweet potato, boiled | 19 | 36 | 75 | 25 |
| White cassava, boiled | 23 | 26 | – | – |
| White ginger, steamed | 320 | 572 | – | – |
| Onion, steamed | 19 | – | 113 | 13 |
| Australian food | | This study | | |
| Blueberry | 560 | 553 | – | – |
| Red cabbage | 140 | 286 | – | – |
| Red onion | 32 | – | – | – |
| Tomato | 26 | – | 47 | 47 |
| Red capsicum | 90 | 281 | – | – |
| Green capsicum | 68 | – | – | – |
| Orange sweet potato | 25 | 36 | – | – |
| White onion | 33 | 102 | – | – |
| Broccoli | 19 | 88 | 84 | – |

– data not available
[1] TEAC data for Fijian food taken from Lako *et al.* (2007)
[2] FRAP data taken from Halvorsen *et al.* (2002)
[3] ORAC data taken from Cao *et al.* (1996) and Wang *et al.* (1996)

three methods. There are some minor discrepancies (e.g. red eggplant, root spices) but these could be due to sampling of different varieties and cultivars of foods available in different localities (Prior and Cao, 2000).

**Group antioxidant assays: total polyphenols (TPP) and total anthocyanins (TAT)**

The determination of group antioxidants such as polyphenols and anthocyanins can be conducted to supplement TAC measurements. The common techniques used are the Folin-Ciocalteu method (Singleton and Rossi, 1965; Singleton *et al.*, 1999) for the measurement of Total Polyphenols (TPP) and the spectrophotometric/pH by difference method (Sellappan *et al.*, 2002) for the measurement of Total Anthocyanins (TAT). Studies have reported high correlation between TAC, TPP and TAT (Pellegrini *et al.*, 2000).

The Folin-Ciocalteu method relies on the Folin-Ciocalteu Reagent (FCR) reacting completely with the oxidisable substances in the samples. Colour development is achieved by the addition of alkali solution at the appropriate time and temperature. Gallic acid is normally used as the standard reference compound and the values are expressed as Gallic Acid Equivalents (mg/100g).

The TAT content of the food was estimated by the pH-differential method using two buffer systems, pH 1.0 and pH 4.5. The absorbance differences read at 700 nm compared to the blank (510 nm) for both solutions gives an estimation of the TAT content. The TAT content is expressed as cyanidin-3-glucoside (mg/100g).

A selection of Australian foods were analysed for TPP and TAT. All samples were analysed in triplicate in the raw state, and the mean of the three determinations reported-Table 2. Reproducibility of results within and between days was acceptable (CV=6%). Blueberry and red cabbage had high amounts of TAT content whereas blueberries, red cabbage, red and green capsicum were all high in TPP content. The data are consistent with literature values (Sellappan *et al.*, 2002; Kaur and Kapoor, 2002). There is a good correlation between the TAC and TPP values for some of the Australian foods (e.g. blueberry, TAC 560 mg/100g TEAC, TPP 310 mg/100g GAE; red cabbage, TAC 140 mg/100g TEAC, TPP 140 mg/100g GAE; red capsicum, TAC 90 mg/100g TEAC, 130 mg/100g GAE).

***Individual antioxidants: carotenoids and flavonoids***

**Carotenoids (e.g. β-carotene)**

HPLC-UV (preferably diode array) detection is the method of choice for quantifying individual carotenoids (e.g. β-carotene, β-carotene and lycopene) in plant extracts. There are a number of different extraction procedures and chromatographic conditions reported in the literature (Eitenmiller and Landen, 1999). For example, a mixture of hexane, acetone and ethanol was used by Teng-Chuen Yu *et al.* (2003) to extract caroteniods from freeze dried broccoli. The extract was assayed by HPLC-UV (diode array detector) using a 5 µm C18 HPLC column and a mobile phase consisting of 75% acetonitrile, 20% 0.05 M ammonium acetate in methanol, 5% dichloromethane, 0.05% triethylamine and 0.1% BHT. β-carotene was detected at 450 nm. The level of β-carotene in broccoli was 76 mg/100g with a 91% recovery of added β-carotene. Lycopene, lutein and β-carotene can also be separated in fruit and vegetable extracts using these conditions. More recently, Lako *et al.* (2007) used acetone to extract lycopene, α-carotene and β-carotene from a wide variety of Fijian foods. The carotenoids were separated on a 5 µm C18 reverse phase HPLC column with a mobile phase consisting of 95% methanol and 5% tetrahydrofuran. Excellent recoveries (96-99%) were also achieved with this procedure.

**Flavonols (e.g. quercetin and kaempferol)**

HPLC-UV (diode array detector) is also used to separate and measure the levels of flavonols (as their aglycones) in plant extracts. The flavonols (e.g. myricetin, quercetin, isorhamnetin and kaempferol) are often present as conjugated glycosides. Pure reference standards of these compounds are not readily available and it is therefore necessary to hydrolyse the flavonol-sugar conjugates to the aglycones with boiling methanolic hydrochloric acid for 2-4 h prior to analysis (Hertog *et al.*, 1992a; Hertog *et al.*, 1992b). These conditions are applicable to a wide range of plant types and matrices. Typically, the flavonols are separated using 5 µm C18 HPLC column and a gradient elution comprising a mixture of water and methanol containing 1% formic acid and measured at 370 nm. The flavonol content of a selection of Australian foods were determined by this procedure and the levels compared favorably with the levels reported elsewhere (USDA, 2003), e.g. brown onion:

**Table 2:** Total polyphenol and total anthocyanin contents of a selection of Australian food

| Food | Total Polyphenol content (TPP) | Total Anthocyanin content (TAT) |
|---|---|---|
| Blueberries | 310 ± 42 (lit. 288-929)[1] | 14 ± 0.1 (lit. 16-197)[1] |
| Red cabbage | 140 ± 1 | 5 ± 0.3 |
| Red onion | 84 ± 0.1 | 0.4 ± 0.0 |
| White onion | 66 ± 0.1 | 0.1 ± 0 |
| Baby spinach leaves | 74 ± 3 | 0.3 ± 0.2 |
| Pumpkin | 22 ± 1 | 0.3 ± 0.0 |
| Tomato | 35 ± 2 | 0.2 ± 0.0 |
| Red capsicum | 130 ± 16 (lit. 67)[2] | 0.1 ± 0.0 |
| Green capsicum | 120 ± 3 | 0.1 ± 0.0 |
| Carrot | 18 ± 1 (lit. 55)[2] | 0.3 ± 0.0 |
| Orange sweet potato | 36 ± 1 | 0.1 ± 0.0 |
| Broccoli | 78 ± 2 (lit. 88)[2] | 0.4 ± 0.1 |

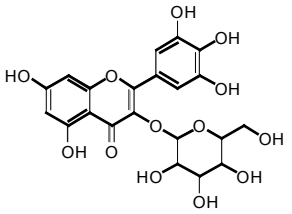
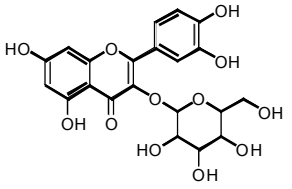
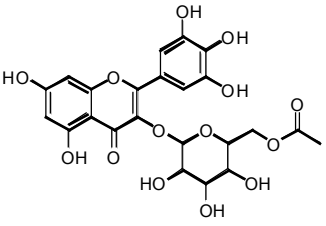
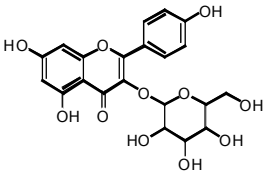
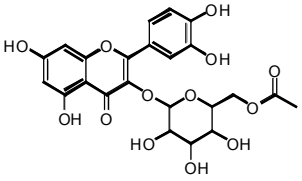
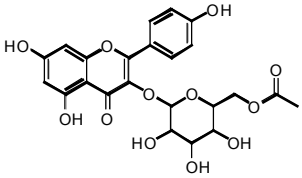
TAT expressed as cyanidin-3-glucoside, mg/100g wet weight
TPP expressed as gallic acid equivalent (GAE), mg/100g wet weight
[1] Sellapan et al. (2002)
[2] Kaur and Kapoor (2002)

quercetin 19 mg/100g, kaempferol 2.5 mg/100g; USDA, quercetin 1.5-41 mg/100g, kaempferol 0-2.4 mg/100g (Caridi *et al.*, 2007).

**Conjugated flavonols (e.g. anthocyanidins and catechins)**

HPLC-DAD-MS/MS using electrospray – ion trap mass spectrometry is a very versatile technique as it not only provides structural information though the $MS^n$ and UV spectral data but is also suitable for quantifying low levels of compounds in complex matrices, provided pure standard compounds are available. Recent examples include those of Llorach *et al.* (2003), Vallejo *et al.* (2004) and Rochfort *et al.* (2006) who identified large numbers of highly glycosylated-acetylated flavonoids in cauliflower, broccoli and Pak Choi respectively. To further demonstrate this capability, we used the technique to tentatively identify a number of flavonol glycosides and anthocyanidins present in a methanol/water extract of freeze dried white clover flowers. The spectral data are displayed in Table 3 and the UV and TIC chromatograms for the flavonol glycosides are displayed in Figure 1. Each compound showed a

**Table 3:** Flavonol glycosides identified in white clover flowers by HPLC-DAD-MS/MS

| Retention Time /min | UV nm | m/z | Structure |
|---|---|---|---|
| 21.2 | 356, 262 | 479, 316/7 |  |
| 22.7 | 354, 266 | 463, 301 |  |
| 23.6 | 350, 266 | 521, 479, 316/7 |  |
| 24.1 | obscured | 447, 325, 284/285 |  |
| 25.2 | 354, 256 | 505, 463, 300/301 |  |
| 26.1 | 320, 296 | 489, 461, 447, 429, 357, 327, 284/5 |  |

characteristic $\lambda_{max}$ and a characteristic molecular ion $[M-H]^-$ with subsequent loss of either a sugar unit (-162 amu) or an acetate unit (-42 amu) followed by a sugar unit (-162 amu). The aglycone structures were confirmed to be myricetin, quercetin and kaempferol by acid hydrolysis of the freeze dried powder (Rochfort *et al.*, 2006). The stereochemistry of the sugar unit and the position of the acetate moiety were not established. However it is likely that the connectivity is as shown and that the sugars are galactose units (Foo *et al.*, 2000).

The two anthocyanidins were tentatively identified as delphinium sambubioside (m/z 597, 355, 300/301) and cyanidin 3-sambubioside (m/z 581, 339, 284). The structure of delphinium sambubioside is displayed in Figure 2. Gallocatechin and epigallocatechin were also quantified (0.7 µg/mg and 0.8 µg/mg respectively) in the sample. A 94% recovery of epigallocatechin, added to the sample matrix before extraction, was achieved using this procedure.

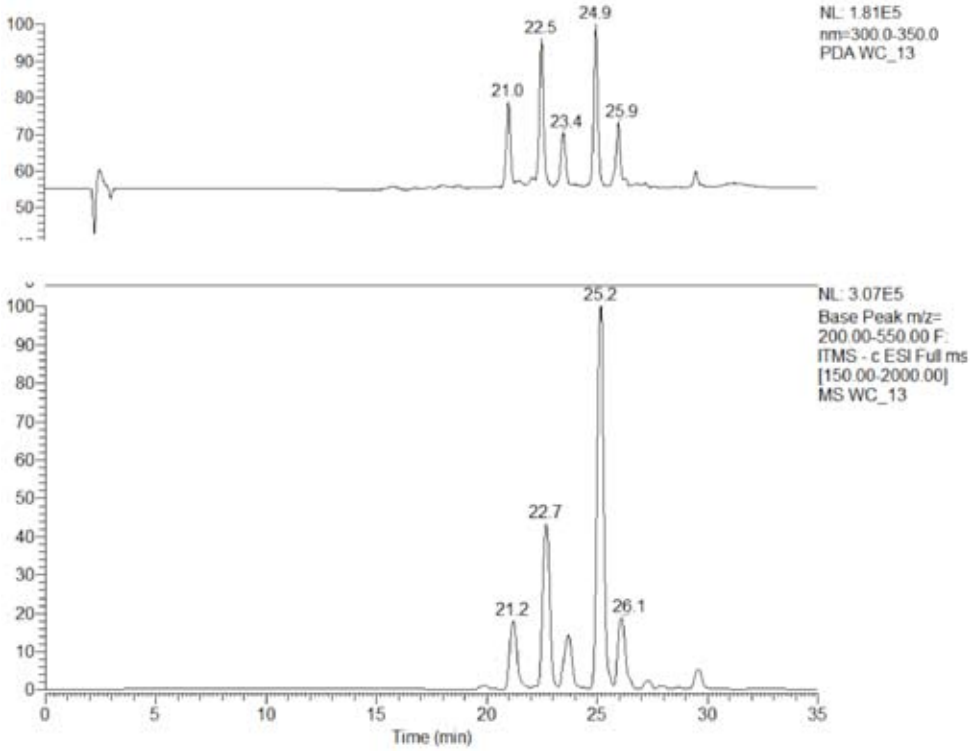



**Figure 1:** UV and TIC chromatograms of white clover flower extract using the conditions described in the Material and Methods. The retention times correspond to the compounds listed in Table 3

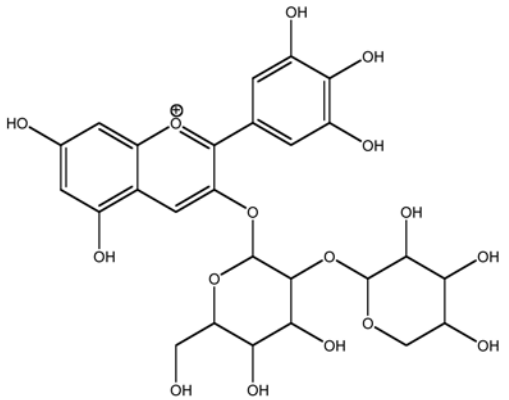


**Figure 2:** delphinidin 3-sambubioside

## CONCLUSION

The generation of reliable antioxidant data in South Pacific regional laboratories is dependent on straight forward fully validated analytical methods. The Total Antioxidant Capacity (TAC) of food can be determined using a variety of methods, e.g. TEAC, ORAC, FRAP, TRAP. Similar values are obtained from all procedures, however, a standardized method, e.g. TEAC, would be useful for a direct comparison of the data. Total Polyphenol (TPP) and Total Anthocyanin (TAT) values are readily determined by wet chemical assays and these data can be used to supplement TAC measurements. HPLC-UV (preferably photodiode array) detection is suitable for quantifying individual antioxidants, e.g. carotenoids and flavonols and HPLC-DAD-MS/MS, in particular ion trap mass spectrometry, provides structural information of unknown compounds as well as quantifying low levels of individual antioxidants. All of these methods are suitable for routine use in South Pacific regional laboratories.

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