ANTIOXIDANTS IN FOOD SYSTEMS – INFLUENCING FACTORS

A thesis submitted for the degree of

Doctor of Philosophy

By

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Declaration

I, Katherine Elizabeth Ann Stockham, declare that this thesis entitled "Antioxidants in food systems – influencing factors" is no more than 100,000 words in length, exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. This research has been conducted in collaboration with a number of partners, whose contributions have been appropriately acknowledged throughout. Thus, except where otherwise indicated, this thesis is my own work.

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PART A:

DETAILS OF INCLUDED PAPERS: THESIS BY PUBLICATION

Please list details of each Paper included in the thesis submission. Copies of published Papers and submitted and/or final draft Paper manuscripts should also be included in the thesis submission

Item/ Chapter No.	Paper Title	Publication Status (e.g. published, accepted for publication, to be revised and resubmitted, currently under review, unsubmitted but proposed to be submitted)	Publication Title and Details (e.g. date published, impact factor etc.)
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6	Antioxidant synergies in food systems - in vitro versus ex vivo studies on eight different classes of chemical components representative of navel oranges	Submitted	**Food Chemistry, Impact factor (4.529)**

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Abstract

For well over 20 years the Oxygen Radical Absorbance Capacity (ORAC) assay has been an important research tool in identifying antioxidant candidates in food and serum samples. The ORAC value is derived from a series of fluorescence intensity measurements taken over a period of time, usually a few hours. The kinetics of this reaction is controlled by the sustained release of peroxyl radicals at 37°C by an azo compound (AAPH). The peroxyl radicals attack the fluorescent probe causing a gradual decrease in fluorescence intensity. Ultimately, the test yields fluorescence decay curves over time, with the presence of any antioxidants in a sample having a protective effect, delaying probe decay and resulting in a greater area under the decay curve. This forms the basis of the ORAC measurement.

Concerns have been raised about the applicability of this assay, chemical interferences influencing the data, and the use of ORAC values to attribute health benefits of foods. In an effort to curb misrepresentation of health benefits from ORAC data, the USDA retracted its ORAC database in 2012 and published a statement by Dr Ronald Prior, a founding researcher for the ORAC assay. The explanatory statement by Dr Prior identified the misapplication of ORAC values and misleading perceived health benefits as major reasons for withdrawal of the database.

Despite this, ORAC remains a popular assay, and the method is widely used as a product development and marketing tool. Some researchers have reported that ORAC values can be influenced by factors such as choice of solvent, chemical constituents in matrices, and pH conditions. However, little has been published on antioxidant synergies in food systems and their contribution to the ORAC value.

Given the predisposition of the assay to interferences, a better understanding of antioxidant synergies is necessary to understand the contributing factors to measurement in real foods, and also to determine how these values may be manipulated. With the USDA caution in mind, this research was carried out to systematically investigate the factors influencing the ORAC measurement and its interpretation.

Firstly, an alternative mode of reporting antioxidant activity to consumers on product labeling was proposed. Calculations needed to convert ORAC units from μM Trolox equivalents (μM T.E/kg or μM T.E/L) to mass units (g T.E/kg or g T.E/L) of Trolox equivalents per kg or per L of foodstuff are provided. We propose that mass units are less misleading to consumers, by not reporting very large and impressive-looking values when a simple conversion reveals most of them to be much more modest when viewed as a mass of vitamin E equivalent. For example, the antioxidant activity of blueberries when measured by the ORAC assay was equivalent to over 71,000 μM of Trolox equivalents. When converted, the blueberries can be said to have the same antioxidant activity as 17.9g of vitamin E per kg of fruit (17.9g T.E/kg). This new mode of reporting was successfully applied to a range of commodities including fruit, confection and beverages.

Influencing factors, including environmental conditions, role of additives and nanoparticles and interactions between classes of chemical constituents were all investigated. Environmental conditions, specifically rainfall, were found to influence the levels of antioxidant compounds/bioactives in Australian wines. Six chemical constituents were identified as warranting further investigation; namely 6-methylcoumarin, protocatechuic acid, vanillic acid, p-coumaric acid, rutin and chlorogenic acid. Significant differences were also observed between the antioxidant capacity of wines by *in vitro* ORAC and *ex vivo* CAA-RBC assays, where wines with similar ORAC values had vastly different bioavailability and activity in the cellular system.

Amino acids and CuNPs additives were found to greatly influence the antioxidant measurements of "superfoods". Results indicated strong enhancements and synergies related to the properties of the amino acids and complexes formed with Cu(I) and essentially matrix independent.. The order of antioxidant enhancement in bilberry, coffee berry, and apple concentrates was Tryptophan > Tyrosine > Methionine ≥ Histidine ≥ 4-Hydroxyproline. This order was also consistent with the order of calculated bond dissociation energies (BDEs), reflecting the inherent antioxidant potentials of the amino acids studied. Density Functional Theory (DFT) was used to support a proposed "substrate

zone" and "antioxidant zone" postulate for amino acids and related additives and this concept assists in demonstrating potential mechanisms involved in achieving such extraordinary enhancements and synergies. Histidine was used as a model system for DFT calculations, and allowable species had homolytic BDEs ranging from high (deactivated) to very low (activated), in the case of species (b) the BDE was at a level well below that of vitamin E, making it an excellent and potentially potent antioxidant. DFT calculations revealed that the histidine-Cu(I) complex had a comparable BDE to that of Trolox, again demonstrating how interactions between chemical constituents can influence, and in this case enhance antioxidant activity measurements.

Synergies and antagonisms were also reported for eight classes of chemical constituents typically found in navel oranges. These mixtures were prepared based on the levels reported in nutritional data tables, and analyzed by ORAC and CAA-RBC assays. A correlation analysis revealed that the ORAC and CAA-RBC data did not correlate overall, however distinct clustering and several interesting outliers were noted. Cluster (a) had low ORAC and low CAA-RBC values, involving combinations of preservatives, sugars and CuNPs. Cluster (b) had low to moderate responses in both assays, and was made up primarily of vitamins in combination with CuNPs, preservatives, sugars and flavonoids. Cluster (c) was dominated by phenolics and their interactions with a number of groups, which gave high antioxidant activity in both ORAC and CAA-RBC assays, and amino acids are the main contributors in cluster (d). Organic acids featured in both outliers, firstly with a high antioxidant activity in both assays when combined with polyphenolics, and secondly as having an auto-oxidation effect in the CAA-RBC assay but a high ORAC value when analysed individually. Antioxidant activities of individual mixtures and combinations of classes of compounds showed antagonism/suppression of antioxidant activity between sugars and vitamins, and between polyphenolics and flavonoids in the ORAC assay. However, these same solutions resulted in antioxidant synergy in the CAA-RBC assay. In fact, the auto-oxidation effect of organic acids was reversed and synergies were noted in interaction with polyphenolics. A number of synergisms *ex vivo*

involved polyphenolics in combination with other constituents such as vitamins, the amino acid Tryptophan, preservatives and CuNPs. These findings support the postulate that interactions at the "substrate zone" are influencing factors of antioxidant capacity at the molecular level. Computational chemistry was used to postulate mechanisms for antioxidant synergy, activation and deactivation of phenolic O-H groups, using quercetin and (-)-epicatechin-3-gallate as examples.

It was concluded that factors including rainfall, amino acid and CuNPs addition, and interactions between common classes of food constituents influenced antioxidant activity in food systems. Computational chemical calculations were used to postulate mechanisms for antioxidant enhancement and synergy, a major influencing factor in antioxidant measurements. This research describes the potential for unlocking new and powerful antioxidant synergies in food systems, nutrition and health and the medical/pharmaceutical fields.

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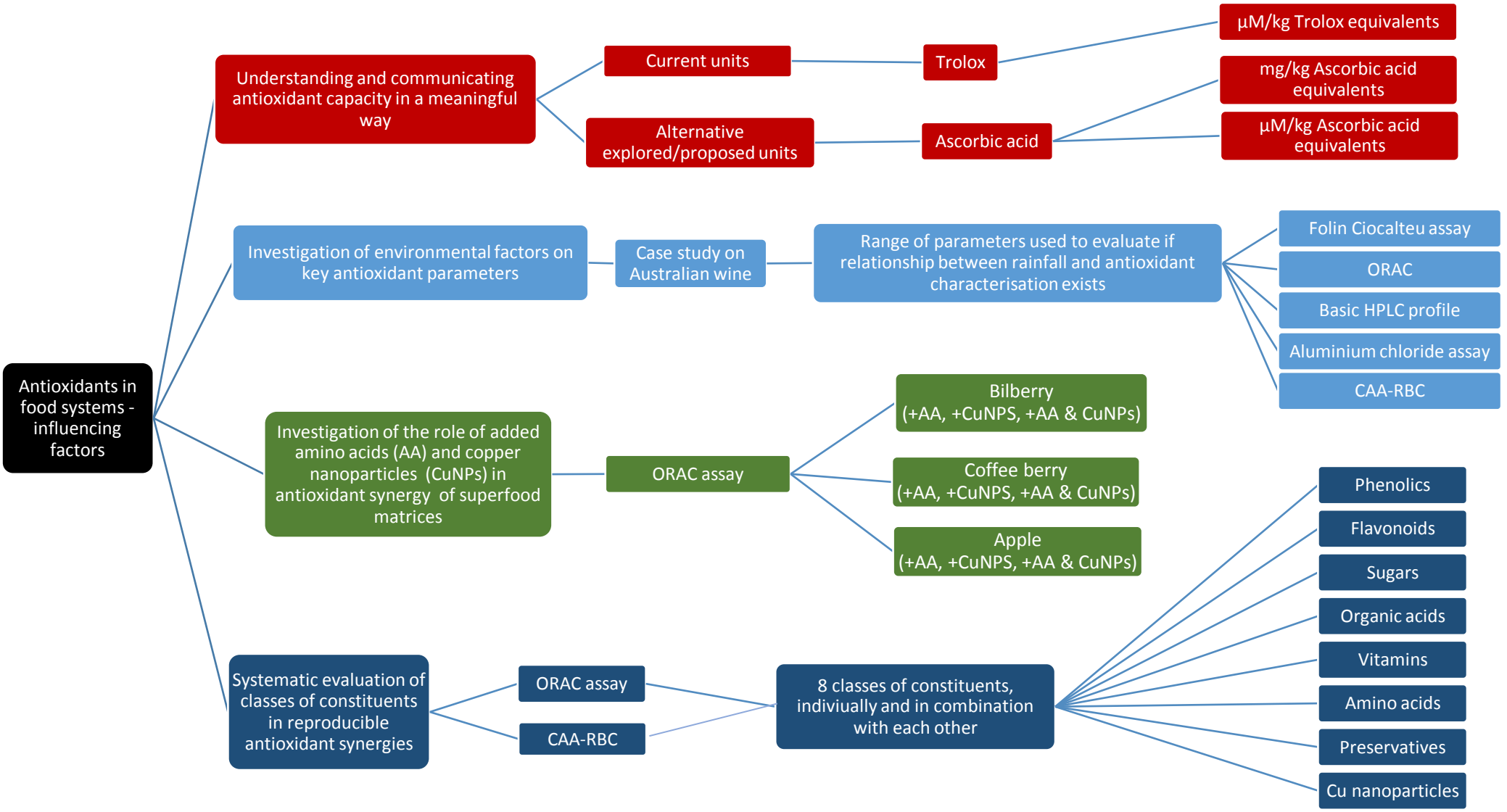
Conceptual Framework



Publications, presentations and professional committees relevant to the scope of this thesis

Refereed Journal Publications

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Stockham, K., Sheard, A., Paimin, R., Buddhadasa, S., Duong, S., and Murdoch, T., 2013, *Comparative studies on the antioxidant properties and bioactive content of wine from different growing regions and vintages*, **Food Chemistry**, 140(3):500-6

Stockham, K., Paimin, R., Buddhadasa, S., and Orbell, J.D., 2017, *Antioxidant synergies in superfoods – role of amino acids and copper nanoparticles in influencing the Oxygen Radical Absorbance Capacity (ORAC) of fruit concentrate powders*, **Food Chemistry**, (submitted)

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Conferences

Australian Food Metrology Symposium (AFMS) (2012), Melbourne, Australia

Symposium Organizer and Participant

Platform Presentation "Reporting food antioxidant capacity – a measurement challenge"

Australian Food Metrology Symposium (AFMS) - Antioxidants Workshop (2012), Melbourne, Australia

Symposium Organizer and Forum Leader

Platform Presentation "Australian case studies and antioxidant measurement issues"

9th International Food Data Conference (9th IFDC) (2011), Norwich, UK

Platform Presentation "Comparative studies on the antioxidant properties and bioactive content of wine from different growing regions and vintages"

12th Government Food Analysts Conference (2011), Brisbane

Platform Presentation "Assessment of automated techniques for performing the oxygen radical absorbance capacity (ORAC) assay, total polyphenolics assay and total flavonoids assay"

Symposium on Antioxidant Measurement (2009), Melbourne

Symposium Organizer and Forum Leader

Workshop on Analysis by Emitted Light (ABEL) Assay for antioxidants (2009), Melbourne

Workshop Organizer and Participant

8th International Food Data Conference (2009), Bangkok, Thailand

Platform Presentation "Evaluation of ascorbic acid as a standard for the Oxygen Radical Absorbance Capacity Assay"

11th Government Food Analysts Conference (2009), Melbourne

Platform Presentation "Investigation of the causes and extent of matrix effects on the Oxygen Radical Absorbance Capacity Assay"

1st Australia OCEANIAFOODS Food Composition course and Meeting (2009), Sydney

Platform Presentation "Principles of ORAC Measurements"

21st Conference of Residue Chemists (2007), Melbourne

Poster Presentation "Oxygen Radical Absorbance Capacity of Benzoic Acid Preservative"

10th Government Food Analysts Conference (2007), Melbourne

Poster Presentation "Analysis of Total Antioxidant Capacity in Common Foods and Beverages Using the Oxygen Radical Absorbance Capacity Assay"

Relevant Professional Committees/Memberships

2012	AOAC Stakeholder Panel for Antioxidants – Member
2012	AOAC Stakeholder Panel for Flavonoids – Member

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Finally, I would like to thank my husband, Jamie, and my grandparents, Ralph and Patience Webb. Words will never be enough to describe how much your love and support has meant to me and how critical it was in helping me to keep going.

Dedication

I wish to dedicate this thesis to my grandparents, Ralph and Patience Webb; and to my husband Jamie Linsdell, for whom I am so thankful and truly blessed.

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4OHC	4-Hydroxycoumarin
4-OH-Pro	4-Hydroxyproline
6-MC	6-Methylcoumarin
A·	Antioxidant radical species
AA	Amino acid
AAC	Area above the decay curve
AAPH	2,2'-azobis(2-amidinopropane) dihydrochloride
ABAP	2,2'-azobis (2-amidinopropane) dihydrochloride
ABTS	2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid)
AES	Aesculin
AFMS	Australian Food Metrology Symposium
AH	Antioxidant
AOAC	Association of Official Analytical Chemists
AOx	Antioxidants in extracts applied to cells
AUC	Area under the decay curve
AWA	Acetone:Water:Acetic acid solution
BDE	Bond dissociation energy
BOM	Bureau of Meteorology, Australia
C	± Catechin
CA	Chlorogenic acid
CAA	Cellular antioxidant activity (HepG2 tumour cell line)
CAA-RBC	Cellular antioxidant activity – red blood cells
CAFF	Caffeine
CE	Catechin equivalents
CFA	Caffeic acid
CG	Catechin gallate
Cin.A	Cinnamic acid
CSIRO	Commonwealth Scientific Industrial Research Organisation
CT	Cycle time (minutes)
CuNPs	Copper nanoparticles (colloidal solution)
DAD	Diode array detector/detection
DCFH	2',7'-dichlorofluorescin
DCFH-DA	2',7'-dichlorofluorescin diacetate
DF	Dilution factor
DFT	Density functional theory
DPPH	2,2-Diphenyl-1-picrylhydrazyl
EC	Epicatechin
ECG	Epicatechin gallate

EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
ET	Electron transfer
FA	Formic acid
FC	Folin-Ciocalteu reagent
FER	Ferulic acid
FL	Fluorescein
Fla	Flavonoid(s)
FRAP	Ferric reducing antioxidant power
FSANZ	Food Standards Australia and New Zealand
GA	Gallic acid
GAE	Gallic acid equivalents
GC	Gallocatechin
GCG	Gallocatechin gallate
GFAC	Government Food Analysts Conference
HAT	Hydrogen atom transfer
HCl	Hydrochloric acid
HES	Hesperetin
His	Histidine
His/Cu(I)	Histidine-copper(I) complex
HPLC	High performance liquid chromatography
IFDC	International Food Data Conference
INMU	Institute of Nutrition, Mahidol University, Thailand
KAEM	Kaempferol
L·	Lipid radical species
LH	Substrate
LOO·	Peroxyl radical species
LUT	Luteolin
MeOH	Methanol
Met	Methionine
MYR	Myricetin
NA	Not applicable
NAR	Naringenin
ND	Not detected
NDL	Nutrient data laboratory
NIP	Nutrition information panel
NMIA / NMI	National Measurement Institute, Australia
NT	Not tested
NUTTAB	Nutritional data tables
ORAC	Oxygen radical absorbance capacity
$ORAC_{(Hydro)}$	Hydrophilic ORAC extract fraction

$ORAC_{(Lipo)}$	Lipophilic ORAC extract fraction
$ORAC_{(TAC)}$	ORAC total antioxidant capacity
PA	Protocatechuic acid
PB	Phosphate buffer
PBS	Phosphate buffer saline
PCA	p-Coumaric acid
PP	Polyphenol(s)
Q.E	Quercetin equivalents
QCE	Quercetin
QCI	Quercitrin
R_2N_2	AAPH radical generator in the ORAC assay
RBC	Red blood cell
RFU	Relative fluorescence units
RMCD	Randomly methylated β-cyclodextrin
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RRLC	Rapid resolution liquid chromatography
RSD	Relative standard deviation
RUT	Rutin
SA	Syringic acid
SCO	Scopoletin
SOI	Southern oscillation index
T.E	Trolox equivalents
T.E	Trolox equivalents
TAC	Total antioxidant capacity
TEAC	Trolox equivalent antioxidant capacity
Tryp	Tryptophan
Tyr	Tyrosine
USDA	United States Department of Agriculture
VA	Vanillic acid
VEA	Vitamin E analogue (Trolox)

1.0 Introduction

General introduction

The investigation of antioxidant capacity is a complex and extensive pursuit. Given their important role in human health and food preservation, antioxidants are often used to promote dietary supplements, neutraceuticals and functional food ingredients (Shahidi and Zhong, 2015). Historically, spices were used to extend the shelf life of foods in warm climates and, gradually, intentional addition of synthetic and natural antioxidants to food became commonplace (Shahidi, 2015).

Introduction to food antioxidants

Antioxidants are found naturally in many foods, and are also added during food processing to delay, control or inhibit lipid oxidation and subsequent food spoilage(Gemili, Yemenicioğlu and Altınkaya, 2010; Oliveira *et al.*, 2011; Shahidi, 2015; Shahidi and Zhong, 2015). Lipid oxidation can be initiated by a number of factors including heat, light, presence of metals and metalloproteins, enzymes and microorganisms, as reported by Shahidi and Zhong (2015). In food systems, this type of oxidation gives rise to unpleasant aromas and flavours as well as loss of important nutrients, amino acids, vitamins and bioactives, reducing shelf life and quality (Shahidi, 2015).

Food antioxidants can be classified based on their mechanism of action as either primary or secondary. Primary antioxidants act sacrificially via hydrogen atom transfer

(HAT) or electron transfer (ET) to neutralize radical species, while secondary antioxidants intercept pro-oxidant catalysts including metal ions (Shahidi, 2015).

Lipid oxidation in biological systems can also have a wide range of cellular effects and has been implicated in a number of serious diseases and inflammatory conditions (Puca *et al.*, 2013; Siti, Kamisah and Kamsiah, 2015). Due to these reports, much has been published on the antioxidant capacity of fruits, vegetables and other plant extracts including tea, herbal and traditional medicines. To a large extent, research appears to be focused on discovering excellent dietary sources of antioxidants to help protect cellular systems from excess radical species (Gramza and Korczak, 2005; Tripoli *et al.*, 2007; Pérez-Jiménez *et al.*, 2008; Huang, Majumder and Wu, 2010; Prior, 2015). The perceived health benefits of foods purported to be high in antioxidants has become increasingly well publicized and so-called "superfoods" and other marketing campaigns are becoming more commonplace.

Assays for determining antioxidants

Considering the importance of lipids in food quality and also biological systems, a range of total antioxidant capacity assays have been developed and reported in the literature (Pérez-Jiménez *et al.*, 2008; Oroian and Escriche, 2015). Below (Table 1.1) is a summary of the main antioxidant capacity assays as reported by Shahidi and Zhong (2015). Of these, ORAC, FRAP, TEAC and DPPH are the most widely reported in literature.

Table 1.1: Major reactive oxygen species (ROS) scavenging – and redox potential-based assays (Shahidi and Zhong, 2015)

Assay	Primary Mechanism	Oxidant	Probe	Detection
ORAC	HAT	Peroxyl radical generated by AAPH	Fluorescein	Fluorometry
TEAC	ET	ABTS radical	ABTS radical cation	Spectrophotometry
FRAP	ET	Fe^{3+}	Ferricyanide	Spectrophotometry
DPPH scavenging	ET	DPPH radical	DPPH radical	Spectrophotometry
Chemiluminescence	HAT	Hydrogen peroxide	Luminol	Fluormetry
CUPRAC	ET	Cu^{2+}	Neocuproine	Spectrophotometry
Ag+ reducing	ET	Ag^{+}	Ag nanoparticle	Surface plasmon resonance
Au+ reducing	ET	Au^{3+}	Au nanoparticle	Cyclic voltammetry
CERAC	ET	Ce^{4+}	Indigo carmine dye	Spectrophotometry
CHROMAC	ET	Cr^{6+}	Cr^{3+} complex	Spectrophotometry

AAPH, 2,2'-azobis(2-amidinopropane) dihydrochloride; DPPH, 2,2-Diphenyl-1-picrylhydrazyl; ABTS, 2.2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid), HAT = hydrogen atom transfer; ET = electron transfer

Oxygen radical absorbance capacity (ORAC) assay

With so much interest in the role of antioxidants in human health and combating disease, the international focus tends to be on two main areas. Firstly, on the medical applications of antioxidants in the treatment of chronic and inflammatory disease and cancer (Siti, Kamisah and Kamsiah, 2015) and, secondly, on the food applications and in particular the use of food as a source of antioxidants for prevention of disease (Prior, 2015). In fact, to carry out a literature search on antioxidants often yields research in one or both of these areas (Zhang and Omaye, 2001; Hou *et al.*, 2004; Kim, Wilson and Lee, 2010).

Due to the costly and lengthy nature of clinical trials, the use of *in vitro* methods is prevalent when trying to determine suitable phytochemical candidates for *in vivo* trials. However, it wasn't until the early 1990's when a new *in vitro* method for measuring total antioxidant capacity of food samples against peroxyl radicals was first published by Cao *et al.* (Cao, Alessio and Cutler, 1993). This method was the oxygen radical absorbance capacity assay (ORAC), and subsequently much research was carried out by Ronald Prior, Dejian Huang, Boxin Ou and Guohua Cao who individually, and in collaboration, published many papers on the ORAC values of different foods (Huang *et al.*, 2002; Ou *et al.*, 2002; Huang, Boxin and Prior, 2005; Huang, Ou and Prior, 2005).

It was earlier work by Alexander Glazer on his method of Phycoerythrin Flurorescence-Based Assay for Reactive Oxygen Species (Glazer, 1990), that paved the way for researchers like Cao to develop the ORAC assay and apply its principles to human

serum and natural antioxidants present therein. Cao's work at the Gerontology Research Centre of the National Institute on Aging in Baltimore, Maryland USA formed the basis for much more research by his group into antioxidants and human health (Cao, Alessio and Cutler, 1993). Cao, like many other researchers used ORAC as a tool to narrow-down the most valuable avenues for further research *in vivo*.

It was Dejing Huang and Boxin Ou who went on to modify and apply ORAC to whole foods, and with modifications by a number of other authors, ORAC was applied to both fat and water soluble antioxidants with the help of solubility enhancers (Huang *et al.*, 2002). Although there are a number of antioxidant capacity assays in use, ORAC remains one of the most widely published, and often misinterpreted, assays (Litescu *et al.*, 2014). In this regard, solvent systems and food constituents, in particular amino acids, are reported as having the greatest influence on the ORAC assay (Pérez-Jiménez and Saura-Calixto, 2006). The role of amino acids in antioxidant capacity measurements will be presented in Chapters 5 and 6 of this research, and subsequent mechanisms for their influence on the ORAC measurement will be proposed.

The ORAC Controversy

Research on the antioxidant capacities of many thousands of foods, natural products and specific phytochemicals has been published (Samaranayaka and Li-Chan, 2011). Based on the ORAC antioxidant capacity measure, these foods and associated by-products have been marketed to consumers by means of many health claims on food labels (Prior, Wu and Schaich, 2005a; Kirakosyan and Kaufman, 2009; Stockham *et*

al., 2011; Pompella *et al.*, 2014). This situation is of great concern for a number of reasons. Firstly, ORAC was developed as a tool to help researchers investigate compounds for their potential antioxidant properties (Huang, Boxin and Prior, 2005; Pinchuk *et al.*, 2012). Secondly, ORAC is a measure of total antioxidant activity of a compound, food or phytochemical against one type of radical species (peroxyl radical), it does not provide a measure of antioxidant activity *in vivo* or against a number of other biologically significant radical species (Blasa *et al.*, 2011; Schaich, Tian and Xie, 2015).

Furthermore, the ORAC assay has been reported to be greatly influenced by interferences from the type of solvent system used to extract food matrices (Pérez-Jiménez and Saura-Calixto, 2006). Due to the wide range of solvent-systems published, and the various modifications to the method reported in literature, comparison of ORAC values in the literature is difficult and often not appropriate unless a standardized method has been used.

Finally, the use of ORAC values for health claims and in food labeling is not fully regulated, particularly in Australia, Asia and the Pacific (Brewer, 2011; Stockham *et al.*, 2011). This is challenging and often misleading for consumers, who must contend with ORAC data being reported in μM Trolox (or vitamin E) equivalents per litre or kilogram of food product. Outside the scientific community, such units are not well known or understood. Large ORAC values are desirable from marketing and labeling perspectives, however this raises great concern over the potential misuse of antioxidant

capacity values to attribute unproven health benefits to food products and is discouraged by researchers working in the field (Pompella *et al.*, 2014).

In response to the exponential growth in ORAC data generation and publication, the USDA withdrew its ORAC database in 2010 citing the following reasons (Prior, 2015):

1. "***There is mounting evidence that the values indicating antioxidant capacity have no relevance to the effects of specific bioactive compounds, including polyphenols on human health***"

2. "***There is no evidence that the beneficial effects of polyphenol-rich foods can be attributed to the antioxidant properties of these foods. The data for antioxidant capacity of foods generated by the in vitro (test-tube) methods cannot be extrapolated to in vivo (human) effects and the clinical trials to test benefits of dietary antioxidants have produced mixed results. We know now that antioxidant molecules in food have a wide range of functions, many of which are unrelated to the ability to absorb free radicals***"

Following this, in 2012, the USDA issued the following revised statement regarding the ORAC database:

"***In 2012 USDA's Nutrient Data Laboratory (NDL) removed the USDA ORAC Database for Selected Foods from the NDL website due to mounting evidence that the values indicating antioxidant capacity have no relevance to the effects of specific bioactive compounds, including polyphenols on human health. There are a number of bioactive compounds which are theorized to have a role in preventing or ameliorating various chronic diseases such as cancer, coronary vascular disease, Alzheimer's, and diabetes. However, the associated metabolic pathways are not completely understood and non-antioxidant mechanisms, still undefined, may be responsible. ORAC values are routinely misused by food and dietary supplement manufacturing companies to promote their products and by consumers to guide their food and dietary supplement choices.***

A number of chemical techniques, of which Oxygen Radical Absorbance Capacity (ORAC) is, one, were developed in an attempt to measure the antioxidant capacity of foods. The ORAC assay measures the degree of inhibition of peroxy-radical-induced oxidation by the compounds of interest in a chemical milieu. It measures the value as Trolox equivalents and includes both inhibition time and the extent of inhibition of oxidation. Some newer versions of the ORAC assay use other substrates and results among the various ORAC assays are not comparable. In addition to the ORAC assay, other measures of antioxidant capacity include ferric ion reducing antioxidant power (FRAP) and trolox equivalence antioxidant capacity (TEAC) assays. These assays are based on discrete underlying mechanisms that use different radical or oxidant sources and therefore generate distinct values and cannot be compared directly.

There is no evidence that the beneficial effects of polyphenol-rich foods can be attributed to the antioxidant properties of these foods. The data for antioxidant capacity of foods generated by in vitro (test-tube) methods cannot be extrapolated to in vivo (human) effects and the clinical trials to test benefits of dietary antioxidants have produced mixed results. We know now that antioxidant molecules in food have a wide range of functions, many of which are unrelated to the ability to absorb free radicals.

For these reasons the ORAC table, previously available on this web site has been withdrawn" (United States Department of Agriculture, 2012).

The USDA statement cited the reason for the removal of the widely accessed database was due to the mounting evidence that ORAC values cannot be used to attribute health benefits for foods (United States Department of Agriculture, 2012). In fact, this statement was not surprising and had been known within the research community since its development and use in the early 1990's.

Problems arose as food product manufacturers began to look for ways to promote products. Perhaps the most damning statement made by the USDA was that:

"***ORAC values are routinely misused by food and dietary supplement manufacturing companies to promote their products and by consumers to guide their food and dietary supplement choices***" (United States Department of Agriculture, 2012).

The use and misuse of ORAC values was widely debated, so much so that in 2012 Ronald Prior made some critical statements regarding ORAC based on his 30+ years experience in antioxidant research. Prior's statements were in response to the removal of the Nutrient Data Laboratory ORAC database from the United States Department of Agriculture (USDA) website for a very large number of foods.

In order to re-focus the scientific community and provide clarity, Ronald Prior made a number of important clarifications as follows (Prior, 2012):

1. ***No in vitro assay that quantifies a characteristic of a nutritional product describes in vivo outcomes and should not be used to suggest such a connection.***
2. ***Vital information about metabolism, bioavailability, mechanisms of action, and efficacy is not measured by any such in vitro assay.***
3. ***There is evidence that polyphenols are connected to beneficial human health outcomes.***
4. ***There is evidence that these beneficial outcomes have antioxidant as well as diverse other mechanisms of action.***
5. ***ORAC has been and remains a valuable analytical tool in connection with other investigative methods.***

Recently, the links between dietary intake and health outcomes have been increasingly published, challenging the above statements from the USDA (Prior, 2015). This includes published data from clinical studies, leaving much more to be investigated in this dynamic field. Many more clinical trials are needed, and risks remain regarding a lack of knowledge on the possible side-effects of ingesting high quantities of specific products, often those which have added specialized plant extracts or fractions.

Cellular Antioxidant Activity – Red Blood Cell (CAA-RBC) assay

Due to the desire to relate antioxidant capacity measurements will cellular systems, an *ex vivo* method was developed using human HepG2 tumor cells (Wolfe and Rui, 2007). This assay was developed in response to the recognized shortcomings of existing *in vitro* antioxidant capacity assays, in particular their inability to predict *in vivo* activity (Honzel *et al.*, 2008; Jensen *et al.*, 2008; Niki, 2010). Other limitations cited include nonphysical pH and temperature of some assays, and lack of information regarding bioavailability of antioxidant species (Blasa *et al.*, 2011).

The most desirable systems are animal and human models which take into account bioavailability, uptake and metabolism of antioxidant species (Havsteen, 2002; Leonard *et al.*, 2003; Sorg, 2004; Liu and Finley, 2005; Chauhan and Chauhan, 2006; Wolfe and Rui, 2007; Frankel and Finley, 2008; Zawia, Lahiri and Cardozo-Pelaez, 2009; López-Alarcón and Denicola, 2013; Siti, Kamisah and Kamsiah, 2015). These are not appropriate for initial screening tests, and are both costly and time-consuming. The CAA assay has been applied to screening of food extracts, and addresses some of the issues raised with *in vitro* tests (Wolfe and Rui, 2007). Unlike more complex biological systems, cell culture models are a relatively quick, inexpensive means of screening for antioxidant activity of foods, phytochemicals and dietary supplements.

In 2011, Blasa *et al.* went on to modify the CAA assay and base it on human red blood cells (RBCs), which are inexpensive, readily available, and cope with reactive oxygen and nitrogen species relevant to human metabolism. Furthermore, RBCs do not have a

nucleus or mitochondria, eliminating radicals generated during transcription or produced in the mitochondria (Finkel, 1998; Buehler, P. W., & Alayash, 2005; Blasa *et al.*, 2011). In their study, Blasa *et al.* compared flavonoids and herbal extracts to investigate how comparable CAA-RBC was with the widely used ORAC assay, and noted some synergies and antagonisms between extracts but an overall poor correlation between ORAC and CAA-RBC. Ultimately, Blasa *et al.* recommended use of both ORAC and CAA-RBC to screen extracts for antioxidant capacity, further supporting earlier statements by Ronald Prior that these types of assays are suitable screening techniques but cannot be taken as direct indications of efficacy *in vivo* (Blasa *et al.*, 2011; Prior, 2012).

Study aims and scope

Given the controversy surrounding antioxidant capacity assays and, in particular, how values are influenced and interpreted, the overall aim of this research is to investigate the factors that influence antioxidant capacity in food systems. The ORAC assay will be used as a benchmark antioxidant capacity method, given its popularity and the fact that it has been identified as the assay most susceptible to influences from solvents and matrices (Pérez-Jiménez and Saura-Calixto, 2006; Michiels *et al.*, 2012). The cellular-based CAA-RBC assay will be used to investigate if antioxidant synergies *in vitro* can also be seen *ex vivo*.

Overall research aim:

To investigate influencing factors on antioxidant capacity measurements in food systems.

Research questions:

Specifically, the following research questions will be addressed:

1. How can antioxidant capacity measurements be communicated in a meaningful way to consumers? *(Chapter 3)*
2. Do environmental factors such as temperature and rainfall influence key antioxidant parameters in a matrix? *(Chapter 4)*
3. Do additives such as amino acids and copper nanoparticles influence the antioxidant capacity of matrices purported to be "superfoods"? *(Chapter 5)*
4. Can common classes of constituents in a matrix be combined to investigate if synergies exist *in vitro* and *ex vivo*? *(Chapter 6)*
5. Can factors at the molecular level be identified, via computational chemistry that lead to the enhancement of antioxidant capacity? *(Chapters 5 & 6)*.

Thesis outline:

This thesis is structured as seven chapters: An introduction, literature review, four chapters (each with their own methodology section), representing published papers (Chapters 3 and 4) and papers submitted for publication (Chapters 5 and 6), as well as a chapter on overall conclusions and future work. Each of the data chapters (3-6) addresses one of the research questions. Much of the information in this thesis has also been presented at international conferences and symposia, as listed elsewhere.

Chapter 1 presents an overall introduction of food antioxidants and a list of some common chemical assays used to determine antioxidant capacity in the literature. Controversy surrounding one of the most widely used *in vitro* assays, ORAC, is discussed, and the merits of an *ex vivo* alternative, CAA-RBC, are summarized briefly. This chapter concludes with a statement of the overall aim and research questions to be addressed.

Chapter 2 details a review of relevant literature, and presents some background information on how the two key antioxidant capacity assays, namely ORAC and CAA-RBC, are performed and interpreted. In this chapter, health implications are discussed with respect to antioxidant capacity assays, and current research into some of the key influencing factors in these types of measurements are presented. Phytochemicals, climate and environmental conditions, amino acids, nutrients and nanoparticles are all implicated as influencing factors in the literature.

Chapter 3 has been published in the *Journal of Food Composition and Analysis* and was also presented at the 8th International Food Data Conference at the Institute of Nutrition, Mahidol University, Thailand. This publication highlights the issues surrounding the use of ORAC values on product labeling, presents an alternate mode (units) for reporting antioxidant capacity and lists a comparison of a number of foodstuffs assessed against the existing Trolox standard scale and the proposed scale. Examples of the calculations needed to convert ORAC units from μM equivalents to mass unit equivalents are provided. This publication addresses the first research question by proposing an alternative approach for communicating antioxidant capacity measurements in a meaningful way to consumers.

Chapter 4 has been published in *Food Chemistry* and presented at the 9th International Food Data Conference at the Institute of Food Research, Norwich, United Kingdom. This paper presents data from a pilot study of the ORAC, CAA-RBC, total phenolic, total flavonoid and HPLC profile of Australian wines from 4 major regions. The pilot study focused on the vintage of highest and lowest rainfall for the regions studied, and in particular the 2008 and 2009 vintages. Warmer climate wines had lower ORAC values but performed well in the CAA-RBC assay. Six bioactive compounds were found to vary greatly within a single grape variety exposed to greater fluctuations in rainfall during key periods. This publication explores the second research question by presenting data which suggests environmental factors such as rainfall do influence key antioxidant parameters in certain grape varieties.

Chapter 5 has been submitted to *Food Chemistry* and the ORAC data has been presented at the 11th Government Food Analysts Conference, Treacy Conference Centre, Parkville, Melbourne, Australia. Density functional theory (DFT) calculations have been carried out and support the experimental data in the submitted manuscript. This paper describes the addition of amino acids and copper nanoparticles (CuNPs) to "superfood" concentrates of bilberry, coffee berry and apple. These concentrate powders all exhibited strong antioxidant activity in the ORAC assay, and extracts of these matrices were supplemented with an amino acid, CuNPs, or a combination of both. Extracts were then compared via the ORAC assay to determine if antioxidant synergies could be observed and to what extent these synergies were reproducible. Finally, the concept of "antioxidant and substrate zones" is proposed and DFT calculations assessed how homolytic bond dissociation energies for phenolic O-H groups can be affected by interactions between the matrix and the substrate zone of an antioxidant and/or an amino acid, hence postulating a mechanism for antioxidant enhancement/synergy at the molecular level. These observations have not been presented elsewhere in literature, and represent novel work and provide a theory for how antioxidant synergy occurs *in vitro*, including the role of amino acids and CuNPs in these mechanisms in response to research question 3.

Chapter 6 has been submitted to *Food Chemistry* and the ORAC and CAA-RBC data were presented at the Australian Food Metrology Symposium, held at Victoria University, Melbourne, Australia. Subsequent DFT calculations were carried out and have been included with the experimental data to complete the submission of the paper. This manuscript describes the preparation of a mixture of components, based on compositional data from the Food Standards Australia New Zealand (FSANZ)

nutritional database (NUTTAB). These mixtures represent eight classes of compounds at the levels described as being typical for navel orange juice. Classes included sugars, vitamins, amino acids, phenolics, flavonoids and preservatives. Based on the experimental observations in Chapter 5, CuNPs were also included in the trial, and all experimental data were compared by *in vitro* ORAC and *ex vivo* CAA-RBC assays. This paper addresses research question 4 by reporting on the major classes of constituents which show antioxidant synergies and describes the structural/electronic properties of the molecules involved in terms of either activating or deactivating O-H sites for hydrogen abstraction as demonstrated by DFT calculations.

Finally, conclusions and future work are proposed. Additional data, copies of presentations, and supplementary materials are contained in the appendices.

ORAC and CAA-RBC will now be reviewed in more detail in Chapter 2, and a summary of relevant literature will be presented.

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2.0 Literature review

Antioxidants in literature

Antioxidants are widely studied and reported in literature (Ishige, Schubert and Sagara, 2001; Pinchuk and Lichtenberg, 2002; Jomova and Valko, 2011; Shahidi and Ambigaipalan, 2015; Siti, Kamisah and Kamsiah, 2015). As mentioned earlier, there are many methods for determining antioxidant capacity of foods, involving usually HAT or ET mechanisms. For the purposes of this review, the two methods to be described are the oxygen radical absorbance capacity (ORAC) assay, and the cellular antioxidant activity – red blood cell (CAA-RBC) assay. This chapter will focus on the mechanisms of these assays, implied health benefits found in literature, and what influencing factors are reported with respect to antioxidant capacity measurements.

Antioxidant measurements in food

As stated, ORAC is one of the most widely reported antioxidant capacity assays (Kim *et al.*, 2002; Armstrong, 2014). In fact, the Association of Official Analytical Chemists (AOAC) published an official method of analysis (OMA) for the ORAC assay due to its popularity and wide use in the scientific community (Ou *et al.*, 2013). This *in vitro* assay has been applied to food extracts and neutraceuticals, as well as serum and plasma (Prior *et al.*, 2003; Prior, Wu and Schaich, 2005b; Prior, 2015).

In an effort to address bioavailability, update and metabolism, Wolfe *et al.* developed a cellular antioxidant assay (CAA) using HepG2 tumor cells (Wolfe and Rui, 2007). This was later modified and applied to human red blood cells which do not require

culturing, are inexpensive and readily available, and do not generate radical species within the cell due to their absence of nucleus and mitochondria (Blasa *et al.*, 2011).

ORAC Assay mechanism

In their review, Huang *et al.* described the chemistry behind antioxidant capacity assays. The chemical reaction monitored in the ORAC assay is based on hydrogen atom transfer (HAT). The method is composed of a free radical generator, and oxidisable probe and an antioxidant standard (Huang, Boxin and Prior, 2005). This type of assay is based on competitive reaction kinetics, and as the radical species degrade the fluorescent probe a loss of fluorescence can be observed. As a result fluorescence decay curves are produced and used to quantitate antioxidant capacity against the Trolox (or vitamin E analogue) standard.

In the case of ORAC, reaction rates of antioxidants or substrates are the main parameter for determining sacrificial antioxidant capacity, and the basic kinetics of the assay are described by Huang *et al.* in Figure 2.1 below (Huang, Boxin and Prior, 2005).

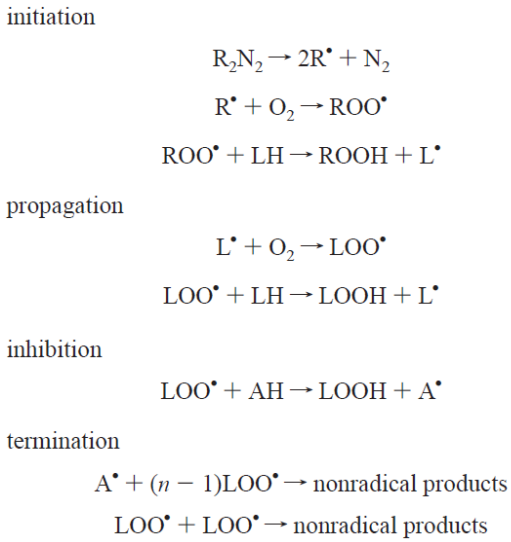


Figure 2.1: Kinetics of auto-oxidation as described by (Huang, Boxin and Prior, 2005). R_2N_2 = azo compound used as a radical generator (2,2'-Azobis(2-amidinopropane) dihydrochloride, AAPH, used in the ORAC assay); LH = substrate; AH = antioxidant. The stages of initiation, propagation, inhibition and termination are demonstrated.

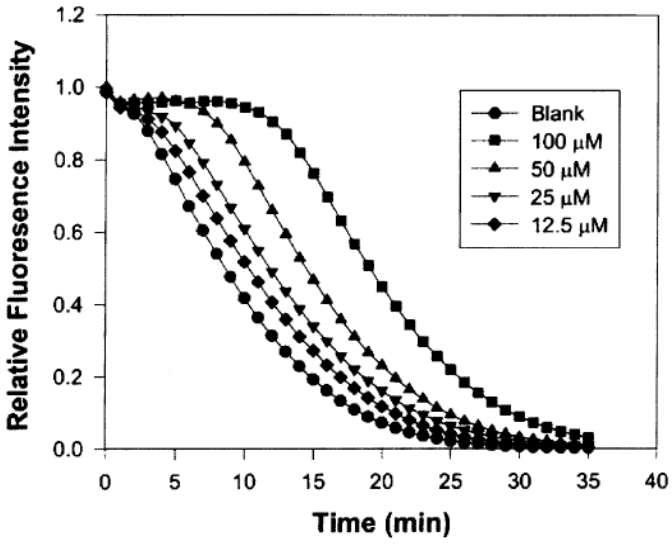
In ORAC, samples, controls and standards are treated with a fluorescein solution (the fluorescent probe used for the assay). These mixtures are then incubated at 37°C, usually for at least 15 minutes, prior to addition of the azo radical generator AAPH. Once added, the AAPH begins to thermally degrade and release peroxyl radicals, which, in turn, attack the fluorescein probe.

Measurements of fluorescence intensity are taken at regular intervals, usually every minute, for a defined number of cycles, usually until the most concentrated antioxidant standard is exhausted. As the reaction progresses loss of fluorescence intensity is seen. Any antioxidants present in extracts act sacrificially via HAT mechanism to delay fluorescein decay. Figure 2.2 displays typical fluorescence decay curves, in this case

of the antioxidant standard, and its associated linear plot used for calculation of antioxidant capacity via ORAC values.

Usually the most predominate source of antioxidant capacity from sample extracts is found in the hydrophilic ORAC extract, usually a mixture of solvents and then addition of phosphate buffer to correct the pH of extracts to 7.4, the optimal pH for the fluorescein probe. Lipophilic antioxidants are extracted with a solvent such as hexane, and then partially dissolved with the aid of a cyclodextrin solubility enhancer, although the efficacy of this is limited (Huang *et al.*, 2002). This yields two ORAC values, the sum of hydrophilic and lipophilic fraction μM T.E/L or /kg being given as the total antioxidant capacity (TAC) for the sample. $ORAC_{(TAC)}$ has been reported in literature, however for this research the hydrophilic fraction was studied as it is the largest contributor to the ORAC values presented.

(a)



(b)

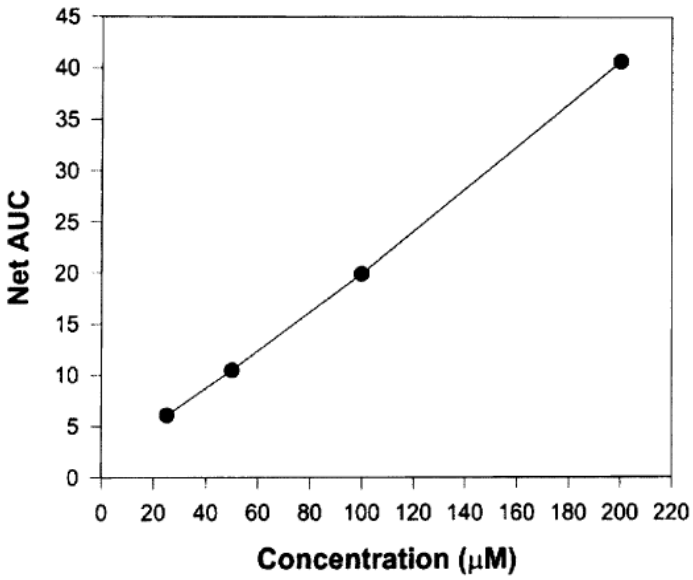


Figure 2.2: (a) Typical fluorescence decay curves of vitamin E standard used in the ORAC assay, and **(b)** use of the net area under the decay curve (Net AUC) to prepare a linear plot for calculation of ORAC value. Units for reporting ORAC are in µM Trolox (or vitamin E) equivalents, typically per litre or per kilogram of sample (for example: µM T.E/kg) (Huang, Boxin and Prior, 2005)

CAA-RBC Assay mechanism

In the initial cellular antioxidant activity (CAA) assay, HepG2 cells were seeded onto a 96 well plate, washed with phosphate buffer saline (PBS), and then treated with extracts of fruit or antioxidant compounds as well as the probe 2',7'-dichlorofluorescin diacetate (DCFH-DA)(Wolfe and Rui, 2007). Following treatment, cells are incubated at 37°C and antioxidants present in extracts either passed through the cell membrane into the HepG2 cells, or bind to the surface, while the DCFH-DA probe diffuses into the cell during this incubation time.

Cells are then treated with an azo compound, 2,2'- azobis (2-amidinopropane) dihydrochloride (ABAP), which diffuses into the cells, decomposing and then generating peroxyl radicals. In the case of CAA, DCFH-DA oxidises to it fluorescent derivative, DCF'H, and an increase of fluorescence is measured at regular intervals. Any antioxidants present in the system will delay DCFH-DA oxidation, leading to a greater area above the curve as an indication of antioxidant capacity (Wolfe and Rui, 2007). The principle of the CAA assay is described by Wolfe and Rui in Figure 2.3 below.

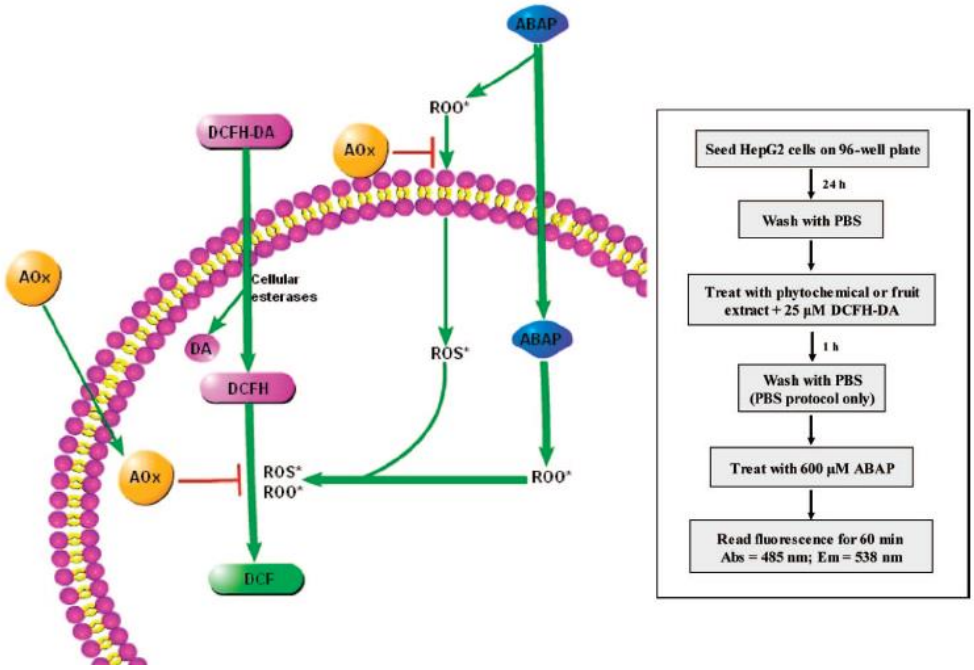


Figure 2.3: Principle of CAA assay as presented by Wolfe and Rui (2007). ABAP = peroxyl radical generator; AOx = antioxidants in the extract applied to cells; DCFH-DA = probe; DCFH = fluorescent product of probe decay in presence of reactive oxygen species (ROS·, ROO·)

Given the presence of auto-initiated radical species in the HepG2 cell line, Blasa *et al.* sought to apply the principles of this CAA assay to human red blood cells. Replacing the HepG2 cells with human red blood cells, the same stages of extract and probe treatment/incubation, followed by radical addition and measurement of fluorescence intensity at regular intervals (Blasa *et al.*, 2011). The antioxidant standard used for this assay is Quercetin, and results are reported in µmol Quercetin equivalents (µmol/QE)(Blasa *et al.*, 2011). Figure 2.4 presents typical area above the curves (AAC) seen in the CAA-RBC assay, and used for quantitation.

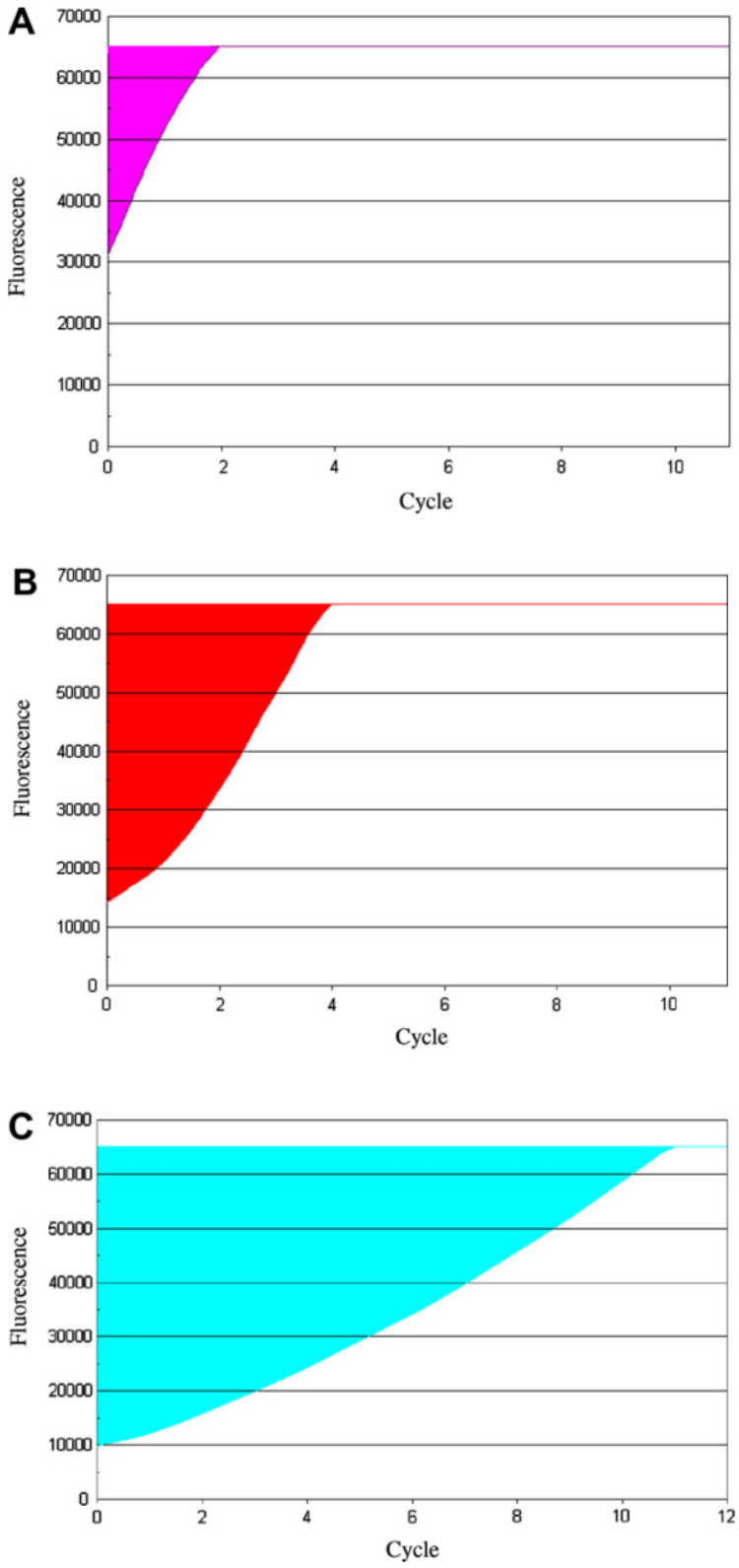


Figure 2.4: Examples of the areas above the fluorescence curve (AAC) used for quantification of CAA-RBC as presented by Blasa *et al.* where (A) = AAC of control (pink); (B) = AAC of 4µmol Quercetin (red); (C) = AAC of 8µmol Quercetin (blue).

Implied health benefits

A large number of papers have been published reporting the antioxidant properties of dietary sources. Studies include citrus flavonoids (Tripoli *et al.*, 2007), antioxidants in wine (Kondrashov *et al.*, 2009; Rodrigo, Andrés and Vergara, 2011; Van Leeuw *et al.*, 2014), eggs (Nimalaratne *et al.*, 2011; Nimalaratne, Schieber and Wu, 2016), spices and herbs (Embuscado, 2015) to name just a few.

The role of dietary antioxidants and health is particularly controversial. There is concern in the scientific community of overly simplistic relationships being promoted, when the underlying mechanisms, metabolites and long term effects are yet to be established. One example might be the promotion of various "superfoods", products, often fruits, which have very high antioxidant capacity measurements by *in vitro* assays such as ORAC.

In a cautionary paper, Pompella *et al.* cited the use of total antioxidant capacity (TAC) as an emerging and prominent aspect of food functionally being used to promote consumption of high TAC foods including "superfruits" (Pompella *et al.*, 2014). The paper goes on to describe the importance of oxidative reactions *in vivo*, including physiological functions and redox regulation of cellular targets inside and outside cells. For these reasons, the authors caution against excessive consumption of dietary antioxidants, so as to avoid interference with natural cellular function.

A range of shortcomings are highlighted, with some of the major issues being that many antioxidant food components undergo major modification during the initial stages of metabolism in the body. This includes flavonoids, phenolics and other phytochemicals, often promoted as antioxidants. Although they may perform antioxidant functions as measured by *in vitro* assays, they do not have this role biologically and many don't function as antioxidants following digestion and metabolism (Pompella *et al.*, 2014).

Regarding the validity of total antioxidant capacity assays, Pompella *et al.* cited ORAC as the only assay whose values were more directly related to specific antioxidants in human plasma samples, although normal serum components were reported as contributing to the ORAC value and may be seen as interferences. Other disease markers in the serum such as bilirubin and uric acid were reported to contribute to the TAC, masking the severity of disease conditions and giving an incorrect assessment of patient health (Pompella *et al.*, 2014).

The paper concludes by discussing the Folin-Ciocalteu assay for total phenolics and its potential for valid TAC assessment of food matrices. Interestingly, ORAC is the only total antioxidant capacity assay to correlate well with the Folin-Ciocalteu assay, as demonstrated in a range of foods including blueberry, spinach and blackberry (Pompella *et al.*, 2014). Despite concerns regarding the use, and rather misuse of TAC values, the ORAC assay has shown good correlation with other valid and well established methods for total phenolic assessment in foods. With this in mind, it is important to consider what factors and reported to influence the ORAC assay.

Influencing factors

It has been reported that factors such as solvent choice and certain food constituents influence the ORAC assay (Pérez-Jiménez and Saura-Calixto, 2006). Measurement of antioxidant activity of wines was reported to be influenced by ethanol content (Fernández-Pachón *et al.*, 2004; Villaño *et al.*, 2005), while choice of extraction solvent effected measurements in wheat bran (Zhou and Yu, 2004). Many different solvent systems have been reported for extracting antioxidants for use in the ORAC assay. Some of these include ethanol, mixtures of ethanol/ water or acetone/water in a variety of ratios, or a combination of acidified methanol/water followed by acetone/water (Pérez-Jiménez and Saura-Calixto, 2006).

Pérez-Jiménez *et al.* suggest that type of solvent and polarity may affect the antioxidant mechanisms, specificially hydrogent atom transfer (HAT), in assays such as ORAC. The authors also postulate that non-antioxidant compounds could interfere with antioxidant capacity measurements. In order to investigate, a range of solvent mixtures were applied to common food consituents including amino acids, pectin, glucose and albumin. A mixture of polyphenol and flavonoid was also trialled to investigate if cumulative effects on antioxidant capcity resulted (Pérez-Jiménez and Saura-Calixto, 2006). In their study, Pérez-Jiménez *et al.* reported that the ORAC assay was the most greatly influenced by solvent choice, in some cases by almost up to 50%. Solvent choice is also implicated in dramatic reduction of H-atom donor activity for phenolic antioxidants (Pedrielli, P., Pedulci, G. F., & Skibsted, 2001). Pérez-Jiménez *et al.* found acidified solvent mixtures were not significantly different from those without acid addition.

Observations were made regarding the amino acid food constituents, namely that Tyrosine and Tryptophan gave measurable ORAC values, even when in very low concentrations. Since amino acids have not been typically regarded as antioxidants the authors listed them as food constituents interfering with the ORAC assay (Pérez-Jiménez and Saura-Calixto, 2006). However, novel work on the intrinsic antioxidant properties of amino acids, their influence on antioxidant capacity measurements and proposed mechanisms will be addressed later in this research (Chapters 5 & 6). Observed high ORAC values for Histidine were also cited (Yilmaz and Toledo, 2005), and this amino acid has also been studied in this research (Chapter 5).

Finally, Pérez-Jiménez *et al.* reported what they termed as polyphenol/food constituent interactions generating new interferences in the ORAC assay. The constituents involved were glucose, arginine and cysteine, when combined with albumin gave rise to inflated antioxidant capacity measurements. Their work concludes that these interactions are interferences, and cites ORAC as the assay most effected by amino acids in combination with other food constituents (Pérez-Jiménez and Saura-Calixto, 2006).

On synergistic interactions between antioxidants, Rong Tsao defines the terms synergistic, additive and antagonistic antioxidant interactions. These definitions are:

"*Synergistic antioxidant interaction or antioxidant synergism: The antioxidant effect of two or more discrete antioxidants when applied together is greater than the sum of the individual antioxidant effects applied separately. The synergistic interaction may be observed at the same or different doses for all individual antioxidants.*

Additive antioxidant interaction: The antioxidant effect of two or more discrete antioxidants when applied together is equal to the sum of the individual antioxidant effects applied separately.

Antagonistic antioxidant interaction: The antioxidant effect of two or more discrete antioxidants when applied together is less than the sum of the individual antioxidant effects applied separately (Tsao, 2015)."

Tsao gives examples of the antioxidant synergies between vitamin C and tocopherols, and cites ferulic acid as acting synergistically with vitamin C, but having antagonistic interactions with α-tocopherol and β-cartone (Tsao, 2015). Interactions between vitamins and polyphenols have been cited, as is the case for interactions of green tea polyphenolics in the presence of vitamin C. Dai *et al.* reported that vitamin C appeared to regenerate green tea polyphenolics, which subsequently regenerated some forms of vitamin E (Dai, Chen and Zhou, 2008).

Synergies between the chlorogenic, vanillic, gallic and protocatechuic phenolic acids in mango pulp have also been reported (Palafox Carlos *et al.*, 2012). Some phenolic components in Chinese herbal medicines, for example those from fragrant rosewood, exhibit antagonistic antioxidant effects in combination with otherwise potent antioxidants including Catechin and Quercetin (Pinelo *et al.*, 2004; Tsao, 2015).

Issues have been raised regarding the correlation between *in vitro* and *in vivo* antioxidant capacity measurements. Silvia *et al.* reported the production of synthesized antioxidants, namely phenolic-amino acid conjugates, which had good antioxidant activity when measured via the ORAC assay, however the efficacy of these conjugates was poor *in vivo* (Silvia *et al.*, 2012). Limited data is available on the correlation of these synergistic and antagonistic influences on antioxidant capacity assays *in vitro* and *ex vivo*. Based on the literature, the antioxidant properties of amino acids warrant further investigation, and some researchers have even recommended that *L*-Histidine supplementation could be an effective and safe method for increasing antioxidant protection from inflammatory disease (Wade and Tucker, 1998; Triantis *et al.*, 2007).

Given these reports of antioxidant synergy, the findings of Pérez-Jiménez *et al.* could be considered to be oberservations of synergy, rather than simply interferences in the ORAC assay. Furthermore, varietal and seasonal variations in whole foods have been reported with respect to antioxidant activity. One matrix that has been extensively studied for antioxidant properties is that of wine. Grape genotype, storage of wines and varietal variations have all been reported as influencing antioxidant capacity of wine (Villaño *et al.*, 2005; De Beer *et al.*, 2006; Iacopini *et al.*, 2008; Kondrashov *et al.*,

2009). Other factors such as the role of environmental conditions on fluctuations in antioxidant content, activity and bioavailability have not been explored or compared in both *in vitro* and *ex vivo* studies.

There are still significant gaps in knowledge of the potential influences on antioxidant capacity measurements in food systems. From a review of literature, environmental factors and potential synergies between chemical constituents of food matrices with a focus on amino acids warrant further investigation. Finally, the interpretation and use of ORAC data on product labelling remains controversial, and should be considered when evaluating factors influencing antioxidant capacity measurements in food systems.

Data resulting from this research will now be presented. The overall aim and research questions are re-stated below:

Statement of research hypotheses

Overall research aim:

To investigate influencing factors on antioxidant capacity measurements in food systems.

Research questions:

Specifically, the following research questions will be addressed:

1. How can antioxidant capacity measurements be communicated in a meaningful way to consumers? (Chapter 3)
2. Do environmental factors such as temperature and rainfall influence key antioxidant parameters in a matrix? *(Chapter 4)*
3. Do additives such as amino acids and copper nanoparticles influence the antioxidant capacity of matrices already purported to be "superfoods"? *(Chapter 5)*
4. Can common classes of constituents in a matrix be combined to investigate if synergies exist *in vitro* and *ex vivo*? *(Chapter 6)*
5. Can factors at the molecular level be identified, via computational chemistry, which lead to the enhancement of antioxidant capacity? *(Chapters 5 & 6)*

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3.0 Modes of handling oxygen radical absorbance capacity (ORAC) data and reporting values in product labelling

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Original article

Modes of handling Oxygen Radical Absorbance Capacity (ORAC) data and reporting values in product labelling

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ABSTRACT

Antioxidant measurement assays are widely used and should be chosen based on their being fit for purpose. Likewise, the mode of reporting antioxidant measurements should also be fit for purpose. The Oxygen Radical Absorbance Capacity (ORAC) assay is widely used internationally for measuring the antioxidant capacity of commodities using the peroxyl radical. However, the current mode of reporting of the ORAC values is not obvious, especially for the consumer groups. In this mode, reporting of the ORAC values is the unit of micromoles of vitamin E analogue (VEA), known commercially as Trolox Equivalents per kilogram or per litre (μM T.E./kg or L). Unlike mass units, molar units are not widely used in nutrition information panels (NIP). This paper presents a simple mathematical model for conversion of ORAC values to mass units to facilitate better understanding of the antioxidant capacity quoted. Additionally, mass values are in keeping with current labelling practice in Australia. Unless legislation is passed for the regulation of ORAC data use in labelling and product marketing, mass units should be considered as a mode of reporting, limiting sensationalism of antioxidant capacity and keeping with current labelling practice.

3.1 PREAMBLE

This work has been published in *Journal of Food Composition and Analysis* (December 2010), and was also presented at the 8th International Food Data Conference, held 1-3 October 2009 at the Institute of Nutrition, Mahidol University, Thailand. This publication highlights the issues surrounding use of ORAC values on product labeling, presents an alternate mode (units) for reporting antioxidant capacity and lists a comparison of a number of foodstuffs assessed against the existing Trolox standard scale and the proposed scale. Examples of the calculations needed to convert ORAC units from μM equivalents to mass unit equivalents of Trolox are provided. This publication addresses the first research question by proposing an alternative approach for communicating antioxidant capacity measurements in a meaningful way to consumers.

3.2 ABSTRACT

Antioxidant measurement assays are widely used and should be chosen based on their being fit for purpose. Likewise, the mode of reporting antioxidant measurements should also be fit for purpose. The Oxygen Radical Absorbance Capacity (ORAC) assay is widely used internationally for measuring the antioxidant capacity of commodities using the peroxyl radical. However, the current mode of reporting of the ORAC values is not obvious, especially for the consumer groups. In this mode, reporting of the ORAC values is the unit of micromoles of Vitamin E analogue (VEA), known commercially as Trolox equivalents per kilogram or per litre (µM T.E/kg or L). Unlike mass units, molar units are not widely used in nutrition information panels (NIP). This paper presents a simple mathematical model for conversion of ORAC values to mass units to facilitate better understanding of the antioxidant capacity quoted. Additionally, mass values are in keeping with current labelling practice in Australia. Unless legislation is passed for the regulation of ORAC data use in labelling and product marketing, mass units should be considered as a mode of reporting, limiting sensationalism of antioxidant capacity and keeping with current labelling practice.

3.3 INTRODUCTION

Naturally occurring radical species arise from various metabolic processes, dietary and environmental factors and exposure to different radiation sources (Huang et al., 2005; Cao et al., 1993; Sies, H., 1997). Radical species are recognised as having a role in cell damage and are associated with disease and cancer (Thiapong et al., 2006; Adom et al., 2002). Given the potential benefits of antioxidants in human health and disease

prevention, measurement of antioxidant capacity in foods is a significant area of international study (George et al., 2005).

Currently, one of the most widely used antioxidant assays is the Oxygen Radical Absorbance Capacity (ORAC) assay (Bisby et al., 2008; Thaipong et al., 2006; Huang et al., 2002a, Alarcón et al., 2008). This assay utilizes a fluorescent probe, fluorescein (FL) (Ou et al., 2001), and a peroxyl radical donor, 2, 2'-azobis (2-amidinopropane) dihydrochloride (AAPH). The controlled generation of peroxyl radicals is achieved thermally, as the entire assay is conducted at 37ºC. Generated radicals then attack the FL probe, leading to a loss of fluorescence intensity over time. The area under the FL decay curve (AUC) is taken as a measure of the antioxidant capacity of a product. The AUC of a product is extrapolated against an antioxidant standard which is a water soluble vitamin E analogue (VEA) known commercially as Trolox (Gomes et al., 2005).

In order to account for fat and water soluble antioxidants, the same sample is extracted using 2 different solvent systems. Fat soluble, or lipophilic, antioxidant compounds are extracted initially, generally with hexane or a similar solvent (Prior et al., 2003) and then the extracts are treated with a solubility enhancer such as methyl-β-cyclodextrin (Mercader-Ros et al., 2010). Water soluble, or hydrophilic, compounds are then extracted using any one of a range of solvents cited in literature. The solvents used vary as researchers tailor their studies, and consequently their solvent systems, to a selected range of products or samples. Commonly used solvents include, but are not limited to, methanol, acetone, water and phosphate buffer (Amorati et al., 2006).

In most techniques, phosphate buffer is used to dilute all extracts, as the FL probe is most stable at pH 7.4 (Gomes et al., 2005). The strength and pH of this buffering solution are important factors, as is solubility, especially of the lipophilic extracts. The final ORAC measurement, known as the Total Antioxidant Capacity ($ORAC_{TAC}$), is given as a sum of the individual ORAC lipophilic and hydrophilic fractions and is reported in micromoles of Trolox equivalents per litre or per kilogram depending on the product (Prior et al., 2003).

The assay has been applied to a wide range of samples including food, beverages and plasma, as was detailed by Prior et al. (2003) and has been successfully applied to samples of a complex nature (Zulueta et al., 2009). Increasingly, ORAC is being applied in the areas of cosmetics and neutraceuticals (Cornelli, 2009), with a twofold aim. Firstly, in product development, to establish the antioxidant affect of specific ingredients and the impact of formulation preparations on these antioxidant properties. The second area is in product marketing, where consumer interest in antioxidants has increased considerably (Mertz et al., 2009).

Any visit to local supermarkets, health food stores or larger grocery chains reveals an increasing number of products being marketed for their antioxidant abilities. In Australia, the main method of reporting antioxidant capacity of a product is by ORAC values. These ORAC values are quoted on product packaging, and sometimes near the base of nutrition information panels (NIP).

A key area of concern is units used for reporting the antioxidant capacity values. As mentioned previously, the units used are micromoles of antioxidant standard equivalent

per litre or per kilogram (μM T.E/L or kg). These units are understood in the scientific community however understanding is more limited in the wider community, in particular the consumer groups. In Australia, most nutritional information for different food components has been reported in mass units, thus, conversion of ORAC values into the mass units is desirable for more effectively communicating antioxidant capacity. Mass units have several advantages in that they increase the communication of antioxidant capacity of foods to the consumer, law makers, manufactures of processed products, and for labelling purposes.

The work presented in this paper involved investigating and measuring the ORAC values of a range of food products. The ORAC values are presented in mass units, in contrast to the conventional presentation of the molar units. In this study, the FLUOstar OPTIMA microplate reader (BMG LabTechnologies) was used in the assay as a semi-automated method to measure the ORAC value of products. The experimental approach used is presented and the results are reported in mass and molar units for comparison purposes.

3.4 MATERIALS AND METHOD

3.4.1 Chemicals. Fluorescein disodium salt (FL), 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), monosodium phosphate monohydrate, disodium phosphate heptahydrate, hydrochloric acid, acetone, acetic acid, hexane and randomly methylated β-cyclodextrin (RMCD). All chemicals were obtained from Sigma-Aldrich (Australia).

3.4.2 Reagent and standard preparation. 75mM aqueous phosphate buffer was prepared to 2L volume with monosodium phosphate monohydrate and disodium phosphate heptahydrate, and the pH adjusted to 7.4 using 1M hydrochloric acid (HCl) prepared from 10M HCl stock. All subsequent working solutions were prepared in phosphate buffer. A stock solution of FL was prepared monthly at 0.7mM concentration, and the final working solution of FL was achieved by serial dilution of the stock with buffer to a final concentration of 70nM. The working FL solution was prepared from stock daily. AAPH peroxyl radical donor was prepared to 35mM concentration by dissolution in phosphate buffer. Due to the thermal sensitivity of AAPH, the working solution was prepared just prior to analysis. The Trolox antioxidant standard stock solution was prepared weekly at a concentration of 10mM and serial dilutions with buffer from the stock were performed daily to achieve a range of 6.25-100μM for preparation of calibration plots. A 7% RMCD solvent solution was prepared in methanol as required to act as a solubility enhancer for extracted lipophilic antioxidants from a given sample (Huang et al., 2002b). An acetone/water/acetic acid (AWA) 70:29.5:0.5 (v/v/v) solvent was prepared for the extraction of hydrophilic antioxidants. Both lipophilic and hydrophilic extracts were diluted in phosphate buffer prior to analysis.

3.4.3 Sample preparation and extraction. Samples were obtained locally from Coles supermarkets in Port Melbourne, Victoria, Australia, and stored according to manufacturer's instructions. In the case of processed products, once opened the products were homogenised and refrigerated at ≤ 4ºC until use. All samples were homogenised and analysed on the same day, to minimise product degradation. All samples were also extracted and analysed in triplicate.

The extraction process was conducted in 2 stages. Firstly, samples to be analysed for both hydrophilic and lipophilic antioxidant capacity were isolated. This involved samples being weighed and extracted for lipophilic antioxidants using hexane as the solvent. This extraction process was repeated twice. The hexane fractions were separated from the remaining sample solids, and combined and blown to dryness at room temperature with nitrogen. This process yielded oily or fatty residue on the inner surface of the collection tube. The residue was redissolved with acetone, followed by dilution with RMCD solvent to enhance solubility. The final solution was dissolved in phosphate buffer prior to analysis. The remaining sample solids were then re-extracted using the AWA solvent system, diluted in phosphate buffer and analysed separately. This allows both lipophilic and hydrophilic fractions to be extracted, buffered and analysed independently.

Snack bar and chocolate samples were homogenised prior to subjecting them to the extraction processes, as described above. Care was taken when solubilising chocolate samples to ensure that even dissolution of the solids was achieved. The lipophilic extraction was conducted first followed by the hydrophilic extraction process. The hydrophilic extraction process was modified to include a pre-dissolution step where the

chocolate sample was dissolved in acetone first, followed by AWA solvent system. The final extract was then diluted in phosphate buffer.

The edible portion of fresh fruit samples (mango, orange, blueberries, and pear) was homogenised and then weighed. This was then subjected to both the lipophilic and the hydrophilic extraction processes. Inedible portions such as seed, stalk or skin were discarded, except in the case of oranges as not all the pith could be completely removed. Dried apple was homogenised and extracted with both hydrophilic and lipophilic solvent systems directly, without rehydration, as the product can be consumed in either the dried or rehydrated form.

Processed or canned fruits were homogenised together with their juice or syrup. The homogenised sample was weighed and subjected to both the hydrophilic and lipophilic extraction processes. Fruit juice samples were centrifuged and an aliquot taken followed by dilution in 75mM phosphate buffer (pH 7.4). This sample was only subjected to the hydrophilic extraction process as the fat content of the sample was known to be negligible.

Tea bags were cut open and the contents from each bag weighed and steeped in 200mL of near boiling water ($\geq$ 92°C) for 5 minutes for infusion. The leaves were strained and the infusion cooled, centrifuged, and then diluted in 75mM phosphate buffer. Upon visual inspection of the bag contents, all teas containing citrus were all subjected to both lipophilic and hydrophilic extraction processes. These teas appeared to have small amounts of peel or rind which were expected to have a measurable lipophilic antioxidant capacity.

3.4.4 Instrumentation. The FLUOstar OPTIMA microplate reader system as used by Volden et al., 2009, was equipped with 2 injector pumps and fitted with FLUOstar OPTIMA software system (version 2.0). Fluorescence intensity measurements were taken at regular intervals using an excitation wavelength of 485nm and an emission wavelength of 520nm. The instrument sensitivity (gain) was set to 1600 as this was found to be optimal for analysing a broad range of samples in the same experimental run. Both pumps were primed with 4mL of reagent (FL for pump 1 and AAPH for pump 2) from beaker reservoirs prior to the assay.

3.4.5 ORAC assay experimental conditions. The FLUOstar OPTIMA instrument was set to a temperature of 37.3 ±0.3ºC. This is the optimum temperature for the production of peroxyl radicals that oxidized the FL probe during the assay to produce decay curves. The area under the curve (AUC) was taken to represent antioxidant capacity. Kinetic windows were programmed to allow the automatic injection of the FL probe and the AAPH radical donor. 25µL of standards and diluted samples were manually pipetted into individual microplate wells. Phosphate buffer was used as a blank, as all solutions were prepared in buffer and all extracts were diluted in buffer prior to analysis. The cycle time was recorded based on the check timing function, as required for the final ORAC calculation. The microplate was then loaded and the run started.

The Trolox FL decay curves reached a minimum Relative Fluorescence Units (RFU) point of approximately 1000 RFU by the 100 minute stage of the assay; therefore, the end-point of the assay was set at 120 minutes, ensuring completion while minimizing the errors resulting from extensive base RFU measurements.

3.5 RESULTS AND DISCUSSION

Figure 3.1 indicates the FL decay curves obtained for the Trolox standard and some selected samples using the FLUOstar OPTIMA. Similar curves were obtained for the rest of the samples investigated in this work.

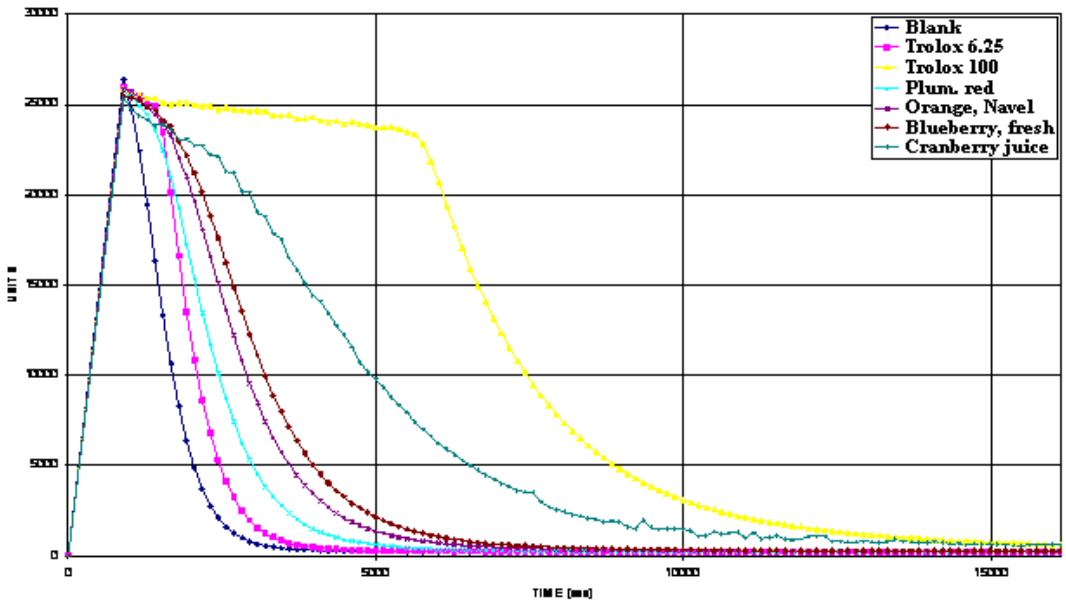

Figure 3.1: FLUOstar OPTIMA software screen plot of FL decay curves for phosphate buffer blank, VEA Trolox at low and high calibration concentration range, and hydrophilic fractions of 4 fruit samples.

The ORAC measurement was calculated, making use of the area under the curve (AUC). The AUC values were calculated using equations 1 and 2.

Equation 1

$$AUC = \left(\left[\frac{f_1}{f_0} + \frac{f_2}{f_0} + \frac{f_3}{f_0} \ldots \frac{f_i}{f_0} \right] + 0.5 \right) \times CT$$

Where, *(f_0* = initial fluorescence reading, f_1, f_2, f_3 *and* f_i = fluorescence reading at cycles 1, 2, 3 and i^{th} respectively, and CT = cycle time in minutes). The AUC is normalised by applying a factor of 0.5 as shown in the equation (Mertz et al., 2009).

Equation 1 was used to calculate for the AUC values for the Trolox and the various samples from the curves obtained in Figure 3.1.

Equation 2

$$Net\ AUC_{(sample)} = \left(\frac{AUC_{sample} - AUC_{blank}}{AUC_{Trolox} - AUC_{blank}} \right)$$

Equation 2 was used to calculate for the net AUC values from the curves obtained in Figure 3.1, however, this equation took into account the correction for the blank (AUC_{blank}) used, which was the phosphate buffer.

The ORAC value for each sample was calculated using Equation 3 as shown below;

Equation 3

$$\frac{ORAC}{kg} = \left[\frac{Net\ AUC_{(sample)} - c}{m}\right] \times DF$$

Where *m* is obtained from the gradient or the slope and *c* is the y-intercept of the plot of *NetAUC* against the concentration of standard (Trolox) as shown in Figure 3.2 and DF = dilution factor of sample.

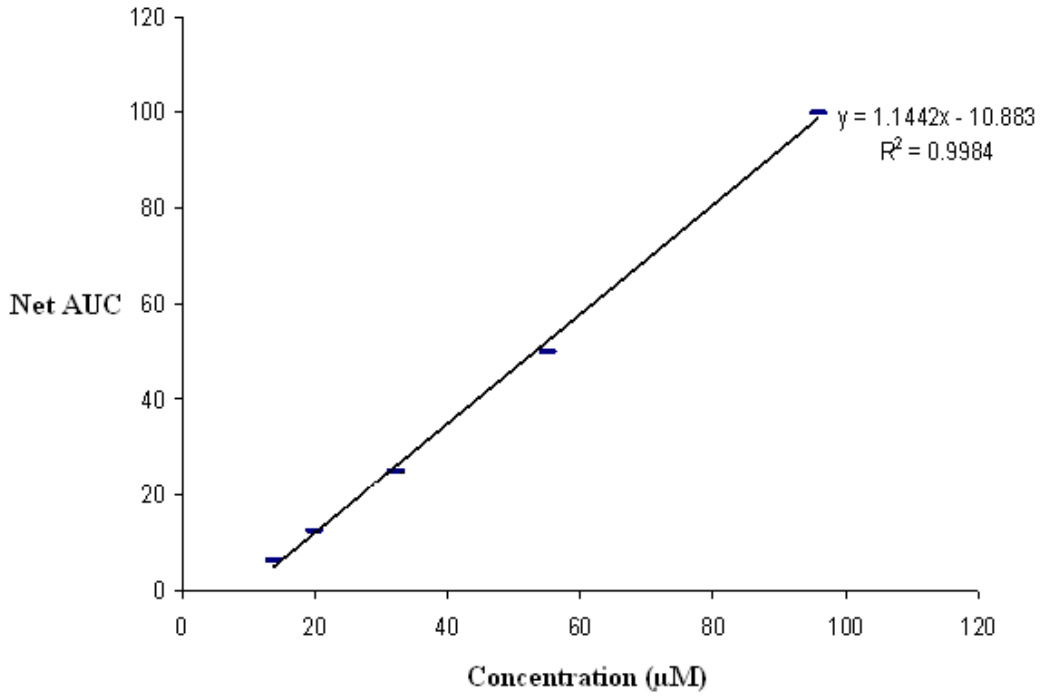

Figure 3.2: Plot of Net $AUC_{(TROLOX)}$ against concentrations (µM) of Trolox

The ORAC measurements were calculated for both the lipophilic and the hydrophilic extracts to produce the $ORAC_{(lipophilic)}$ and $ORAC_{(hydrophilic)}$ values respectively. The sum of these values produced the values for the $ORAC_{(total)}$, as shown in Equation 4 below;

Equation 4

$ORAC_{(total)} = ORAC_{(lipophilic)} + ORAC_{(hydrophilic)}$

The unit used for the ORAC values obtained from the calculation above is the *μM Trolox Equivalents/kg*.

For dense liquid samples for example, honey, calculation of the ORAC values require the density factor to be taken into account. Let the ORAC value be

$$x\mu M\ Trolox\ Equivalents \big/ kg$$

Firstly, converting the unit kg to g, as follows;

$$\frac{x\mu M\ Trolox\ Equivalents}{kg} \times \frac{1kg}{1000g} = \frac{x\mu M\ Trolox\ Equivalents}{g}$$

Followed by;

$$\frac{x\mu M\ Trolox\ Equivalents}{\left(\frac{g}{density(g/mL)}\right)} = x\mu M\ Trolox\ Equivalents \times \frac{density}{1mL}$$

From the above equations, the ORAC value of dense liquid samples was calculated using Equation 5 as shown below.

Equation 5

$$ORAC_{(sample)} = x\ \mu M\ Trolox\ Equivalents\ x\ \frac{density}{1mL}$$

Results in Table 3.1 show the ORAC values for both the lipophilic and the hydrophilic extracts, and the total ORAC values for the various samples. When equations 3 and 5 were utilised, all the values for the $ORAC_{(lipophilic)}$, $ORAC_{(hydrophilic)}$ and $ORAC_{(total)}$ made use of the unit *μM Trolox Equivalents/kg*.

As previously discussed, this unit is understood in the scientific community; however the significance of this unit is unclear by the wider community, in particular the consumer groups. In Australia, most nutritional information has been reported in the mass units, thus, conversion of ORAC values into the mass units is desirable for more effectively communication of the antioxidant capacity and also in line with the NIP standard.

Conversion to mass units

Let $ORAC_{(sample)}$ = *xμM Trolox Equivalents/kg*

The equation to be used to convert *μM Trolox Equivalents/kg* to the g/kg unit is shown below (Equation 6);

Equation 6

$$\frac{y\mu M\ Trolox\ equivalents}{kg} = \frac{y\mu M\ x\ 1M\ Trolox\ Equivalents}{1x10^{6}\ \mu M\ x\ kg}$$

$$= \frac{ymol\ Trolox\ Equivalents\ x\ V(L)}{L\ x\ 1\ x\ 10^{6}\ kg}$$

$$= \frac{ymol\ x\ MMg\ Trolox\ Equivalents\ x\ V}{mol\ x\ 1\ x\ 10^{6}\ kg}$$

$$= \frac{y\ x\ MMg\ Trolox\ Equivalents\ x\ V}{1\ x\ 10^{6}\ kg}$$

Where V = Volume of Trolox in litre (L), MM = molar mass of Trolox in g/mol.

Table 3.1 indicates the equivalent values of the ORAC in both molar and mass units.

Table 3.1: Samples reported in µM and mass (g) of Trolox equivalents for comparison purposes. The $ORAC_{(Total)} = \sum(ORAC_{(Hydrophilic)} + ORAC_{(Lipophilic)})$. Each sample tested was extracted and analysed in triplicate (*n=3*), with the % relative standard deviation for each sample set ≤ 10%.

Commodity Grouping (Reporting units)	Sample Tested	Brand / Manufacturer / Supplier	ORAC Hydro µM T.E/kg or µM T.E/L	ORAC Lipo µM T.E/kg or µM T.E/L	ORAC Total µM T.E/kg or µM T.E/L	ORAC Total g/kg T.E or g/L T.E
Stone Fruit (per kg)	Plum, red	Coles	35400	870	36270	9.1
	Plum, red	Coles	45100	540	45640	11.4
	Plum, red	Coles	38300	1150	39450	9.9
	Plum, red	Coles	42300	650	42950	10.7
	Plum, red	Coles	49000	900	49900	12.5
	Plum, red	Coles	32100	100	32200	8.1
	Plum, red	Coles	32200	100	32300	8.1
	Whole plum in juice	Coles	82200	200	82400	20.6
	Whole plum in juice	Coles	91100	230	91330	22.9
	Cherry, red	Coles	39600	400	40000	10.0
	Apricot halves in juice	Coles	7200	300	7500	1.9
	Peach halves in juice	Coles	4800	400	5200	1.3
	Peach halves in juice	Coles	4300	200	4500	1.1
	Peach slices in juice	Coles	7600	100	7700	1.9
	Peach, white	Coles	6800	400	7200	1.8
	Peach, white	Coles	7200	400	7600	1.9
	Whole Peach in syrup	Coles	12700	500	13200	3.3
	Whole Peach in juice	Coles	26200	500	26700	6.7
Berries (per kg)	Blueberry, fresh	Coles	71000	660	71660	17.9
Tea (per kg)	Lemon tea	Lipton	9000	2500	11500	2.9
	Lemon tea	Lipton	9600	2700	12300	3.1
	Green tea with citrus	Lipton	7900	1900	9800	2.5

(TAC = total antioxidant capacity; T.E = Trolox equivalents; NT = not tested. Results are taken as mean of n=3 replicates. Mass results are reported to 1 decimal place, and rounded up where necessary.)

Table 3.1: *continued*

	Raspberry tea	Lipton	9500	NT	9500	2.4
	Black tea	Lipton	2200	NT	2200	0.6
	Black tea	Lipton	3100	NT	3100	0.8
	Green tea	Lipton	16000	NT	16000	4.0
	Green tea	Lipton	14000	NT	14000	3.5
Citrus fruit (per kg)	Orange, Navel	Navel	41000	840	41840	10.5
Juice (per L)	Tropical juice	Golden circle	13000	NT	13000	3.3
	Tomato juice	Farmland	16000	NT	16000	4.0
	Cranberry juice	Bickfords	16000	NT	16000	4.0
	Pineapple juice	Golden circle	7600	NT	7600	1.9
	Grapefruit juice	Original Juice Company	34000	NT	34000	8.5
	Mango and banana juice	Berri	11000	NT	11000	2.8
Snacks (per kg)	Berry muesli bar	Coles	64800	1250	66050	16.5
	Berry muesli bar	Coles	61700	1230	62930	15.8
Confection (per kg)	Chocolate, dark	Black and Green's Organic	108300	10600	118900	29.8
	Chocolate, milk	Black and Green's Organic, 40%	113800	9400	123200	30.8
	Chocolate, dark	Lindt, 70%	163000	8800	171800	43.0
	Chocolate, dark	Lindt, 90%	272000	18000	290000	72.6
Dried Fruit (per kg)	Dried apple with peel	Coles	188500	5800	194300	48.6
Apple juice and blends (per L)	Apple juice	Berri	28000	NT	28000	7.0
	Apple juice	Berri	31000	NT	31000	7.8
	Apple and blackcurrant juice	Just Juice	22000	NT	22000	5.5
	Apple and blackcurrant juice	Just Juice	23000	NT	23000	5.8
	Apple and guava juice	Original Juice Company	20000	NT	20000	5.0
	Apple, mango and banana juice	Berri	17000	NT	17000	4.3
	Apple and mango juice	Berri	34000	NT	34000	8.5
Pears, fresh and processed (per kg)	Pear in syrup	SPC	4100	220	4320	1.1
	Pear in juice	Coles	11400	250	11650	2.9
	Pear, brown	Packham, Coles	16200	260	16460	4.1
Tropical fruit (per kg)	Mango	Kensington Pride, Coles	12000	NT	12000	3.0

(TAC = total antioxidant capacity; T.E = Trolox equivalents; NT = not tested. Results are taken as mean of n=3 replicates. Mass results are reported to 1 decimal place, and rounded up where necessary.)

Variations in the antioxidant activity of multiple sample sets, such as for plums, were observed as can be seen in Table 3.1. These variations between sample groups are to be expected, as variety, harvest time, ripeness, transport and storage were all contributing factors to these variations. In the case of plums, samples were taken on different days over a period of 2 weeks. All samples were handled in the same manner, and care was taken to avoid heavily bruised or damaged fruit. The inherent heterogeneity in a crate of fruit is also likely to contribute to the variations observed.

Samples that are more heavily processed, such as tinned fruits or fruit juices showed a more consistent $ORAC_{(Total)}$ value. Processed fruit such as pears or peaches in syrup had lower $ORAC_{(Total)}$ values compared with those in natural juice. This is to be expected, as natural fruit juices contain many nutrients and antioxidant compounds that contribute to the overall $ORAC_{(Total)}$ value of a commodity. Fruit juice had moderate $ORAC_{(Total)}$ values, possibly due to the use of additives or preservatives, or due to their preparation from fruit concentrates. In general, the combination of fruit flesh and juice following processing was found to result in higher $ORAC_{(Total)}$ values.

While the level of fat contained in the products varies depending on seasonal and processing variations, the $ORAC_{(Total)}$ values indicate that more potent antioxidants are usually found in the hydrophilic extract. Samples with higher fat content, such as the chocolate samples, had proportionally greater $ORAC_{(Lipophilic)}$ values.

The results shown in Table 3.1 can be readily compared in either mass or molar units. Comparatively, mass units are straightforward to deal with and mathematically sound. Reporting data in this manner is in keeping with current Australian labelling

conventions. It should be noted that in Australia regulations are not set out for ORAC antioxidant capacity labelling on products. In the absence of regulation or guidelines, mass unit reporting is a mode that allows consumers to conceptualise the antioxidant capacity information that is used in product marketing and health claims. $ORAC_{(Total)}$ values in the tens or hundreds of thousands lend themselves to product marketing and health claims. Mass units are open to less sensationalism, more readily accepted and understood by consumers and regulators, and easier to deal with on product packaging. The data significance is unchanged; however it is reported in a modest manner in keeping with current NIP labelling practice.

3.6 CONCLUSION

The ORAC assay was successfully applied to a range of commodities found in local supermarkets. Samples were extracted using 2 solvent systems to produce lipophilic and hydrophilic antioxidant capacity data. This data was then summed to provide $ORAC_{(Total)}$ values and these values were successfully converted to mass units for easy integration with current labelling practice in Australia, until such time as legislation is passed to mandate antioxidant capacity claims on products.

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4.0 Comparative studies on the antioxidant properties and polyphenolic content of wine from different growing regions and vintages; a pilot study to investigate chemical markers for climate change

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GRADUATE RESEARCH CENTRE

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1. PUBLICATION DETAILS (to be completed by the candidate)

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Amanda Sheard	15	Experimental design, laboratory support, manuscript drafting and review	[redacted]	1/12/17
Rohani Paimin	5	Manuscript review	[redacted]	5/12/17
Saman Buddhadasa	5	Manuscript review	[redacted]	28/11/17
Samantha Duong	5	Laboratory support, manuscript review	[redacted]	30/11/17
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Comparative studies on the antioxidant properties and polyphenolic content of wine from different growing regions and vintages, a pilot study to investigate chemical markers for climate change

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ABSTRACT

Many health benefits of wine result from specific polyphenolic compounds. Factors such as climate, CO_2 levels and region are known to affect polyphenolic compounds in wine; therefore a pilot study was conducted to focus on the Australian climate which has shifted from El Niño to La Niña. This research paper presents the influence of climate conditions and growing regions on the *in vitro* and *ex vivo* antioxidant capacity of red and white wine and the profile and concentration of polyphenols in these wines from the 2008 and 2009 vintages. The ORAC and polyphenolic data show that warmer climate wines had lower *in vitro* antioxidant capacity values but retained good bioavailability based on data from the RBC *ex vivo* assay compared to cool climate wines. Based on this pilot study, further research is being conducted at the National Measurement Institute, Australia (NMIA) with the goal of determining more polyphenolic compounds which appear to be affected by climate conditions.

4.1 PREAMBLE

This research has been published in *Food Chemistry* (January 2013) and presented at the 9th International Food Data Conference held 14-17 September 2011 at the Institute of Food Research, Norwich, United Kingdom. This paper presents data from a pilot study of the ORAC, CAA-RBC, total phenolic, total flavonoid and HPLC profile of Australian wines from 4 major regions. The pilot study focused on the vintage of highest and lowest rainfall for the regions studied, and in particular the 2008 and 2009 vintages. Warmer climate wines had lower ORAC values but performed well in the CAA-RBC assay. Six bioactive compounds were found to vary greatly within a single grape variety exposed to greater fluctuations in rainfall during key periods. This publication explores the second research question by presenting data which suggests environmental factors such as rainfall do influence key antioxidant parameters in certain grape varieties.

4.2 ABSTRACT

Many health benefits of wine result from specific polyphenolic compounds. Factors such as climate, CO_2 levels and region are known to affect polyphenolic compounds in wine; therefore a pilot study was conducted to focus on the Australian climate which has shifted from El Niño to La Niña. This research paper presents the influence of climate conditions and growing regions on the *in vitro* and *ex vivo* antioxidant capacity of red and white wine and the profile and concentration of polyphenols in these wines from the 2008 and 2009 vintages. The ORAC and polyphenolic data show that warmer climate wines had lower *in vitro* antioxidant capacity values but retained good bioavailability based on data from the RBC *ex vivo* assay compared to cool climate wines. Based on this pilot study, further research is being conducted at the National Measurement Institute, Australia (NMIA) with the goal of determining more polyphenolic compounds which appear to be affected by climate conditions.

4.3 INTRODUCTION

Much has been published regarding the health benefits of wine, many of which result from specific polyphenolic compounds (Iacopini *et al.* 2008). These compounds often display antioxidant activity and some, such as quercetin, have been found to have numerous functions including anti-inflammatory, antimicrobial and anticarcinogenic properties (Bisht *et al.* 2010; Katalinić *et al.* 2010; Kim *et al.* 2011). Often it is the many complex interactions between these functional compounds which result in a net benefit to health. For example, studies by Yang *et al.* (2008) showed a relationship between quercetin and resveratrol in the inhibition of fat cell production, thereby exerting

potential anti-obesity effects. Bioactivity is also attributed to grapes and their resultant wines as measured by 1,1-Diphenyl-2-picrylhydrazyl (DPPH) *in vitro* assay by Iacopini *et al.* (2008).

All polyphenolics, both flavonoid and non-flavonoid, contribute to the characteristics of wine (Iacopini *et al.* 2008). Given that factors such as climate, CO_2 levels and region are known to affect polyphenolic compounds in wine (Orduña 2010), a study was conducted to focus on the Australian climate. Specific wine growing regions were chosen, namely Margaret River (Western Australia), Yarra Valley (Victoria), Hunter Valley (New South Wales) and Barossa Valley (South Australia).

Data sourced from the Australian Government, Bureau of Meteorology (BOM) shows that Australia has undergone climate change trends over the last decade (2000-2010), with a transition from El Niño to La Niña resulting in increased rainfall in some wine growing regions.

Regions most notably affected by La Niña in 2008 were the Western Australia (WA) and New South Wales (NSW) regions, which experienced rainfall significantly higher (30%) than their ten year averages. This trend was also seen in the Margaret River and Hunter Valley region-specific BOM data. Conversely, the Yarra Valley in Victoria (VIC) and the Barossa Valley in South Australia (SA) were approximately 10% drier than the decade average. This is consistent with earlier Australian climate trends showing El Niño conditions on the continent between 2001 and 2005 and for much of

2007. The natural southern oscillation index (SOI) trends for the 2000-2010 decade show periods of both El Niño and La Niña, however the overall trend for the decade is El Niño (BOM 2011). To further compound this rainfall variation, overall temperature trends for Australia have increased which is a critical factor for grape quality (Orduña *et al.* 2010). In fact, 2001-2010 was the warmest decade on record, being on average 0.5ºC warmer than previous decades according to the BOM. This is part of a warming trend observed over the last 6 decades by the BOM, Australia.

Previous BOM data showed El Niño and La Niña events being up to 12 months long, however the period between 1990 and 2010 showed more El Niño events and more prolonged El Niño and La Niña events occurring.

Climate change models by Webb *et al.* (2007) and Anderson *et al.* (2008) have projected that a warmer climate will result in earlier harvest and have a negative impact on grape quality. In the past 10 years, irregular rainfall patterns in key wine producing regions have lead to concerns over the overall quality and palatability of Australian wines for domestic and export use. Climate change affects the quality of wine grapes and presents a harvesting challenge for producers. According to the Commonwealth Scientific Industrial Research Organisation (CSIRO) in 2010 (Davies & Thompson 2010), grape ripening is occurring earlier, resulting in shorter harvest seasons. This early maturation presents a challenge for the Australian wine industry, as harvest scheduling must be adjusted to maximise important quality aspects of grape varieties.

Given that 2001-2010 was the warmest Australian decade and that quality and grading are often based on polyphenolics such as anthocyanins, resveratrol, quercetin and catechins, a pilot study was conducted. This pilot study of the 2008 and 2009 vintages was carried out to test the hypothesis that polyphenolic compounds and resultant antioxidant activity could be used as chemical markers for climate change in Australian wines. This research paper presents data on climate conditions, *in vitro* and *ex vivo* antioxidant capacity of red and white wine and the concentrations of polyphenolics in these wines.

In order to test this hypothesis, 18 wines were purchased from the 2008 and 2009 vintages across 4 major growing regions in Australia. Growing regions include the Margret River in Western Australia, Yarra valley in Victoria, Barossa valley in South Australia and Hunter valley region in New South Wales. The 2008 vintage was chosen based on its extremes in rainfall pattern based on BOM data, and the 2009 vintage due to its rainfall being representative of the 2000-2010 decade averages overall. Wines were analysed as purchased for antioxidant capacity using the widely published oxygen radical absorbance capacity (ORAC) *in vitro* method.

In order to investigate bioavailability and *ex vivo* antioxidant capacity, the same wine samples were then freeze dried to remove alcohol content prior to being applied to human red blood cells. This cellular antioxidant activity – red blood cells (CAA-RBC) was first published by Blasa *et al.* (2011), and is a suitable assay for measuring antioxidant capacity of food extracts in human red blood cells. Wines were also analysed by two colorimetric methods, the Folin-Ciocalteu total polyphenolics assay

(PP) and the aluminium-chloride total flavonoids assay (Fla). Finally, an overall profile of 31 polyphenolic compounds was obtained for all wine samples in order to evaluate concentrations of specific compounds between growing regions. This pilot study provides data on polyphenolic profiles of wine produced under conditions of climate change, and presents data on antioxidant activity *in vitro* and *ex vivo*. The use of polyphenolic profiling as chemical markers of climate change can potentially be used to understand the of climate change on wine quality with implications for its grading, potential use and impact on human health.

4.4 METHODOLOGY

4.4.1 Chemicals

4.4.1.1 ORAC assay; Fluorescein disodium salt (FL), 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox™), quercetin dihydrate, monosodium phosphate monohydrate, disodium phosphate heptahydrate, hydrochloric acid (HCl), acetone and acetic acid.

4.4.1.2 Flavonoids (Fla) assay; sodium nitrite, aluminium-chloride (hydrated) and sodium hydroxide (NaOH).

4.4.1.3 Polyphenolic (PP) assay; Folin-Ciocalteu's phenol reagent (FC) (2N with respect to acid), sodium carbonate (Na_2CO_3).

4.4.1.4 Cellular antioxidant activity – red blood cell (CAA-RBC); phosphate buffered saline solution (PBS) (x10 concentrate solution), reverse osmosis water (RO), 2',7'Dicholorofluorescien diacetate (DCFH-DA), 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH), human red blood cells (RBC) (AB group) glutaraldehyde treated, and quercetin dihydrate.

4.4.1.5 Polyphenol profiling; formic acid (FA), methanol (MeOH), gallic acid (GA), protocatechuic acid (PA), chlorogenic acid (CA), vanillic acid (VA), caffeic acid (CFA), syringic acid (SA), ferulic (FER), p-coumaric acid (PCA), cinnamic acid (Cin. A), ± catechin (C), catechin gallate (CG), epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), epigallocatechin gallate (EGCG), gallocatechin (GC), gallocatechin gallate (GCG), rutin (RUT), myricetin (MYR), quercitrin (QCI), quercetin (QCE), naringenin (NAR), luteolin (LUT), hesperetin (HES), kaempferol (KAEM), aesculin (AES), scopoletin (SCO), 4-hydroxycoumarin (4OHC), 6-methylcoumarin (6MC) and caffeine (CAFF) were obtained from Sigma-Aldrich (Australia). All solvents were purchased from Merck™ Australia. Aluminium chloride ($AlCl_3$ hydrated) was purchased from BDH™.

4.5 MATERIALS

Black (sterile) 96-well microplates were used for the CAA-RBC assay, and black (non-sterile) 96-well microplates were used for the ORAC assay. Clear 96-well microplates were used for the PP and Fla assays. Falcon™ extraction tubes, 15mL and 50mL were used, (non-sterile) tubes for the ORAC, Fla and PP assays and (sterile) tubes for the CAA-RBC assay.

4.6 INSTRUMENTATION

4.6.1 FLUOstar OPTIMA

In this study, the FLUOstar OPTIMA microplate reader (BMG LabTechnologies) was used to obtain results for the ORAC, CAA-RBC, PP and Fla assays. The OPTIMA microplate reader system was equipped with 2 on board injector ports (with software version 2.0) for FL and AAPH addition in the ORAC assay. FI measurements were recorded over 120 cycles using wavelengths of 485nm for excitation and 520nm for emission (Stockham *et al.* 2011). Calculations were carried out using the OPTIMA MARS (version 1.2) software package. Briefly, the area under the FI decay curve was calculated, normalised, and compared to that of the Trolox calibration standards in the range of 6.25-100µM. Dilution factors for the wine were applied accordingly using MARS software (version 1.2), and hydrophilic ORAC values in µM T.E (Trolox equivalent) and µM Q.E (Quercetin equivalents) per litre were determined.

For the PP assay, a 30µL aliquot of each sample and standard was loaded onto a clear 96-well microplate. FC reagent and sodium carbonate addition was carried out using OPTIMA on-board injectors 1 and 2. Similarly for the Fla assay, samples, standards and nitrate solution additions were loaded manually onto the microplate. Again, on-board injectors 1 and 2 were used to add $AlCl_3$ and NaOH for the Fla assay. The experimental conditions for both polyphenolics and flavonoids follow those published by Marinova *et al.* (2005). CAA-RBC method; samples and standards were manually transferred to a sterile, black 96-well microplate and only AAPH addition was carried out by the OPTIMA injector 2. FI measurements were recorded over 60 cycles using wavelengths 485nm for excitation and 520nm for emission with antioxidant activity calculations as per Blasa *et al.*(2011).

4.6.2 Rapid Resolution Liquid Chromatography (RRLC) for polyphenolic profiling

An Agilent 1200 Rapid Resolution Liquid Chromatography (RRLC) system was used to obtain polyphenolic profiles of wine samples. Polyphenolic standards were prepared in methanol according to Novakova *et al.* (2010). An Agilent Technologies, Zorbax Eclipse Plus C18 50mm, 4.6mm I.D, 1.8µm column was used for this work. The polyphenolic LC screen used a gradient program with mobile phase (A) 0.1% formic acid (in deionised water) and mobile phase (B) methanol. The gradient program was carried out over 30 minute run time. Initially, 88.5% of mobile phase B was held from 0 – 4 minutes, then reduced to 85% by 5 minutes and 80% by 9 minutes. Mobile phase B was held at 80% from 9 – 18 minutes, after which B was reduced to 50% by 22.5 minutes. Finally the gradient was returned to starting conditions of 88.5% mobile phase

B between 22.5 and 24 minutes and held at 88.5% mobile phase B until the end of the run.

4.7 EQUIPMENT

A Thermo centrifuge and Thermo BB15 bench top carbon dioxide incubator were used for the CAA-RBC assay.

4.8 REAGENT AND STANDARD PREPARATION

4.8.1 ORAC assay

All solutions for the ORAC assay were prepared immediately prior to analysis according to Stockham *et al.* (2011). Hydrophilic extracts were diluted in phosphate buffer (PB) prior to analysis on the FLUOstar OPTIMA. PB was prepared according to Stockham *et al.* (2011).

4.8.2 Polyphenolic (PP) assay

FC reagent was used as supplied (2N w.r.t acid) and a 7.5% (w/v) solution of sodium carbonate was prepared in MilliQ water. FC reagent and sodium carbonate solution were delivered through injector 1 and 2 of the OPTIMA system respectively. A 200 mg

l^{-1} Gallic acid standard was prepared in 80% methanol and serially diluted to a concentration range of 12.5-200 mg l^{-1}.

4.8.3 Flavonoids (Fla) assay

A 10% (w/v) solution of aluminium-chloride, a 5% sodium nitrite solution and a 1M sodium hydroxide solution were prepared separately in MilliQ water. A 200 mg l^{-1} ± Catechin solution was prepared in 80% methanol, and serially diluted to a range of 12.5-200 mg l^{-1}.

4.8.4 CAA-RBC assay

A DCFH-DA stock solution was prepared in methanol and diluted to a working concentration of 25µM in 15mL Falcon™ tubes. A 10mM Quercetin stock was prepared (Blasa *et al.* 2011) and working standards made in sterile Falcon™ tubes post-RBC preparation. PBS stock solution was diluted 1:10 in RO water, with a pH 7.4. Human RBC (AB group) in glutaraldehyde solution was purchased from Sigma-Aldrich Australia. RBC's were rinsed in PBS at a ratio of 1:5 RBC to PBS, centrifuged, and the supernatant removed. Washing the RBC's removed the residual buffy coat and glutaradehyde. The RBC's were transferred to Falcon™ tubes where sample or antioxidant standard and DCFH-DA were added, and the contents made to a final volume of 10mL with PBS. The tubes were incubated at 37^0C for one hour. The RBC's were rinsed twice, made to final volume and transferred to a black 96 well microplate, again following the Blasa protocol.

4.9 SAMPLE PREPARATION AND EXTRACTION

18 wine samples (merlot, cabernet sauvignon, shiraz, chardonnay, and pinot noir) were purchased from liquor markets in Wyndham Vale, Victoria, Australia, and stored in the refrigerator until use. All samples were extracted and analysed in replicate (n=10) on the same day. All wine extracts were diluted in PB to the appropriate dilution factor and analysed by the OPTIMA microplate reader. Wines labelled as freeze dried have had alcohols removed and wines labelled as neat are as extracted as purchased with alcohol contents ranging from 9-15%. This extraction protocol was suitable for hydrophilic antioxidant compounds; therefore only ORAC (hydro) results are reported.

Wine aliquots for use in the PP and Fla assays were diluted in 80% methanol solution. Absorbance measurements were carried out by the OPTIMA microplate reader as per Marinova *et al.* (2005). Portions of each wine were freeze dried in an OPERON bench top freeze drier for 48 hours at -50ºC. 200 mL of each wine was freeze dried in 10mL aliquots. Half of the aliquots were reconstituted in PBS and the other half in ORAC phosphate buffer (PB) resulting in *n*=10 replicates of each wine for each antioxidant assay. All sample replicates were extracted and analysed on the same day. Extraction for RRLC profiling was carried out using 80% methanol solution. Wine samples were analysed by RRLC with diode array detection (DAD) of individual polyphenolic compounds and comparison to analytical standards.

4.10 RESULTS AND DISCUSSION

For the purposes of this paper, the following aspects of the study will be presented. The effect of alcohol and buffer choice on *in vitro* antioxidant capacity measurements in wine, a comparison of antioxidant capacity measurements of wine by *in vitro* and *ex vivo* methods, and the relationship between temperature profile and rainfall patterns of the regions compared and overall affect of climate on antioxidant activity of wines. Anomalies noticed in polyphenolic profiling and future work will also be discussed.

4.10.1 Effect of alcohol and buffer choice on in vitro antioxidant capacity measurements in wine

Due to the repeatability limitations of the *in vitro* ORAC assay, a comparison of the ORAC values for wines in the presence and absence of alcohol was conducted. Data from this comparison is shown in Table 4.1. Overall, removal of alcohol by freeze drying increased the ORAC values for both Trolox and Quercetin indices when prepared in PB, an interesting observation compared with earlier work by Pérez-Jiménez *et al.* (2006), who found that alcohols such as methanol do increase ORAC values. Although no study was conducted on the effects of ethanol on ORAC results, it is reasonable to hypothesize that this alcohol content in wine would affect ORAC values in a similar manner to methanol.

Table 4.1: Comparison of antioxidant capacity measurements of VIC wines by *in vitro* methods. ORAC values reported are the mean of *n*=10 replicates. Neat wines contain alcohol, freeze dried wines have had alcohol removed prior to extraction and analysis.

		Chardonnay			**Shiraz**			**Merlot**		
		ORAC	SD	%RSD	ORAC	SD	%RSD	ORAC	SD	%RSD
Freeze-dried (PB)	µM TE/L	6100	840	14	42400	5300	14	44300	6900	15
	µM QE/L	510	80	15	2900	500	17	3600	610	17
Neat (PB)	µM TE/L	4900	390	8	43200	4600	11	37800	2200	6
	µM QE/L	450	40	8	2600	280	11	3000	210	7
Neat (PBS)	µM TE/L	5600	1100	19	41000	4400	10	29600	4800	16
	µM QE/L	300	60	20	6300	900	14	3700	450	12

(PB = phosphate buffer for ORAC; PBS = phosphate buffer saline for CAA-RBC; %RSD = % relative standard deviation, SD = standard deviation; µM T.E/L = micro molar Trolox equivalents per litre; µM Q.E/L = micro molar Quercetin equivalents per litre)

Ethanol content of wine commonly ranges from 5-15% depending on variety, a much higher level than trace amounts of methanol by-product. It is therefore likely that ethanol would also exhibit similar inflation of the ORAC value. However, the opposite is seen. Wine samples were freeze dried under vacuum to remove all moisture including alcohols. The residue was then redissolved in PB back to original volume, therefore no concentration effects exist. Increased ORAC values after the removal of alcohol may be attributed to stronger synergistic effects of polyphenolic compounds following the removal of alcohol. Commercial low-alcohol wines should also be investigated to note

any trends. Given that both *in vitro* and *ex vivo* methods were used to study the antioxidant properties of wines, a comparison of the effects of buffer on overall antioxidant capacity values was also carried out. The PBS at pH of 7.4 had to be used for red blood cell work; therefore a trial of PBS for diluting neat wines for ORAC assays was also carried out. These results are also shown in Table 4.1 and can be compared with the ORAC values of neat wines prepared in PB. Overall, shiraz and merlot ORAC values are lower in both indices when prepared in PBS. This may be due to the higher impurities present in the PB than the PBS used for cell work. Impurities such as metals, and in particular iron and copper, can interrupt peroxyl radical attack on the FL probe, potentially having a net effect of protecting the FL probe and slowing the loss of fluorescence. This would result in an overall greater ORAC value for the wines, and is a possible reason for the result observed.

Another observation is that of the % relative standard deviations (%RSD) under different conditions. The hypotheses that PB impurities and alcohol removal affect antioxidant capacity appears to be supported. For example, both neat PBS and freeze dried PB extracts had an average %RSD of 15%, whereas the neat PB extract had a much lower %RSD of only 8%. It is noteworthy that neat PBS extracts do have alcohol present, however lower impurities of metals, the opposite is true for the PB extracts. In the case of neat PB extracts, both alcohol and metal impurities may affect the ORAC value. Put simply, the presence of either alcohol or metal impurities appears to result in higher variations in the ORAC value. The presence of both alcohol and metal impurities appears to give a more reproducible ORAC measurement overall. This further supports earlier reports of the affect of alcohols and metals on the ORAC assay (Pérez-Jiménez *et al.* (2006) and Nkhili *et al.* (2011)).

Furthermore, bioavailability of antioxidant compounds appears to be a more reproducible parameter than *in vitro* ORAC assay results. This is evidenced by the %RSD values in Table 4.2, which show that the ORAC %RSD are approximately 15-17%, whereas CAA-RBC *ex vivo* results are in the range of 1.3 to 7.2% RSD.

Table 4.2: Comparison of antioxidant capacity measurements of wine by *in vitro* and *ex vivo* methods. Results are reported as the mean of *n*=10 replicates. All wines in this comparison were freeze dried to remove alcohol.

	Chardonnay			Shiraz			Merlot		
Freeze-dried (PB)	µM QE/L	SD	%RSD	µM QE/L	SD	%RSD	µM QE/L	SD	%RSD
ORAC	510	80	15	2900	500	17	3600	610	17
CAA-RBC	76	1.8	2.5	606	44	7.2	390	5.4	1.3

(PB = phosphate buffer for ORAC; PBS = phosphate buffer saline for CAA-RBC; %RSD = % relative standard deviation)

It is unlikely that differences in buffering capacity between buffers would result in PBS ORAC values being lower, as the FL probe performance in both assays was comparable. This indicates that the optimal pH of 7.4 was maintained in both ORAC assays, and therefore other impurities or factors may contribute to this variation between PB and PBS results.

The wines analysed in this part of the study were all from the same growing region in Victoria (VIC), and are therefore subject to the same climate factors including temperature, rainfall and soil conditions. The primary variables in this part of the study were therefore buffer choice and alcohol content.

4.10.2 Comparison of antioxidant capacity measurements of wine by *in vitro* and *ex vivo* methods

In order to evaluate trends in antioxidant estimation with bioavailability, a comparison of the same wine samples from VIC was conducted. Wines were freeze dried and analysed by the traditional ORAC method for hydrophilic antioxidants (as per Stockham *et al.* 2011) using PB. The one variation on the Stockham *et al.* method was the use of quercetin as a standard for comparing ORAC values. This was done in an effort to express values using the same standard for equivalence. Separate aliquots of these wines were also freeze dried and then redissolved in PBS and applied to human red blood cells. The CAA-RBC method was then followed according to Blasa *et al.* (2011). Data from this study is presented in Table 4.2. It can be seen that although the shiraz and merlot *in vitro* ORAC values are very similar, they differ significantly in bioavailability in the *ex vivo* "cell model", e.g. the shiraz bioavailability is twice that of the merlot. This demonstrates that although ORAC values are widely used in literature, cell models are ultimately the most appropriate methods for understanding the antioxidant activity in biological systems and the ability of these compounds to permeate cell membranes. Furthermore, RBC data from these studies shows an overall lower variation in results for a number of replicates (n = 10).

4.10.3 Relationship between temperature profile and rainfall patterns of the regions compared and overall affect of climate on antioxidant activity of wines

The annual temperature averages are shown in Figure 4.1. Briefly, the annual average temperatures for the 2000-2010 decade were similar for the VIC, WA and SA wine growing regions; however the NSW Hunter Valley region average temperature was approximately 3 – 4ºC warmer for the decade.

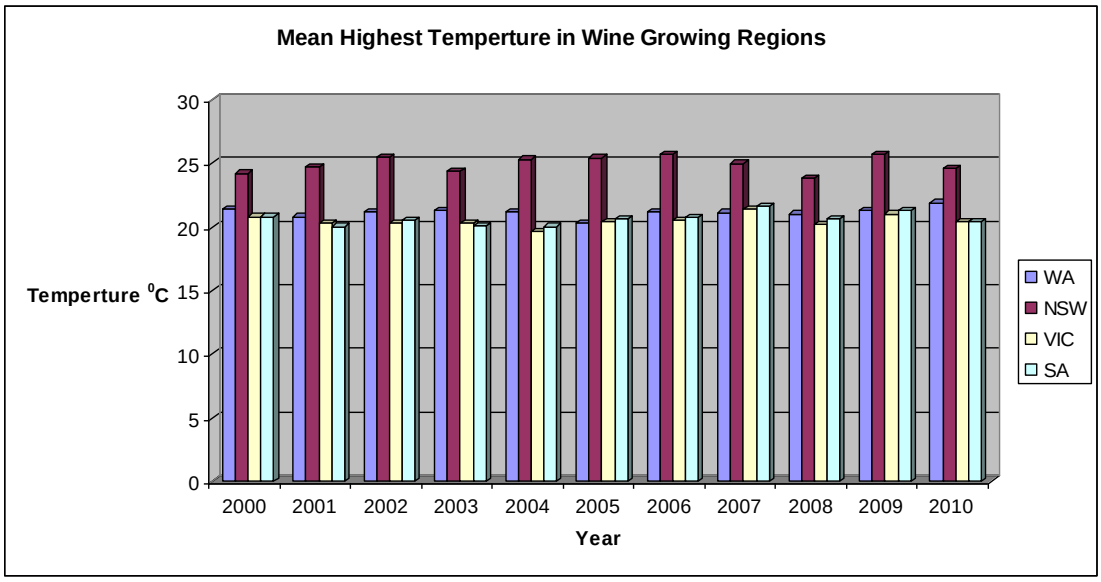

Figure 4.1: Chart of temperature patterns over the 2000 – 2010 decade for WA, VIC, SA and NSW.

Figure 4.2 shows the annual rainfall data trends for the 2000-2010 decade for the 4 regions in this study. It can be seen that the WA and NSW wine growing regions were wetter in the 2008 vintage, and VIC and SA wine growing regions were drier in the 2008 vintage. Figure 4.2 also shows the rainfall trends for 2009 being representative of the decade average rainfall across all 4 wine growing regions, providing an appropriate comparison point for this pilot study.

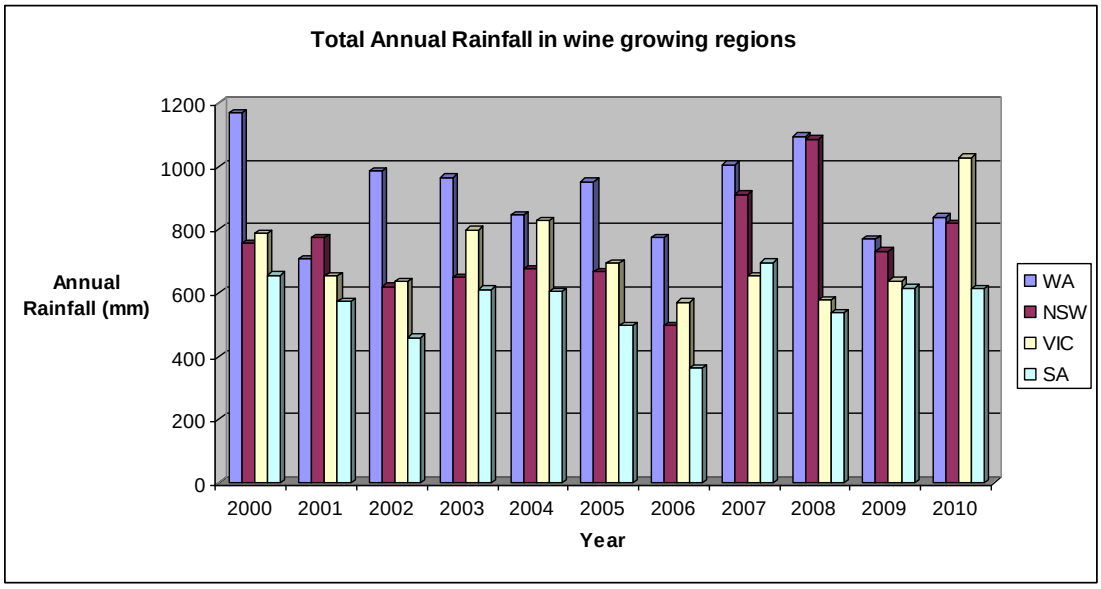

Figure 4.2: Chart of rainfall patterns over the 2000 – 2010 decade for WA, VIC, SA and NSW.

The relationship between rainfall trends and antioxidant activity are summarised in Table 4.3. Despite having very similar rainfall patterns, NSW and WA ORAC values showed the greatest variation, with WA shiraz having a 26% higher ORAC value than NSW shiraz. This trend is reversed when examining the CAA-RBC data, where although having a lower ORAC result, the NSW shiraz appeared to have higher bioavailability with respect to antioxidant compounds.

Table 4.3: Comparison of annual rainfall trends in key growing regions and the antioxidant activity of wines from these regions.

	ORAC	CAA-RBC	PP	Fla	Annual Rainfall
	µM TE/L	µM QE/L	g/L GAE	g/L CE	(mm)
2008					
NSW Shiraz	36400	750	2.9	1.7	1086
WA Shiraz	49400	600	1.7	1.2	1095
SA Shiraz	41900	650	2.9	1.9	539
VIC Shiraz	40600	700	3.5	1.7	579
2008					
NSW Chardonnay	5100	150	0.4	0.34	1086
WA Chardonnay	3800	80	1.2	0.38	1095
SA Chardonnay	5800	80	1.2	0.43	539
VIC Chardonnay	5800	100	1.2	0.43	579
2008					
SA Cabernet Sauvignon	30400	400	3.4	1.1	539
VIC Cabernet Sauvignon	31900	620	2.8	1.4	579
2008					
SA Merlot	28000	640	1.1	1.4	539
VIC Merlot	32300	670	2.8	1.9	579
2009					
NSW Cabernet Merlot	32700	540	3.2	1.9	
NSW Cabernet Sauvignon	26300	640	2.7	1.9	731
NSW Shiraz	38700	750	3.0	3.1	
NSW Pinot Noir	31900	670	1.1	1.8	
VIC Pinot Noir	41500	600	3.3	2.6	638
WA Pinot Noir	45900	700	2.8	1.8	769

(GAE = gallic acid equivalents; CE = catechin equivalents)

With the exception of WA Margaret River wine growing region, all other regions had very similar ORAC results for chardonnay, irrespective of rainfall or temperature patterns for the 2008 vintage. Differences are mostly noted between the VIC and WA pinot noirs when compared to the NSW pinot noir. In this instance, the ORAC value for the NSW pinot noir was significantly lower than both the VIC and WA varieties. Again, bioavailability, as measured by the RBC assay was not significantly affected. Total PP and Fla data is also represented, however these colorimetric methods are susceptible to interferences, making trend analysis difficult for this study. Data is included as these colorimetric assays are widely used and may be used for comparison purposes by other researchers working in the area of viniculture. Briefly, total polyphenolic content by PP assay appears to be lower for all chardonnays when compared to the red wines. PP data from VIC also appears to be slightly higher for shiraz and merlot than for SA in the 2008 vintage.

4.10.4 Anomalies noticed in polyphenolic profiling and future work

During the RRLC analysis of the 18 Australian wines, polyphenolic profiles from wines of the same variety showed similar profiles. Table 4.4 summarises the data obtained. Interestingly, CFA was detected in all red wines from the VIC region, but was absent from all red wines from the NSW, WA and SA wine growing regions. GCG and QCI were not detected in any of the wines studied. Furthermore, RUT appeared to be most variable. This is likely due to the variation in natural sugar profiles which affect the glycosidic moieties associated with RUT. Sugar profile in wine is influenced by climate, region and variety, and is another factor which should be considered for future work.

Table 4.4: Comparison of polyphenolic content across WA, VIC, SA and NSW targeted wine growing regions for 2008 and 2009 vintages.

	Region	Wine	mg/L															
			GA	PA	AES	VA	CA	CFA	PCA	GCG	SCO	EGC	CG	4OHC	RUT	NAR	QCI	6MC	QCE
2008	VIC	Cabernet Sauvignon			0.30	0.31		0.13	0.13									0.17	
	SA	Cabernet Sauvignon			0.35		0.14	0.16							0.19				
	NSW	Chardonnay	0.13	0.31		0.32			0.17										
	SA	Chardonnay							0.16										
	VIC	Chardonnay		0.31		0.31			0.19									0.16	0.07
	WA	Chardonnay	0.08				0.12		0.15									0.09	0.18
	VIC	Merlot			0.34	0.31		0.17	0.21		0.10				0.22	0.42		0.17	
	SA	Merlot			0.34	0.31	0.14	0.16							0.14			0.19	
	VIC	Shiraz			0.33	0.31		0.19	0.28						0.13			0.10	
	SA	Shiraz			0.32	0.32	0.14	0.19				0.20			0.39				
	NSW	Shiraz	0.08			0.32	0.15	0.24										0.20	
	WA	Shiraz			0.33	0.31	0.14	0.20							0.06			0.19	
2009	NSW	Cabernet Merlot			0.33	0.31	0.16	0.23			0.09							0.18	
	NSW	Cabernet Sauvignon		0.32		0.31	0.15		0.21		0.09			0.03				0.18	
	NSW	Pinot Noir		0.31		0.32	0.14		0.19									0.17	
	WA	Pinot Noir																0.17	
	VIC	Pinot Noir				0.32	0.14		0.19					0.04		0.39		0.16	
	NSW	Shiraz		0.32		0.31	0.15		0.22				0.33						

(VIC = Yarra Valley, Victoria; SA = Barossa Valley, South Australia; NSW = Hunter Valley, New South Wales; WA = Margaret River, Western Australia)

In the case of NSW and WA, both wine growing regions share similar rainfall patterns for the 2008 vintage, though NSW Hunter Valley temperature is higher. Despite these similarities, WA Margaret River pinot noir from the 2009 vintage only had detectable levels of 6MC. In contrast, NSW Hunter Valley pinot noir from the same vintage has more detectable phenolics such as PA, VA, CA and PCA. NSW and WA shiraz had similar levels of VA, CA, CFA and 6MC for the 2008 vintage, however WA shiraz also had AES and RUT which where not detected in the NSW variety.

Cooler climate wines from SA Barossa Valley and VIC Yarra Valley were also studied. The 2008 vintage cabernet sauvignon from SA and VIC were compared and AES and CFA concentrations were similar. VA, PCA and 6MC were only detected in VIC Yarra Valley cabernet sauvignon, while CA and RUT were unique to the SA Barossa Valley variety for the 2008 vintage. PCA was also detected in VIC Yarra Valley shiraz but not in any other shiraz from the 2008 vintage.

The polyphenolic profiling data show that while the majority of polyphenolics in a variety of wine are similar, there are notable exceptions such as 6MC, PA, VA, CA, PCA and RUT. These polyphenolic compounds should be investigated further as chemical markers for climate change as they appear to vary the most by wine growing region. Further studies on the effect of soil composition on polyphenolic content in Australian wines under El Niño and La Niña conditions are needed.

4.11 CONCLUSION

Prolonged El Niño and La Niña events over the last decade have been observed in Australia. El Niño has been reported to have a negative impact on grape quality. In this study the ORAC and polyphenolic data show that the warmer NSW climate wines had lower antioxidant capacity values *in vitro* but still retained good bioavailability based on data from the RBC *ex vivo* assay. It was noted that %RSD was greatest in the *in vitro* assay, and that buffer impurities and alcohol content of wines also appeared to contribute to variation in measurement. The %RSD was lower for the *ex vivo* CAA-RBC assay, which was attributed to the fact that cell models are more appropriate for understanding and reporting antioxidant ability, as they account for permeability of antioxidants across cell membranes.

Cell models also provide a better representation of synergistic effects of antioxidants. Data from the CAA-RBC model showed that although shiraz and merlot wines had very similar *in vitro* results, their *ex vivo* bioavailability was vastly different. Data from the polyphenolic profiling of wine showed that 6MC, PA, VA, CA, PCA and RUT are potential chemical markers for climate change due to their variation by wine growing region. This variation was noted in regions with similar climate profiles, leading to the need for further studies on the effect of soil composition on polyphenolic content.

Based on this pilot study, further research is being conducted at the National Measurement Institute, Australia (NMIA) with the goal of determining more polyphenolic compounds which appear to be affected by climate conditions.

4.12 ACKNOWLEDGEMENTS

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5.0 Antioxidant synergies in superfoods – role of amino acids and copper nanoparticles in influencing the Oxygen Radical Absorbance Capacity (ORAC) of fruit concentrate powders

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I declare that the publication above meets the requirements to be included in the thesis as outlined in the HDR Policy and related Procedures – policy.vu.edu.au.

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Name(s) of Co-Author(s)	Contribution (%)	Nature of Contribution	Signature	Date
John Orbell	20	Experimental design, manuscript drafting and review	[redacted]	27/11/17
Rohani Paimin	5	Manuscript review	[redacted]	05/12/17
Saman Buddhadasa	5	Manuscript review	[redacted]	27/11/17

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5.1 PREAMBLE

Chapter 5 has been submitted to *Food Chemistry* (June 2018) and the ORAC data has been presented at the 11th Government Food Analysts Conference, held 22-24 February 2009 at the Treacy Conference Centre, Parkville, Melbourne, Australia. Density functional theory (DFT) calculations have been carried out and support the experimental data in the submitted manuscript. This paper describes the addition of amino acids and copper nanoparticles (CuNPs) to "superfood" concentrates of bilberry, coffee berry and apple. These concentrate powders all exhibited strong antioxidant activity in the ORAC assay, and extracts of these matrices were supplemented with an amino acid, CuNPs, or a combination of both. Extracts were then compared via the ORAC assay to determine if antioxidant synergies could be observed and to what extent these synergies were reproducible. Finally, the concept of "redox and substrate zones" is proposed and DFT calculations assessed how homolytic bond dissociation energies for phenolic O-H groups can be affected by interactions between the matrix and the substrate zone of an antioxidant and/or an amino acid, hence postulating a mechanism for antioxidant enhancement/synergy at the molecular level. These observations have not been presented elsewhere in literature, and represent novel work and provide a theory for how antioxidant synergy occurs *in vitro*, including the role of amino acids and CuNPs in these mechanisms in response to research question 3.

5.2 ABSTRACT:

The oxygen radical absorbance capacity (ORAC) assay is one of the most widely used assays for measuring the antioxidant capacity of food. Increasingly, so-called "superfoods" are promoted, sometimes with ORAC values reported on their labelling. In an effort to understand the influencing factors on the ORAC values of typical "superfoods" – here, bilberry, apple and coffee berry concentrate powders, the antioxidant effect of adding potential supplements, either individually or in combination, has been investigated. These include histidine, 4-hydroxy-proline, methionine, tyrosine, tryptophan and copper nanoparticles. The addition to the "superfood" matrices of the amino acids, the copper nanoparticles and a mixture of the latter two has a dramatic effect on antioxidant amplification. Thus, antioxidant synergies were observed in all cases, with ORAC values being enhanced 3 to 25 fold, yielding unusually high ORAC values. Possible mechanisms for the extraordinary enhancement of antioxidant activity have been investigated via Density Functional Theory molecular modelling.

Keywords; antioxidants, superfoods, amino acids, copper nanoparticles, density functional theory

5.3 INTRODUCTION

Much has been reported on the antioxidant properties of so-called "superfoods", many of which are fruits or fruit concentrates (Havsteen, 2002; López-Alarcón and Denicola, 2013). Antioxidant properties are often attributed to phytochemicals readily found in fruits, such as polyphenolics, flavonoids and vitamins (Tripoli *et al.*, 2007; de Marino, Iorizzi and Zollo, 2008; Jensen *et al.*, 2008; Michiels *et al.*, 2012). A steady increase in the reporting of "superfoods" in product labelling and advertising has also led to efforts to increase antioxidant capacity of food products (Kirakosyan and Kaufman, 2009; Shahidi and Ambigaipalan, 2015). Often this can be achieved by the addition of "superfood" concentrates to existing foods and beverages.

These foodstuffs are increasingly popular, and advertising campaigns are often aimed at consumers who are health conscious. Much effort is invested in product development to enhance antioxidant properties of existing products as well as to develop entirely new ones (Ishige, Schubert and Sagara, 2001; Silván *et al.*, 2006; Shahidi and Chandrasekara, 2010; Nimalaratne *et al.*, 2011; Sandhir *et al.*, 2015). Furthermore, there are many examples in the literature of the antioxidant properties of fruits and vegetables, with some well-known examples such as goji berries, blueberry, acai berries and cocoa products such as dark chocolate (Wu *et al.*, 2004).

The continual expansion of the "superfoods" industry raises the question of how antioxidant properties or measurements can indeed be enhanced and what classes of compounds might be involved. In this study, the antioxidant capacity of a number of amino acids and copper nanoparticles were measured by the oxygen radical absorbance capacity (ORAC) assay both for baseline antioxidant capacity and for their ability to enhance the antioxidant capacity of "superfoods". ORAC is one of the most widely used antioxidant capacity assays, and the results

continue to be used in product labelling internationally. Amino acids were chosen for this study as they are crucial to muscle recovery after exercise (Williams, 2005). Health conscious individuals are likely to be attracted to amino acid supplements but little is published on their actual contributions to antioxidant measurements. Similarly, copper nanoparticle suspensions are sold and marketed as a dietary supplement.

Garret et al. (2014) reported baseline ORAC values of 20 amino acids, citing tryptophan, tyrosine and methionine as the only three with antioxidant activity. In their study they further investigated the role of amino acid structure, and reported that carbon ratio and conjugated double bond length were important structural features which contributed to antioxidant property of these amino acids. To date, very little is published on the role of free amino acids and copper nanoparticle dietary supplements with respect to their influence on antioxidant measurements, particularly in food systems (Sandhir *et al.*, 2015; Vilela, Gonz??lez and Escarpa, 2015). This study explores the relationship between amino acids, copper nanoparticles and antioxidant measurements in real "superfood" systems.

As stated, ORAC is one of the most widely used antioxidant capacity assays. This study investigated the baseline ORAC values of all 20 amino acids, as well as a copper nanoparticle solution. Amino acids which responded to the ORAC assay, or whose chemical structures were deemed to make them good candidates, were subjected to trials with fruit concentrate powders. The trials were carried out in the presence and absence of amino acids, copper nanoparticles, and fruit concentrate powders.

This systematic study presents the antioxidant synergies observed in the ORAC assay for histidine, 4-hydroxyproline, methionine, tyrosine and tryptophan, in the presence of

"superfood" fruit concentrate powders, and copper nanoparticles. Amino acids that did not respond to the ORAC assay are not presented. All extract experiments were carried out in replicate (*n=10*), and only the hydrophilic extraction protocol is reported, as this fraction yields the overwhelming majority of antioxidant capacity measurable by the ORAC assay in this study.

5.4 MATERIALS AND METHOD

5.4.1 Chemicals

5.4.1.1 ORAC assay: The following chemicals were all obtained from Sigma-Aldrich (Australia) - fluorescein disodium salt (FL); 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH); 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox™); monosodium phosphate monohydrate; disodium phosphate heptahydrate; hydrochloric acid (HCl); acetone; acetic acid; and the following amino acids were obtained as neat analytical standards: *L*-Histidine; *L*-Methionine; 4-Hydroxy-*L*-Proline; *L*-Tyrosine and *L*-Tryptophan. Fruit concentrate powders of apple, coffee berry and bilberry were sourced from Nutradry Australia and were stored unopened at -20°C until use. A copper nanoparticle colloidal suspension (7.4mg/L) was sourced from Australian Natural Colloids and stored at room temperature.

5.4.2 Reagent and standard preparation

5.4.2.1 Phosphate buffer (PB): 2L of 75mM aqueous phosphate buffer was prepared with monosodium phosphate monohydrate and disodium phosphate heptahydrate and the pH adjusted to 7.4 using 1M hydrochloric acid (HCl) prepared from 10M HCl stock. All subsequent working solutions were prepared in phosphate buffer.

5.4.2.2 Fluorescein solution (FL): A stock solution of FL was prepared monthly at 0.7mM concentration. The final working solution of FL was achieved by serial dilution of the stock with buffer to a final concentration of 70nM. The working FL solution was prepared from stock on a daily basis.

5.4.2.3 AAPH peroxyl radical solution: AAPH solution was prepared to a concentration of 35mM in phosphate buffer. Due to the thermal sensitivity of AAPH, the working solution was prepared just prior to analysis.

5.4.2.4 Trolox antioxidant standard: A 10mM stock solution was prepared weekly. The stock solution was serially diluted daily with PB. A concentration range of 6.25-100µM was used for the preparation of calibration plots for quantitation.

5.4.2.5 Hydrophilic extraction solution: Acetone/water/acetic acid (AWA) 70:29.5:0.5 (v/v/v) solvent was prepared for the extraction of hydrophilic antioxidants. Hydrophilic extracts were diluted in PB prior to analysis on the FLUOstar OPTIMA instrument.

5.4.2.6 Amino acid standard solutions: A 20nmol/mL solution of each of the five amino acids was prepared fresh daily in PB.

5.4.2.7 Computational chemistry: Relative homolytic bond dissociation energies (BDEs) were computed by Density Functional Methods at the B3LYP/6-311++G(2DF,2P)/PM3 level of theory using Spartan'16 (Wavefunction, Inc, 18401 Von Karman Avenue, Suite 370, Irvine, CA 92612 USA, www.wavefun.com). Such energies are a recognized measure of a molecule's antioxidant capacity (Wijtmans *et al.*, 2003).

5.4.3 Materials

Black (non-sterile) 96-well microplates were used for the ORAC assay. 15mL and 50mL Falcon™ extraction tubes were employed.

5.4.4 Sample preparation and extraction

5.4.4.1 Extraction of fruit concentrate powders: A mass of 0.5g of each sample was weighed into 50mL extraction tubes in replicate (*n = 10*). The samples were then extracted with hydrophilic extraction solution (AWA), and serially diluted in PB. A range of dilution levels were analysed using the ORAC assay, and the optimal dilution level determined for each matrix.

5.4.4.2 Supplementation of extracts: Replicate extracts at appropriate dilution were supplemented with either (a) amino acid standard solution, (b) copper nanoparticle solution, or both (a) amino acid and (b) copper nanoparticle solution. This was achieved by replacing some of the PB volume with amino acid or copper nanoparticle solution at the final stage of sample extract dilution. The final extracts contained either; (I) no addition, (II) added amino acid (at 10% of the extract volume), (III) added copper nanoparticle solution (at 10% of the extract volume) or (IV) both added amino acid and copper nanoparticle solution (both at 10% of the extract volume). For example, apple concentrate powder extract was diluted 10,000 fold. For the final dilution stage, 1mL of 1,000 fold diluted extract was added to a 10mL extraction tube. Then, 1mL of copper nanoparticle solution (7.4mg/L concentration, as sold), 1mL of 20nmol/mL amino acid standard solution, or 1mL of both solutions were added. The extract was then made to 10mL final volume using PB. The ORAC results could then be compared to the baseline values of the sample extract at appropriate dilution level. Using this approach, the influence of amino acids and copper nanoparticle solution on antioxidant capacity measurements in the "superfood" extracts could be determined and quantified. The relative standard deviation of ORAC value between replicates was < 10% where *n=10*.

5.4.5 Instrumentation

5.4.5.1 FLUOstar OPTIMA: In this study, the FLUOstar OPTIMA microplate reader (BMG LabTechnologies) was used to obtain results for the ORAC assays. The OPTIMA microplate reader system was equipped with two on-board injector ports (with software version 2.0) for FL and AAPH addition in the ORAC assay. The incubation temperature was set to 37.3°C (±0.3°C) for the duration of the assay. Following addition of FL to all wells, a 15 minute incubation time was observed. APPH was then added to all wells and fluorescence intensity (FI) measurements were recorded over 120 cycles using wavelengths of 485nm for excitation and 520nm for emission (Stockham *et al.* 2011). Calculations were carried out using the OPTIMA MARS (version 1.2) software package. Briefly, the area under the FI decay curve was calculated, normalised, and compared to that of the Trolox calibration standards in the range of 6.25-100µM. Dilution factors for the "superfood" extracts were applied accordingly using MARS software (version 1.2), and hydrophilic ORAC values in µM T.E (Trolox equivalent) per kg were determined.

5.5 RESULTS AND DISCUSSION

Throughout the study, consistent elevation of ORAC values were observed for supplement-enhanced "superfood" matrices, Table 5.2. Initially, a total of 20 amino acids were analysed by ORAC assay to assess their individual baseline antioxidant properties. None of these exhibited significant ORAC values except for methionine, tyrosine and tryptophan. Therefore, these were selected as potential supplement additives, as were histidine and 4-hydroxy-proline, since a consideration of their molecular structures suggested that they could have antioxidant

potential under certain conditions. For the above selected amino acids, the BDEs for potentially labile hydrogen atoms are given in Table 5.1 and compared to their ORAC values. The calculated BDEs that are less than 100 kcal/mol could suggest some antioxidant potential[1]. Notably, the combination of CuNPs and His is predicted to have high antioxidant activity at 78.7 kcal/mol, although the ORAC values suggest that the presence of His actually dampens the capacity of the CuNPs themselves. However, these results are suggestive of the CuNPs interacting with an amino acid to affect the antioxidant behavior. This is consistent with our subsequent investigations on the effect of amino acid/CuNP additives to "superfood" matrices compared to just amino acid additives alone – see below.

Table 5.1. Calculated BDEs for relevant O(N)-H in His, Tyr, Tryp and His/Cu(I) compared to experimentally derived ORAC values. Amino acids are assumed to be in the zwitterionic form.

	BDE (kcal/mol)	ORAC (μM T.E/L)
4-OH-Pro	109.3 / 102.6	ND
His	104.2	ND
Met	-	45
Tyr	91.3	70
Tryp	96.4	177
CuNPs	-	243
4-OH-Pro/CuNPs	-	58
His/CuNPs	78.7	98
Met/CuNPs	-	126
Tyr/CuNPs	-	257
Tryp/CuNPs	-	343

Table 5.2 presents the observed antioxidant synergies/enhancements (as measured by ORAC) for the addition of 4-OH-Proline, Histidine, Methionine, Tyrosine, Tryptophan and Copper

[1] Note that, under the same level of theory our calculated BDE for Trolox is 80.4 kcal/mol (the experimentally determined value is 80.9 ± 1 kcal/mol, (Bordwell and Liu, 1996).

Nanoparticles, individually and in combination, with three different "superfood" matrices, namely Bilberry, Coffee Berry and Apple. This data is also represented in Figure 5.1.

From Table 5.2 and Figure 5.1, it is evident that this data is highly replicable and distinct trends are apparent.

Table 5.2. Changes in the ORAC values of three "superfoods" as a result of the addition of **(a)** five selected amino acids; His = Histidine; 4-OH-Pro = 4-Hydroxyproline; Met = Methionine; Tyr = Tyrosine and Tryp = Tryptophan **(b)** copper nanoparticles; CuNPs **(c)** the five selected amino acids plus copper nanoparticles. Each ORAC value is the mean of n=10 replicates and is in units of μM TE/kg. Errors represent 95% confidence intervals. The numbers in green type represent the % enhancement over the baseline and the single red entry represents a suppression.

(a)	*"Superfood"*	*Baseline*	*+ 4-OH-Pro*	*+ His*	*+ Met*	*+ Tyr*	*+ Tryp*
	Bilberry	264592 (±3622)	232401 (±3437)	315370 (±2318)	1137774 (±6301)	1580450 (±34606)	3998151 (±78138)
	%Change		**-12**	**19**	**330**	**497**	**1411**
	Coffee berry	418659 (±6408)	421039 (±7995)	575186 (±24139)	1351487 (±32531)	1820695 (±24553)	4394772 (±13960)
	%Change		**0.6**	**37**	**223**	**335**	**950**
	Apple	8832912 (±54406)	9297050 (33940)	18105035 (±675441)	17641166 (±692303)	23173382 (±1180419)	42683881 (±1974498)
	%Change		**5**	**105**	**100**	**162**	**383**
(b)	*"Superfood"*	*Baseline*	*+ CuNPs*				
	Bilberry	264592 (±3622)	1738650 (±74350)				
	%Change		**557**				
	Coffee berry	418659 (±6408)	2390421 (±38234)				
	%Change		**471**				
	Apple	8832912 (±54406)	28932234 (±740719)				
	%Change		**228**				
(c)	*"Superfood"*	*Baseline*	*+ CuNPs/4-OH-pro*	*+ CuNPs/His*	*+ CuNPs/Met*	*+ CuNPs/Tyr*	*+ CuNPs/Tryp*
	Bilberry	264592 (±3622)	1611706 (±81623)	2380255 (±99609)	2456940 (±41642)	5463782 (±131083)	6558145 (±231762)
	%Change		**509**	**800**	**829**	**1965**	**2379**
	Coffee berry	418659 (±6408)	2036738 (±117813)	3026068 (±156290)	3095682 (±63244)	5858722 (±169357)	7398893 (±159973)
	%Change		**386**	**623**	**639**	**1299**	**1667**
	Apple	8832912 (±54406)	26760545 (±1290549)	34891212 (±1943363)	35005759 (±1707750)	62921329 (±2514191)	69322442 (±3245239)

%Change			203	295	296	612	685

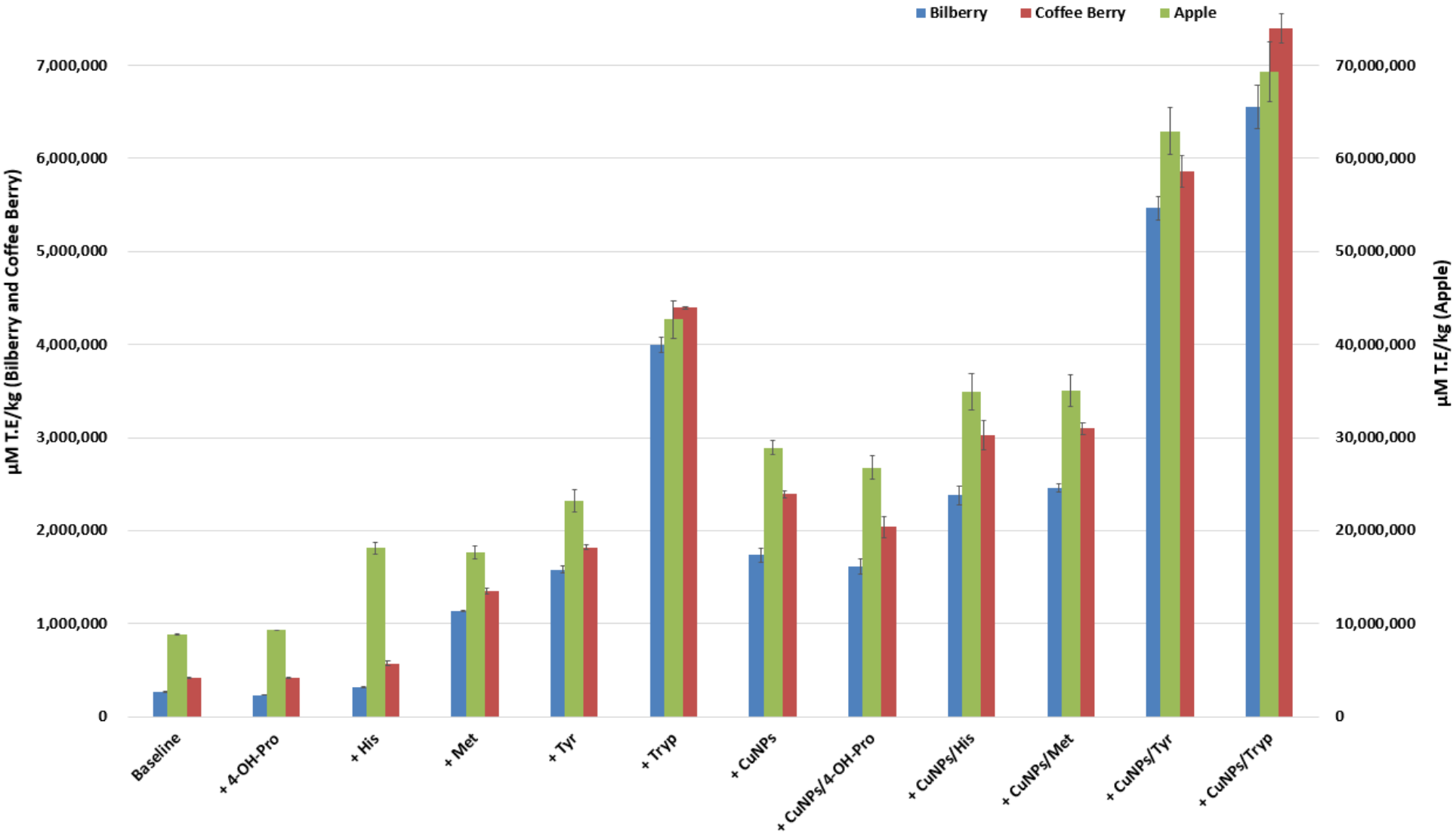


Figure 5.1: Trends in antioxidant activity/synergy in "superfood" matrices as a result of amino acid or CuNPs addition

Firstly it may be observed that significant antioxidant enhancement effects are evident when the amino acids, CuNPs and amino acid/CuNP are added, with the latter being the most potent. More specifically, for all three matrices, the order of enhancement for *both* the amino acids and the amino acid/CuNPs is:

$$\text{Try} > \text{Tyr} > \text{Met} \geq \text{His} \geq \text{4-OH-Pro}$$

This remarkable trend is clearly evident in Figure 5.1, both for the amino acids alone and for the amino acid/CuNPs and suggests that the enhancement effect is more an inherent property of the additives rather than of the matrix itself, since it appears to be matrix independent. Interestingly, this order is also consistent with the order of calculated BDEs shown in Table 5.1 - that reflect the relative inherent antioxidant potentials.

These results raise the question as to how a molecule such as an amino acid or an amino acid/CuNP can have its antioxidant potential magnified so dramatically by interacting with its molecular environment. We suggest that such molecules can be viewed as consisting of an "redox zone" and a "substrate zone". For example, the molecule Tyr, Figure 5.2, clearly has its antioxidant zone in the vicinity of the phenolic OH. The substrate zone of such molecules frequently have considerable interactive potential with other molecules via hydrogen bonding, deprotonation, metal chelation etc. that can be shown to dramatically affect the relevant BDEs even though they are remote.

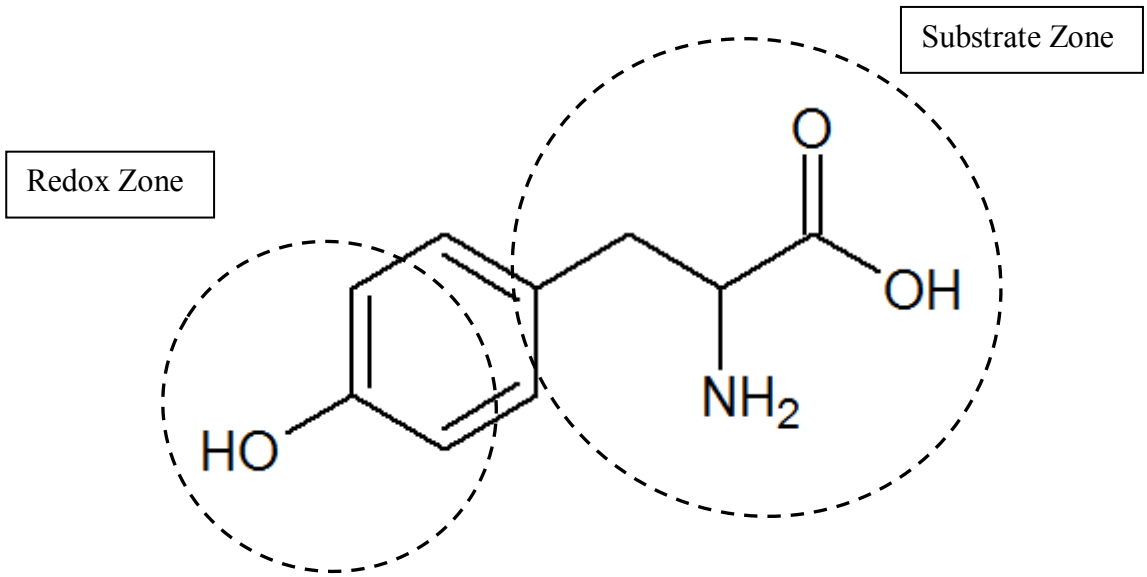
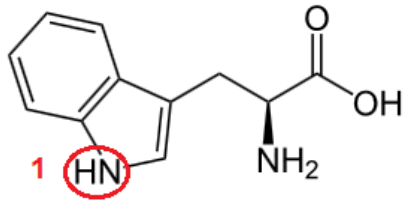
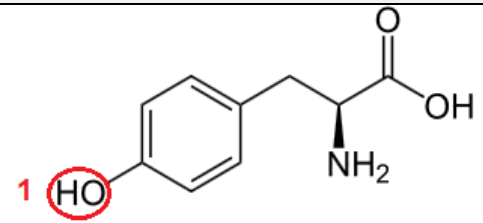
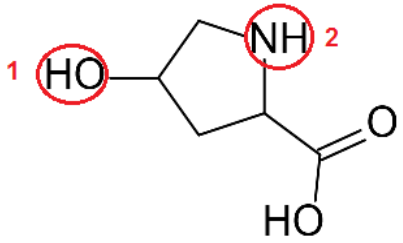
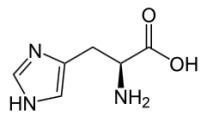
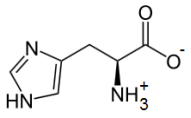
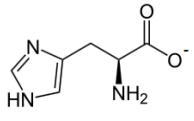
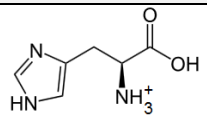
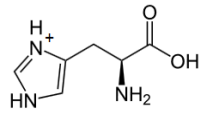
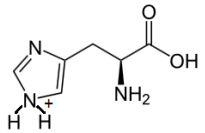
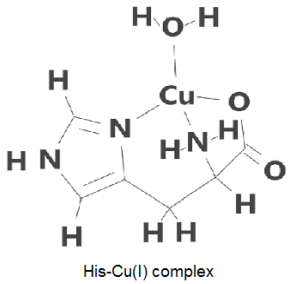


Figure 5.2: The "redox" and substrate zones" of the tyrosine molecule.

In this regard, we have calculated, Table 5.3, that the BDE of Tyr in its anionic form is considerably reduced to from 91.3 (zwitterion) to 80.1 kcal/mol – now comparable to trolox. In addition, we have calculated that the BDE of a potential Cu(I) – His complex reduces the N-H DBE of this amino acid from 104.2 (zwitterion) to 78.7 kcal/mol. Furthermore, we have considered perturbation(s) within the redox zone itself. For example, an allowable protonation or equivalent structural/electronic change to the His molecule, such as that depicted in Figure 5.3, results in an extraordinary labialization of the N-H bond with an extremely low BDE of 56.2 kcal/mol. This kind of event could result in enormously enhanced antioxidant potential.

Table 5.3: Calculated homolytic BDEs for amino acids in neutral, zwitterion, anion and cation species. Cu(I) complexation with Histidine is also shown.

Tryptophan (B3LYP/6-311++G(2DF,2P)/PM3)	**1**	**Neutral**	**Zwitterion**	**Anion**	**Cation**
	**Parent**	-430841.2	-430830.8	-430497.5	-431080.3
	Radical	-430430.6	-430419.3	-430097.1	-430657.7
	H·	-315.2	-315.2	-315.2	-315.2
	BDE	95.4	96.4	84.8	107.4
Tyrosine (B3LYP/6-311++G(2DF,2P)/PM3)	**1**	**Neutral**	**Zwitterion**	**Anion**	**Cation**
	**Parent**	-395476.7	-395459.2	-395135.4	-395699.4
	Radical	-395071.4	-395052.7	-394740.2	-395284.7
	H·	-315.2	-315.2	-315.2	-315.2
	BDE	90.2	91.3	80.1	99.5
4-hydroxy-proline (B3LYP/6-311++G(2DF,2P)/PM3)	**1**	**Neutral**	**Zwitterion**	**Anion**	**Cation**
	**Parent**	-299030.1	-299020.3	-298685.9	-299261.3
	Radical	-298602.9	-298595.8	-298265.0	-298831.8
	H·	-315.2	-315.2	-315.2	-315.2
	BDE	112.1	109.3	105.7	114.3
	2	**Neutral**	**Zwitterion**	**Anion**	**Cation**
	Parent	-299030.1	-299020.4	-298685.9	-299261.3
	Radical	-298618.5	-298602.6	-298290.0	-298832.0
	H·	-315.2	-315.2	-315.2	-315.2
	BDE	96.5	102.6	80.7	114.1

Histidine (B3LYP/6-311++G(2DF,2P)/PM3)	**1 - 4**	**Neutral**	**Zwitterion**	**Anion**	**Cation**
 Neutral	**Parent**	-344472.7	-344464.5	-344126.8	-344708.2
 Zwitterion	**Radical**	-344055.1	-344045.1	-343721.5	-344289.4
 Anion	**H·**	-315.2	-315.2	-315.2	-315.2
 Cation	**BDE**	102.5	104.2	90.1	113.6
 Species (a)	**5 - 7**	**Species (a)**	**Species (b)**	**His-Cu(I) Complex**	
 Species (b)	**Parent**	-344701.3	-344660.8	-1517759.1	
 His-Cu(I) complex	**Radical**	-344269.5	-344289.4	-1517365.3	
	H·	-315.2	-315.2	-315.2	
	BDE	116.7	56.2	78.7	

Note: In both species (a) and (b), the protonated *L*-His charge is delocalized over the imidazole ring

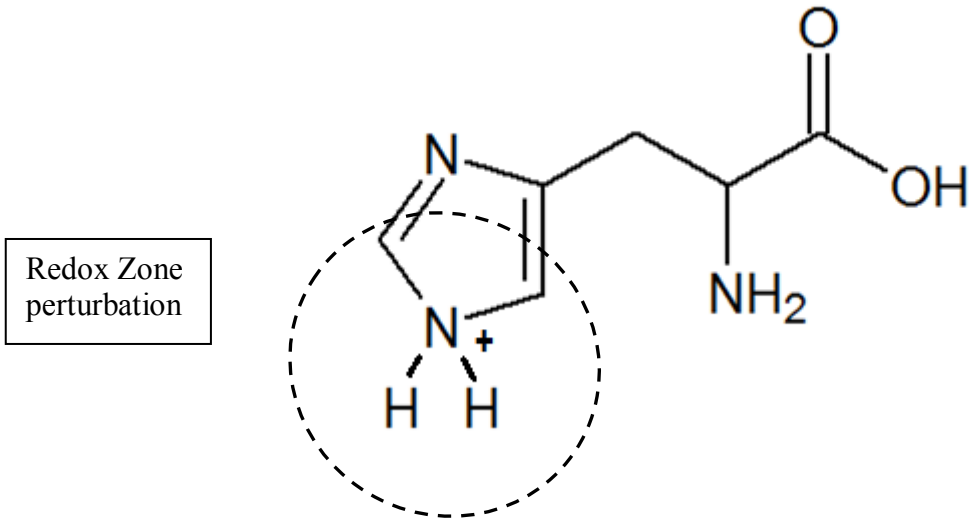


Figure 5.3: A possible redox zone perturbation for the His molecule.

Of course, it could also be possible for a perturbation within the redox zone to occur in concert with an intermolecular interaction with the substrate zone, further modifying the antioxidant potential. However, the specific intermolecular interactions that are occurring in the "superfood" matrices presented within this paper remain to be investigated.

5.6 CONCLUSIONS AND FUTURE WORK

In this study, consistent elevation of ORAC values were observed for supplement-enhanced "superfood" matrices. CuNPs and the amino acids 4-Hydroxyproline, Histidine, Methionine, Tyrosine and Tryptophan were added to bilberry, coffee berry and apple concentrate matrices, and remarkably strong synergies were observed. Distinct trends were noted; in particular, significant antioxidant enhancement effects were evident when the amino acids, CuNPs and amino acid/CuNP were added, with the latter being the most potent. The order of enhancement for both the amino acids and the amino acid/CuNPs was Try > Tyr > Met ≥ His ≥ 4-OH-Pro in all three matrices, suggesting the enhancement effect is an inherent property of the additives and appears to be essentially matrix independent. In this regard, this order is also consistent with the order of calculated BDEs reflecting the relative inherent antioxidant potentials of the amino acids studied.

We propose the presence of an "redox zone" and a "substrate zone" as the molecular features of significance involved in influencing the antioxidant capacity measurements so dramatically. Using DFT calculations, interactions at the substrate zone were shown to have a dramatic effect on the relevant BDEs, even though they are remote. Furthermore, using histidine-Cu(I) complex as a model, we have demonstrated how allowable protonation or equivalent structural/electronic change to an amino acid results in extraordinary labilization of the N-H bond, with the potential to enormously enhance antioxidant potential. It could also be possible for a perturbation within the redox zone to occur in concert with an intermolecular interaction with the substrate zone, further modifying the antioxidant potential. However, the specific intermolecular interactions that are occurring in the "superfood" matrices presented within this paper remain to be investigated.

These discoveries are an important step in identifying new classes of compounds with potential for antioxidant synergies. More experimental trials are needed to elucidate trends for other amino acids which do not show baseline antioxidant activity. As was demonstrated for 4-hydroxy-proline and histidine, the absence of baseline ORAC measurement is not a clear indication of absence of antioxidant synergistic potential.

Antioxidant synergies involving amino acids, copper nanoparticles and copper ions (that are presumably formed on the surface of the particles) should be investigated further. Emphasis should be given to cellular systems in order to understand if the antioxidant species formed have in vivo activity and warrant further investigation. There is great potential for future work in this area, and a need for more hypotheses and data to demonstrate synergies between other classes of compounds and antioxidants in food.

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6.0 Antioxidant synergies in food systems – *in vitro* versus *ex vivo* studies on eight different classes of chemical components representative of navel oranges

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1. PUBLICATION DETAILS (to be completed by the candidate)

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2. CANDIDATE DECLARATION

I declare that the publication above meets the requirements to be included in the thesis as outlined in the HDR Policy and related Procedures – policy.vu.edu.au.

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Updated: June 2015

6.1 PREAMBLE

Chapter 6 has been submitted to the journal *Food Chemistry* (June 2018) and the ORAC and CAA-RBC data were presented at the Australian Food Metrology Symposium, held 23rd October 2012 at Victoria University, Melbourne, Australia. Specific DFT calculations were carried out on selected molecules in an attempt to better interpret the experimental data in relation to the submission of this paper. This manuscript describes the preparation of a synthetic juice matrix, based on compositional data from the Food Standards Australia New Zealand (FSANZ) nutritional database (NUTTAB). This synthetic matrix contained eight classes of compounds at the levels described as being typical for navel orange juice. Classes included sugars, vitamins, amino acids, phenolics, flavonoids and preservatives. Based on the experimental observations in Chapter 5, CuNPs were also included in the trial and all experiments were compared via *in vitro* ORAC and *ex vivo* CAA-RBC assays. This paper addresses Research Question 4 by reporting on the major classes of constituents that show antioxidant synergy and describes the structural/electronic properties of selected molecules in terms of activating or deactivating O-H sites for hydrogen abstraction, as demonstrated by the DFT calculations.

6.2 ABSTRACT:

Eight classes of chemical components were analyzed by ORAC and the cellular antioxidant assay – red blood cells (CAA-RBC) to investigate potential for antioxidant synergy and correlation between *in* vitro and *ex* vivo methods. Sugars, vitamins, amino acids, polyphenolics, flavonoids, preservatives and organic acids were prepared as mixtures and evaluated for antioxidant activity, both of individual mixes and combinations of classes. Copper nanoparticle solution (CuNPs) was also evaluated. Antagonisms between many mixtures containing amino acids and CuNPs were seen, as was the mixture of sugars and vitamins in the ORAC assay. When the same solutions were applied to the CAA-RBC assay synergies were observed. Finally, computational studies of Quercetin and Catechins are presented to demonstrate activation/deactivation of antioxidants upon perturbations of the substrate zone. Homolytic bond dissociation energies (BDEs) are presented for major antioxidants studied, with chlorogenic acid and caffeic acid identified as good candidates for further studies *ex vivo/in vivo*.

Keywords; antioxidants, amino acids, copper nanoparticles, density functional theory

6.3 INTRODUCTION:

Antioxidants synergies in food systems have been hypothesized and demonstrated experimentally(De Beer *et al.*, 2006; Iacopini *et al.*, 2008; Blasa *et al.*, 2011; Tsao, 2015). Despite this, a lack of understanding exists surrounding the extent of such synergies and the molecular basis for them. Antioxidant capacity assays attempt to measure the antioxidant properties of various food systems and there are a range of different antioxidant assays that can be investigated. The oxygen radical absorbance capacity (ORAC) assay is one of the most popular methods used to evaluate antioxidant capacity of foods and beverages.

Despite some limitations, ORAC values are reported for many foods with the most predominant antioxidant contributions thought to be from polyphenolics, flavonoids and some vitamins (Boots, Haenen and Bast, 2008; Shahidi and Chandrasekara, 2010; Brewer, 2011). In chapter 5 we reported that other nutrients and chemicals can greatly influence antioxidant activity *in vitro*. Even in the case of amino acids whose structural composition did not appear to inherently support antioxidant activity, synergies were shown in fruit concentrate powders. These synergies yielded increased antioxidant capacity in the ORAC assay by up to 25-fold in some cases.

Freeman *et al.* made similar attempts to understand how matrix components influence the ORAC assay (Freeman, Eggett and Parker, 2010). Using orange juice, their study was based solely on specific phenolic standard compounds, combining standard solutions to observe the effect on ORAC. In their study, Freeman et al. (2010) modelled some of the potential causes of

the interactions observed between different phenolic compounds commonly found in navel oranges, however they did not extend their work to looking a real navel orange juice.

This paper presents a pseudo juice model system and compares observed effects in this system to observed effects in a real food system with the same macronutrient composition profile. Sugars, organic acids, vitamins, preservatives, amino acids, polyphenolics, flavonoids and addition of commercial metal additives, such as a copper colloidal solution, were all considered in this study. The experimental design took into account the interactions of these groups as a whole in the final pseudo juice product, as well as one-on-one interactions between groups to identify specific synergies, antagonisms, sources of bias and uncertainty in the overall ORAC measurement.

To date, no research of this kind has been published. However, preliminary investigations by Wang et al. (2011) and Freeman et al. (2010) have been reported in the literature. Wang et al. (2011) focused on food mixtures, combining a number of different foods and analysing antioxidant capacity by 4 different assays, one of them being ORAC. Wang et al. (2011) also carried out phytochemical profiling, but did not look at major sources such as vitamins and amino acids, which have been shown in this thesis to be major contributors to the ORAC assay when added to the matrix.

Notably, synergy and matrix effects as observed with ORAC have not been reported in cellular systems (Liu and Finley, 2005). The cellular antioxidant assay, using human red blood cells (CAA-RBC), has been applied to food extracts as reported (Blasa *et al.*, 2011). The present

work attempts to address this next phase of investigation into antioxidant synergy. Thus, in this work we present the findings of a systematic study of eight classes of chemicals, all which are commonly found in navel orange juice. This study was conducted using *in vitro* (ORAC) and *ex vivo* (CAA-RBC) antioxidant capacity assays in order to determine if the same synergies observed from ORAC studies of food systems are also seen in cellular systems via the CAA-RBC assay and to what extent such synergies can be measured.

Phenolics, flavonoids, vitamins, sugars, amino acids, preservatives and organic acids All constituents were prepared and combined to create a synthetic juice matrix. The chemical composition was based on the Food Standards Australia New Zealand nutritional table database (NUTTAB). As such, the synthetic juice matrix was prepared to match, as closely as possible, the NUTTAB compositional information for Australian navel orange juice (FSANZ, 2010).

Specifically, the nutrients and chemicals found in navel orange juice were; fructose, glucose, sucrose, malic acid, citric acid, thiamine, pantothenic acid, folic acid, ascorbic acid, tocopherol, tryptophan, gallic acid, quercetin, caffeic acid, chlorogenic acid, ferulic acid, catechin. The ORAC and CAA-RBC values of these chemicals were measured individually, in combination with one another, and in comparison with real navel orange fruit juice matrices which had undergone various degrees of treatment prior to being purchased at retail outlets. Australian navel orange juice processed by a variety of methods was obtained commercially. Cold-pressed, reconstituted, fresh juice, and fresh juice squeezed from navel orange fruit were also analyzed by both ORAC assay and CAA-RBC assay.

6.4 METHODOLOGY

6.4.1 ORAC assay

6.4.1.1 Chemicals for the ORAC assay. Fluorescein disodium salt (FL), 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox™), monosodium phosphate monohydrate, disodium phosphate heptahydrate, hydrochloric acid (HCl), acetone and acetic acid were purchased from Sigma-Aldrich (Australia). Similarly, all neat analytical standards for fructose, glucose, sucrose, malic acid, citric acid, thiamine, pantothenic acid, folic acid, ascorbic acid, tocopherol, *L*-tryptophan, gallic acid, quercetin, caffeic acid, chlorogenic acid, ferulic acid and catechin were obtained from Sigma-Aldrich (Australia). Australian navel oranges, cold-pressed navel orange juice, re-constituted navel orange juice and fresh orange juice were purchased commercially from Coles supermarkets, Port Melbourne, Australia. All purchased juices listed Australian navel oranges as the fruit source for the product. Copper nanoparticle solution (concentration of 7.2mg/L) was sourced from Australian Natural Colloids, and stored at room temperature.

6.4.1.2 ORAC assay protocol. Phosphate buffer (PB) 75mM was prepared to 2L volume with monosodium phosphate monohydrate and disodium phosphate heptahydrate, and the pH adjusted to 7.4 using 1M hydrochloric acid (HCl) prepared from 10M HCl stock. All subsequent working solutions were prepared in phosphate buffer. A stock solution of FL was prepared monthly at 0.7mM concentration. A final working solution of FL was achieved by serial dilution of the stock with buffer to a final concentration of 70nM. The working FL solution was prepared from stock daily. AAPH peroxyl radical solution was prepared to a concentration of 35mM in phosphate buffer. Due to the thermal sensitivity of AAPH, the working solution was prepared

just prior to analysis. Hydrophilic extraction solution comprised of acetone/water/acetic acid (AWA) 70:29.5:0.5 (v/v/v) solvent was prepared for the extraction of hydrophilic antioxidants. Hydrophilic extracts were diluted in PB prior to analysis on the FLUOstar OPTIMA instrument.

Trolox antioxidant standard, 10mM stock solution, was prepared weekly. The stock solution was serially diluted daily with PB. A concentration range of 6.25-100µM was used for preparation of calibration plots for quantitation. All other standard solutions were prepared in milliQ water to the concentrations listed below in Table 1. These standard solutions were prepared fresh daily.

6.4.2 CAA-RBC assay

6.4.2.1 Chemicals for the CAA-RBC assay. Phosphate buffered saline solution (PBS) (x10 concentrate solution), reverse osmosis water (RO), 2',7'-Dicholorofluorescien diacetate, (DCFH-DA), 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH), human red blood cells (RBC) (AB group) - glutaraldehyde treated, and quercetin dihydrate, were purchased from Sigma-Aldrich (Australia).

6.4.2.2 CAA-RBC assay protocol. A DCFH-DA stock solution was prepared in methanol and diluted to a working concentration of 25µM in 15mL Falcon™ tubes. A 10mM Quercetin stock was prepared (Blasa *et al.* 2011) and working standards made in sterile Falcon™ tubes after RBC preparation. PBS stock solution was diluted 1:10 in RO water, with a pH 7.4. Human RBC (AB group) in glutaraldehyde solution were rinsed in PBS at a ratio of 1:5 RBC to PBS,

centrifuged, and the supernatant removed. The RBC's were transferred to Falcon™ tubes where sample, chemical or antioxidant standard and DCFH-DA were added, and the contents made to a final volume of 10mL with PBS. The tubes were incubated at 37°C for one hour. The RBC's were rinsed twice, made to final volume and transferred to a black 96 well microplate, again following the Blasa protocol (Blasa *et al.*, 2011; Stockham *et al.*, 2013).

6.4.3 Sample preparation and extraction

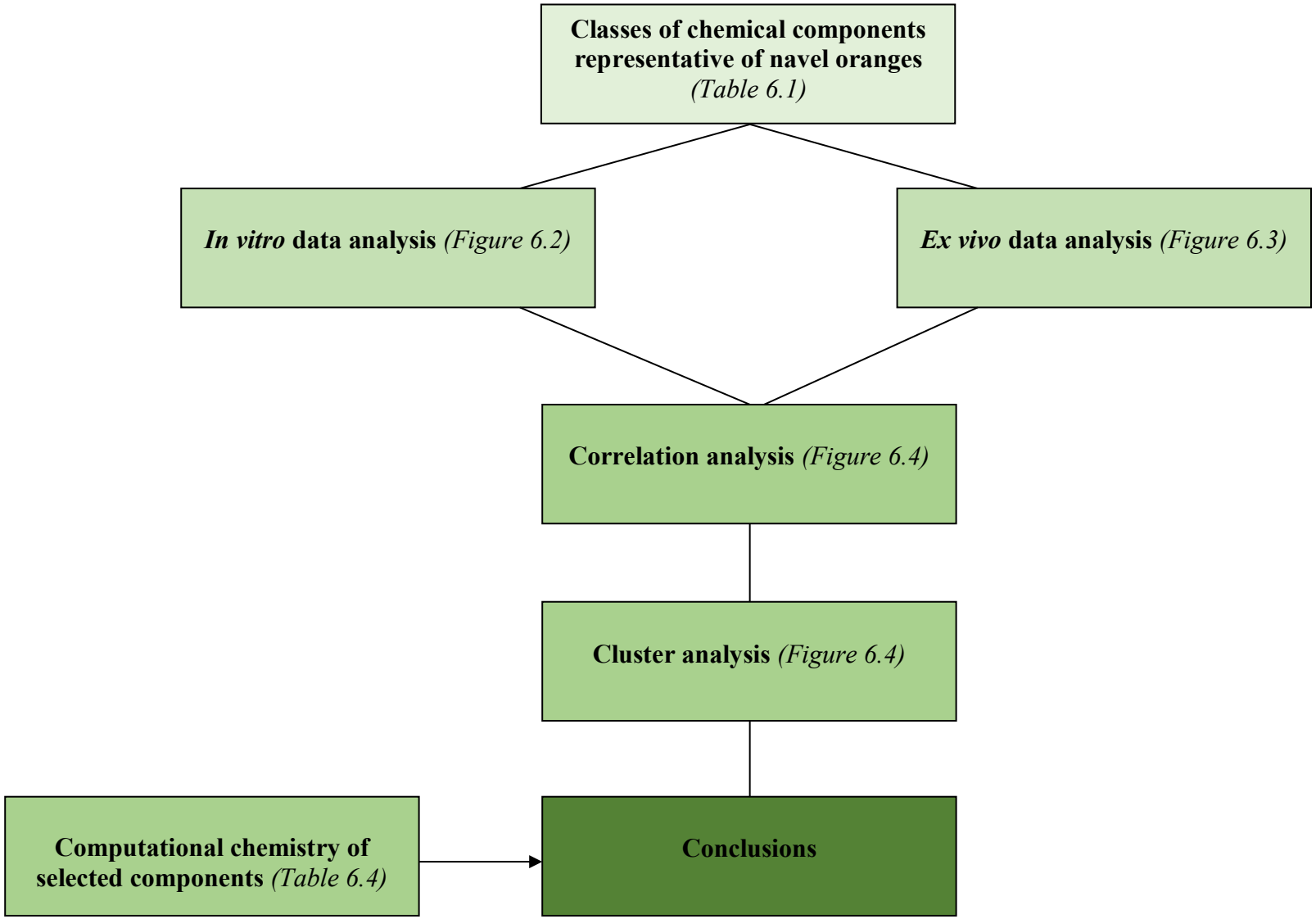
6.4.3.1 Preparation of solutions for eight classes of chemical components found in navel oranges.

Eight solutions were prepared according to the composition found in the NUTTAB database. Mixtures of sugars, organic acids, vitamins, amino acid Tryptophan, polyphenolics (PP), flavonoids (FL), copper nanoparticles (CuNPs/cu) and preservative benzoic acid (benz) were prepared. These solutions were prepared to the concentrations listed (g/10mL) and are summarized as follows in Table 6.1. These solutions were prepared in milliQ water, and then diluted with phosphate buffer (ORAC) or PBS (CAA-RBC). Combinations of mixtures are summarized in Figure 6.1 below, and were applied to ORAC and CAA-RBC assays. The eight classes of components described in Table 6.1 have been further combined to make a total of 36 mixtures for antioxidant analysis experiments (ORAC and CAA-RBC), see the horizontal axes of Figures 6.2 and 6.3 respectively. Also see the Schematic in Figure 6.1.

Table 6.1: Solutions prepared for ORAC and CAA-RBC study

Mixture	Abbreviation	Description (concentration g/10mL)
Sugars	"sugars"	Fructose (1.6) Glucose (1.7) Sucrose (2.7)
Organic acids	"org acids"	Malic acid (0.1) Citric acid (0.8)
Vitamins	"vitamins"	Ascorbic acid (0.011) Tocopherol (0.001) Pantothenic acid (0.0013) Thiamin (0.0011)
Amino acid	"aminos"	Tryptophan (0.0101)
Polyphenolics	"PP"	Gallic acid (0.002) Chlorogenic acid (0.0035) Ferulic acid (0.0019) Caffeic acid (0.002)
Flavonoids	"Fla"	Quercetin (0.0031) Catechin (0.0035)
Copper nanoparticles	"Cu"	1mL of 7.2mg/L solution was used (0.000072)
Preservative	"benz"	Benzoic acid (0.0478)

Figure 6.1: Schematic of experimental design



6.5 RESULTS AND DISCUSSION

Antioxidant activities of classes of compounds found in navel oranges were evaluated by ORAC and CAA-RBC. These solutions were prepared to concentrations reported in the NUTTAB nutritional database (FSANZ, 2010). The solutions that were analyzed were made up from various combinations of the eight classes of mixtures described in Table 6.1 and are given in the horizontal axis captions of Figures 6.2 and 6.3. Thus Figures 6.2 and 6.3 show the antioxidant capacity of these mixtures, individually and in combination, for the ORAC and CAA-RBC assays respectively.

Antagonisms were seen in the ORAC assay, with the sum of antioxidant capacity groups in combination being less than the individual mixtures. For example, “aminos” and “PP” both had similar antioxidant activity; however the combination of “aminos” and “PP” mixtures did not yield a synergistic or even fully additive effect (Figure 6.2). Similarly, the combination of “org acids” and “PP” would be expected to be high; however the effect on antioxidant activity was negligible as results weren’t synergistic and only marginally additive. The addition of “sugars” to “vitamins” resulted in a suppression of antioxidant activity. The same is shown for many other combinations including “aminos” and “Cu”, which is consistent with the discussion in Chapter 5. Other notable suppressions include “PP” and “Fla”, whose activity could be expected to be additive given their good performance as antioxidants in many other ORAC papers. This result suggests that the presence of a mixture of some “PP” and “Fla” are not necessarily potent antioxidant solutions.

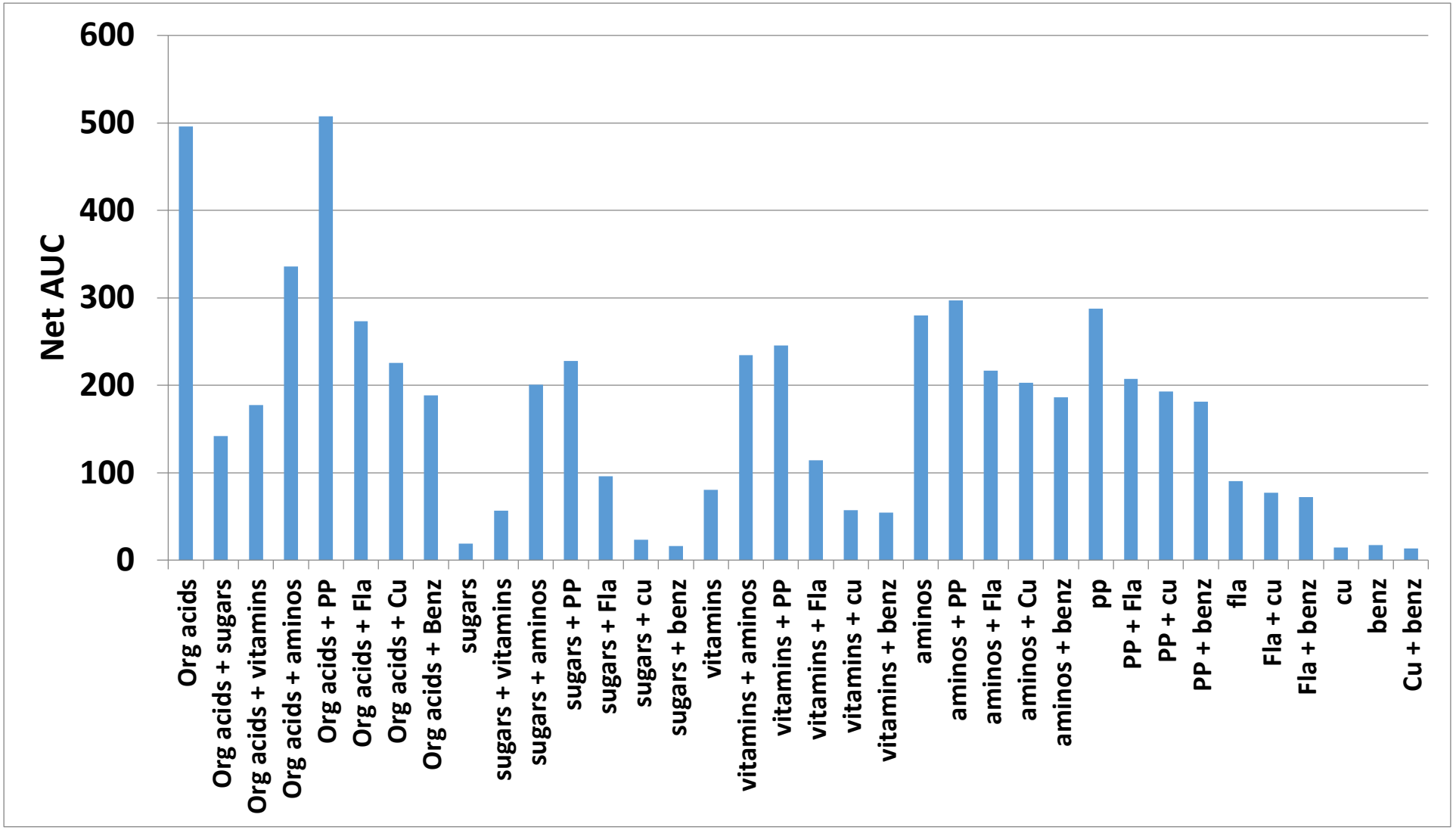


Figure 6.2: Relative ORAC data for the 36 mixtures derived from the 8 classes of chemical substances described in Table 6.1 above.

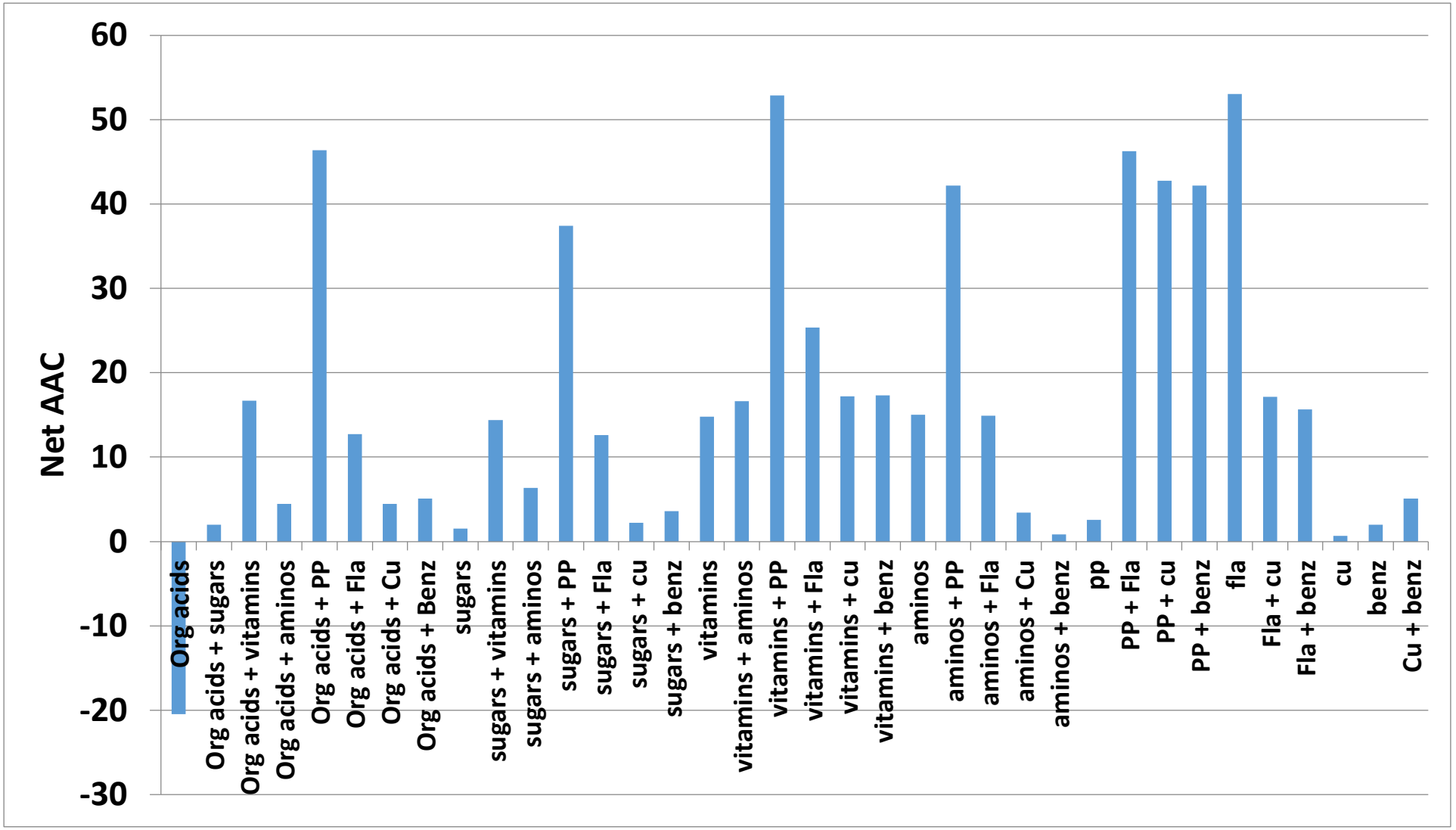


Figure 6.3: Relative CAA-RBC data for the 36 mixtures derived from the 8 classes of chemical substances described in Table 6.1 above.

In the case of the analogous CAA-RBC assays, Figure 6.3, synergies were observed. This is interesting as it highlights the limitations of using one assay to evaluate total antioxidant capacity of foods (TAC). Foods that may seem to have little or no antioxidant activity by the ORAC assay may still exhibit antioxidant activity and bioavailability when applied to cellular systems. Strong synergies were seen between polyphenolics ("PP") and most other classes of compounds. The most notable example is that of "PP" and "org acids", where an auto-oxidation effect of "org acids" was reversed and the overall antioxidant activity was greatly enhanced in combination with the "PP" mix. Synergies between "PP" and "sugars", "vitamins", "aminos", "benz" and "Cu" were all demonstrated. These observations support our earlier reports of interactions in the "substrate zone" at the molecular level, Chapter 5, and how these interactions can dramatically lower the bond dissociation energies (BDEs) of O-H groups in the "antioxidant zone", thereby boosting antioxidant capacity. The mechanisms for enhancement/synergy postulated in Chapter 5 are consistent with the results seen in this chapter, both in the ORAC assay and now in the CAA-RBC assay. These findings suggest that antioxidant synergies can be demonstrated in the cellular assay.

Suppressions were also noted in the CAA-RBC assay, in particular for interactions of "Fla" with many of the other groups including "benz", "Cu", "PP", "aminos" and "vitamins", where antioxidant activity of "Fla" were more than halved in combination with these other mixtures.

In order to investigate a potential correlation between the ORAC and the CAA-RBC data, a correlation plot was calculated, Figure 6.4.

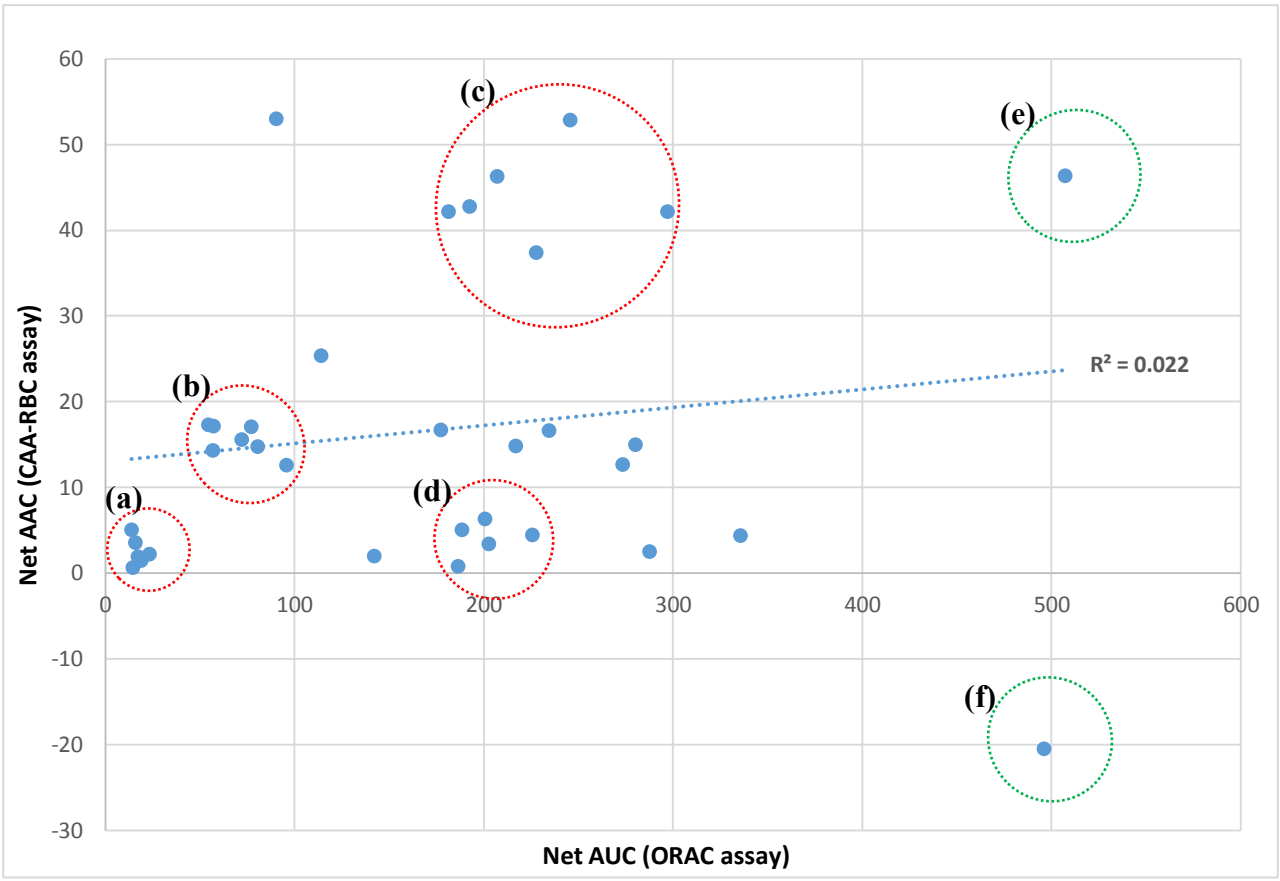


Figure 6.4: Scatter plot of the correlation between the ORAC values of Figure 6.2 and the CAA-RBC values of Figure 6.3 demonstrating the clustering (a) to (d) and several extreme outliers.(e) and (f).

It can be seen that, overall, the data between the two methods are not correlated (R = 0.022). However, a clear clustering is apparent (red dotted circles (a) – (d) as well as two extreme outliers (e) and (f). The components involved in these clusters and outliers are summarized in Table 6.2.

From Figure 6.4, in relation to the outliers, it can be seen that a combination of "org acids" and "PP" (e) showed a uniquely high antioxidant activity in both ORAC and CAA-RBC, whilst "org acids" had an auto-oxidation effect in the CAA-RBC, but a high ORAC value (f).

In relation to the clusters themselves, (a) involves benzoic acid sugars and CuNPs. These appear to be uniquely low in antioxidant response for both methods. This would suggest that ORAC alone would suffice as a predicator of antioxidant potential (or the lack thereof) for these classes of compounds. Cluster (b) involves mostly vitamins combined with Cu, benzoic acid, sugars and flavonoids. For these combinations, it would appear that both methods give an adequate representation of the antioxidant potential. Cluster (c) is dominated by polyphenolics in association (interaction) with other components. The predicted antioxidant activity is significant for both methods but is more enhanced for the cellular assay. Possible mechanisms, at the molecular level, for the enhancement of the antioxidant activity of polyphenolics have been explored using computational chemistry (DFT), with respect to Quercetin and a Catechin, later on this this Chapter. Cluster (d) is dominated by amino acids and organic acids. Here we have moderate ORAC values but low cellular values suggesting low activity within a cellular matrix.

Table 6.2: The components involved in the clusters (a) to (d) and the extreme outliers (e) and (f) as seen in Figure 6.4[2]

(a)	(b)	(c)	(d)	(e)	(f)
"benz"	"vitamins + cu"	"PP + benz"	"org acids + benz"	"org acids + PP"	"org acids"
"cu"	"vitamins + benz"	"PP + cu"	"sugars + aminos"		
"sugars"	"sugars + vitamins"	"PP + Fla"	"aminos + cu"		
"cu + benz"	"Fla + cu"	"vitamins + PP"	"org acids + cu"		
"sugars + benz"	"Fla + benz"	"PP + aminos"	"aminos + benz"		
"sugars + cu"	"vitamins"	"sugars + PP"			
	"sugars + Fla"				

[2] Abbreviations are described in Table 6.1.

Observed synergies or antagonisms for all combinations are summarized in Table 6.3 (for ORAC) and Table 6.4 (for CAA-RBC).

Table 6.3: Observed enhancements/synergies for all combinations for the ORAC assay

	Sugars	Organic acids	Vitamins	Amino acid	Polyphenols	Flavonoids	Preservatives	Cu
Sugars		-	-	-	-	N/A	N/A	N/A
Organic acids	-		-	-	N/A	-	-	-
Vitamins	-	-		-	-	-	-	-
Amino acid	-	-	-		N/A	-	-	-
Polyphenols	-	N/A	-	N/A		-	-	-
Flavonoids	N/A	-	-	-	-		N/A	N/A
Preservatives	N/A	-	-	-	-	N/A		N/A
Cu	N/A	-	-	-	-	N/A	N/A	

Where + represents synergistic effects (an increase in antioxidant activity, greater than the summation of the activity of the individual classes); - represents antagonism (a suppression of antioxidant activity); and A represents and additive effect

Table 6.4: Observed enhancements/synergies for all combinations for the CAA-RBC assay

	Sugars	Organic acids	Vitamins	Amino acid	Polyphenols	Flavonoids	Preservatives	Cu
Sugars		N/A	N/A	-	+	-	A	N/A
Organic acids	N/A		N/A	-	+	-	+	+
Vitamins	N/A	N/A		N/A	+	-	N/A	N/A
Amino acid	-	-	N/A		+	N/A	-	-
Polyphenols	+	+	+	+		-	+	+
Flavonoids	-	-	-	N/A	-		-	-
Preservatives	A	+	N/A	-	+	-		N/A
Cu	N/A	+	N/A	-	+	-	N/A	

Where + represents synergistic effects (an increase in antioxidant activity, greater than the summation of the activity of the individual classes); - represents antagonism (a suppression of antioxidant activity); and A represents and additive effect

For clusters (a) and (b), Figure 6.4, ORAC appears to be a reasonable predictor of CAA-RBC activity for these compounds, and could be used for screening purposes.

6.5.1 Quercetin as a model for antioxidant enhancement (or synergy) at the molecular level

Figure 6.5 shows two forms of the Quercetin molecule. The molecule in (a) is considered to be in the most stable form, with the intramolecular O(5) - H - - - - O(4) intact. The molecule in (b) has this particular intramolecular hydrogen bond broken by a hypothetical interaction with a substrate. The substrate zone of this molecule can be considered to be a strong candidate for such interactions due to its rich hydrogen bonding acceptors and donors.

As discussed in Chapter 5, potential antioxidant molecules may be considered to possess a "substrate zone" and an "antioxidant zone". This was based on the notion that intermolecular interactions with the substrate zone (and also the antioxidant zone) could have a profound effect on the antioxidant characteristics of the molecule by influencing the homolytic BDEs of O-H groups (and also N-H groups). The BDE of particular interest is the one that has the lowest value since this corresponds to the more rapid release of hydrogen atoms. In this regard, it may be seen from Figure 6.5 (a) that the BDEs of all five of the O-Hs in this polyphenolic molecule are different, the lowest being the O-H at the 3' position (78.0 kcal/mol). It is assumed that this is the O-H that is primarily involved in the antioxidant activity. Notably, the loss of this hydrogen atom subsequently deactivates the other O-H groups. However, it may be seen from Figure 6.5 (b) that when the O(5) - H - - - - O(4) is disengaged (hypothetically via an intermolecular interaction), that the BDEs within the molecule change dramatically. In particular, it should be noted that the new lowest value is now associated with the OH at the 4' position and this is also considerably lowered to 73.5 kcal/mol. The loss of this hydrogen atom is also deactivating with respect to the other O-H groups. It is worth noting that this antioxidant enhancement is due to an effect that is quite remote from the antioxidant site. This demonstrates

how a molecule such as Quercetin may undergo a significant enhancement of antioxidant activity by interacting with another molecule or molecular matrix.

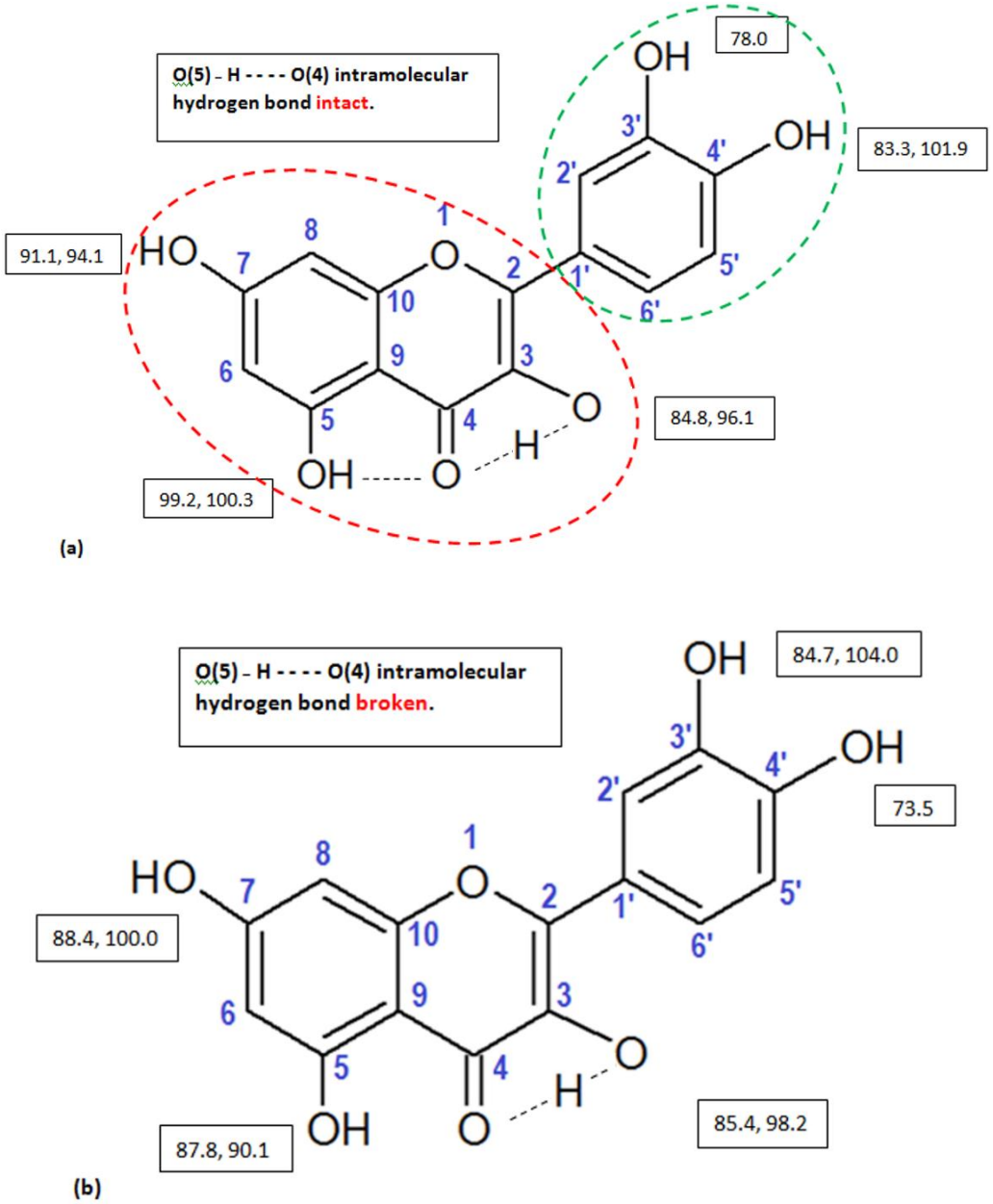


Figure 6.5: The Quercetin molecule showing the "substrate zone" – red dotted line and the "antioxidant zone" – green dotted line. Interaction with the substrate zone is represented by the breakage of the O(5) - H - - - - O(4) intramolecular hydrogen bond. **(a)** Hydrogen bond intact and the numbers in the boxes are the computed homolytic BDEs for the adjacent O-H bonds. The second numbers represent the BDEs for the radical species formed from the loss of the most labile H atom. **(b)** Intramolecular hydrogen bond broken.

6.5.2 Antioxidant activity of Catechin polyphenolics

Following on from the previous discussion of Quercetin as a polyphenolic, it was decided to investigate (computationally) the antioxidant characteristics of a representative Catechin structure. The outcomes of this experiment are depicted in Figure 6.6. As with Quercetin, all of the O-H groups display different BDEs with one of them being the lowest in value (85.0 kcal/mol). Interestingly, unlike Quercetin, when this hydrogen atom is lost, four out of the six remaining O-H groups are activated for antioxidant activity. These are depicted in green; two of the remaining groups are deactivated and are depicted in red. This further activation could help to explain why green tea is considered to be extraordinary as an antioxidant foodstuff - since it is rich in these molecules. This also represents a potential mechanism for antioxidant enhancement.

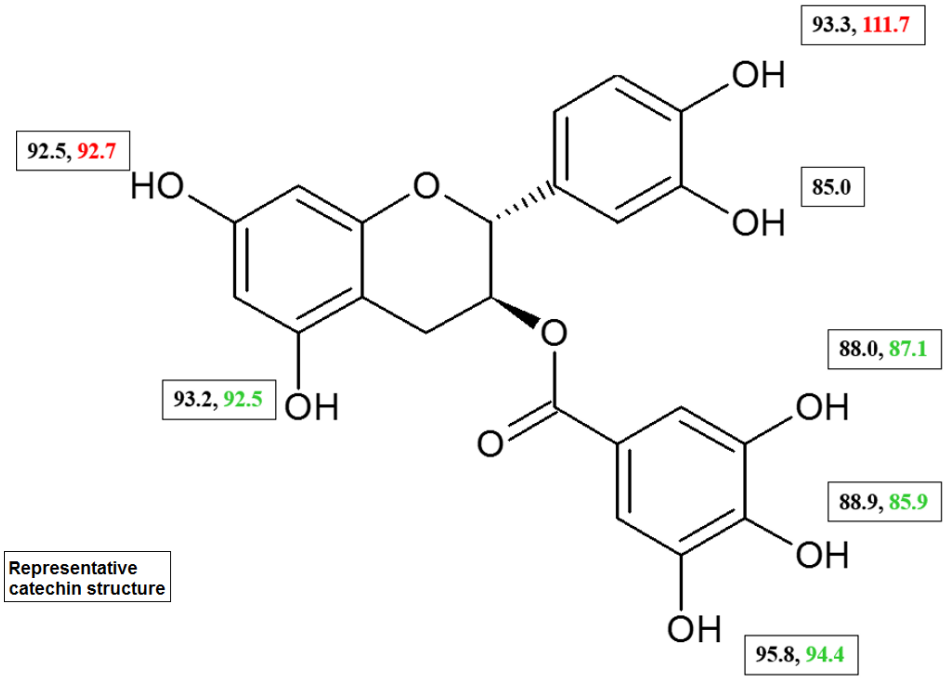


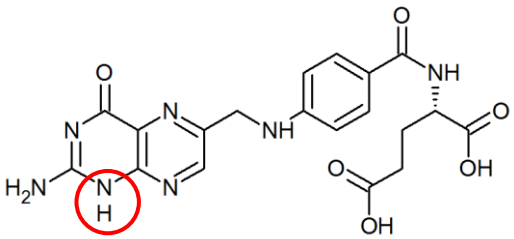
Figure 6.6: Computational analysis of a representative Catechin structure showing how the homolytic cleavage of the O - H bond with the lowest BDE activates four out of the six remaining O-H groups.

As presented earlier, the "Fla" solution had the single highest antioxidant activity in the CAA-RBC assay (Figure 6.3). It is notable that when other classes of compounds were added to the "Fla" solution, the antioxidant activity decreased significantly in many cases. This "Fla" solution was comprised of both Catechin and Quercetin, and these computational studies suggest that whilst both are flavonoids, the influence of substrates has a significant effect on their antioxidant capacity. Catechins have unique structural and intramolecular properties that could allow each molecule to donate multiple H atoms to neutralize radical species, making them a "multi-barreled gun".

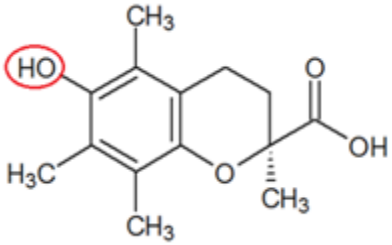
Finally, the relative homolytic BDEs for some of the other potential antioxidant molecules used in these experiments have been calculated and are presented in Table 6.5. Here, calculations were performed using density functional theory (DFT) at the *B3LYP/6-31G*/PM3* level using *Spartan06* software. These calculations were carried out at a lower level of theory due to computational restrictions and since only relative values were of interest. The antioxidant potential of chlorogenic acid and caffeic acid were found to be excellent, and comparable to the Vitamin E analogue, Trolox.

Table 6.5: Calculated relative BDEs for some of the molecules studied. The BDEs in green text represent Trolox, Chlorogenic acid and Caffeic acid. These molecules have excellent antioxidant capacity comparable to vitamin E, of which Trolox is an analogue. Structures and sites of hydrogen abstraction shown below.

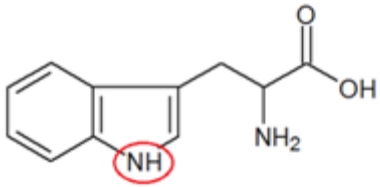
	Folic acid	Trolox	Tryptophan	Ferulic acid	Chlorogenic acid	Caffeic acid	Gallic acid	Quercetin
Parent molecule	-985267.323	-530391.458	-430687.391	-431699.897	-814206.408	-407035.983	-405665.273	-692863.02
Parent radical	-984846.229	-530000.482	-430279.961	-431303.651	-813815.574	-406646.446	-405271.171	-692463.801
Hydrogen radical	-313.926173	-313.296173	-313.296173	-313.296173	-313.296173	-313.296173	-313.296173	-313.296173
BDE 1	**107.2**	**77.7**	**94.1**	**83.0**	**77.5**	**76.2**	**80.8**	**85.9**



Folic acid



Trolox



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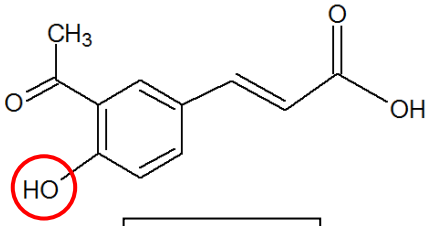
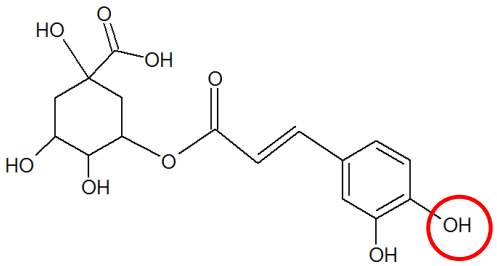
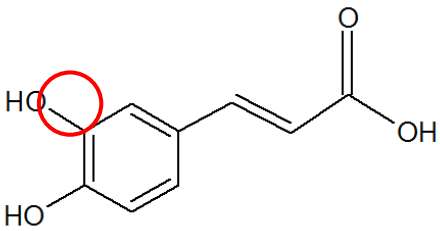


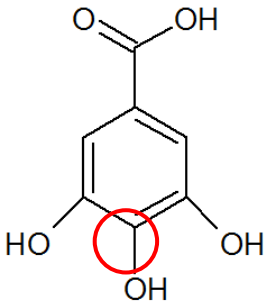
Ferulic acid



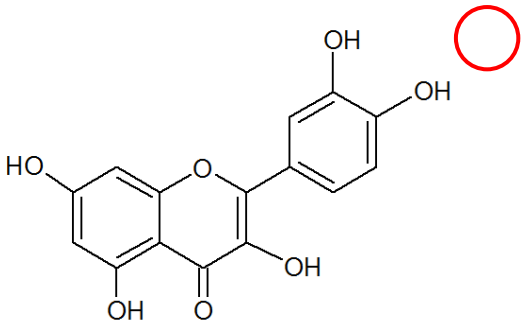
Chlorogenic acid



Caffeic acid



Gallic acid



Quercetin

6.6 CONCLUSIONS

Antioxidant activities of individual mixtures and combinations of classes of compounds found in navel oranges were evaluated by ORAC and CAA-RBC assays respectively. Antagonisms or suppressions were seen in the ORAC assay, with the combination of "sugars" and "vitamins" and the same was seen for many other combinations including "aminos" and "Cu". This is consistent with what was observed in Chapter 5. Other notable suppressions include "PP" and "Fla", suggesting that even though, individually, these are good potential antioxidants a mixture may not be as reflected in the ORAC assay.

When the same solutions were applied to the CAA-RBC assay synergies were observed, highlighting the limitations of using any single assay to evaluate the total antioxidant capacity of foods (TAC). Strong synergies were seen between polyphenolics ("PP") and most other classes of compounds. The most notable example was that of "PP" and "org acids", where an auto-oxidation effect of "org acids" was reversed and the overall antioxidant activity was greatly enhanced in combination with the "PP" mix. Synergies between "PP" and "sugars", "vitamins", "aminos", "benz" and "Cu" were all demonstrated. These observations support our earlier postulate of interactions in the "substrate zone" at the molecular level, and how these interactions can dramatically lower the bond dissociation energies (BDEs) of O-H groups in the "antioxidant zone", thereby boosting antioxidant capacity. The mechanism for synergy postulated in Chapter 5 is consistent with the results seen in this Chapter, both in the ORAC assay and now in the CAA-RBC assay. These findings suggest that antioxidant enhancement/synergies can be measured in the cellular assay, even when screening via the ORAC assay does not yield such synergies.

Suppressions were also noted in the CAA-RBC assay, in particular those involving interactions of “Fla” with many of the other groups, including “benz”, “cu”, “PP”, “aminos” and “vitamins”. A correlation plot was produced and it was found that even though, overall, the ORAC and CAA-RBC data are not correlated (R = 0.022), some remarkable clustering and several interesting outliers are apparent. From an analysis of this data, ORAC appears to be a reasonable predictor of CAA-RBC activity for these compounds, and could be used for screening purposes.

Computational studies of quercetin and catechin, clearly demonstrate how antioxidants can be activated or deactivated upon perturbation of the substrate zone. Finally, chlorogenic acid and caffeic acid were shown via their BDEs to be inherently similar to the Vitamin E analogue Trolox and should be further investigated for their antioxidant activity *in vitro* and *ex vivo/in vivo*.

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7.0 Conclusions and Future Work

Overall research aim

Antioxidant capacity assays are controversial with respect to how values are influenced and interpreted. The overall aim of this research was to investigate influencing factors on antioxidant capacity measurements in food systems. Influencing factors were evaluated for the ORAC and CAA-RBC assays involving a range of food systems including fruit, wine and "superfood" concentrates. Furthermore, a number of approaches are proposed including new modes of reporting antioxidant capacity on food labelling, and postulations of mechanisms for antioxidant synergies in food systems. Below is a summary of findings in response to research questions.

Responses to research questions

QUESTION 1: How can antioxidant capacity measurements be communicated in a meaningful way to consumers?

RESEARCH OUTCOMES: An alternative mode of reporting antioxidant capacity by the ORAC assay is proposed in Chapter 3. In summary, an alternate mode of reporting ORAC values is presented. Calculations needed to convert ORAC units from µM Trolox equivalents (µM T.E/kg or µM T.E/L) to mass unit (g T.E/kg) Trolox equivalents per kg or per L of foodstuff are provided. Antioxidant capacity reported as g T.E/kg or g T.E/L are more easily understood by consumers, unlike µM units of Trolox equivalents which are not commonplace on food labels. Furthermore, results in mass units are visually smaller than units in tens or hundreds of thousands of µM equivalents. Mass units are therefore purported to be less misleading to consumers, by not reporting very large and impressive-looking values when a

simple conversion reveals most of them to be much more modest when viewed as a mass of vitamin E equivalent. For example, the antioxidant activity of blueberries was reported to be equivalent to 71,660μM of Trolox (vitamin E analogue). When converted, the blueberries have the same antioxidant activity as 17.9g of vitamin E per kg of fruit. Using this approach, the new mode of reporting was successfully applied to a range of commodities found in local supermarkets, demonstrating an alternative approach for communicating antioxidant capacity measurements in a meaningful way to consumers for easy integration with current labelling practice in Australia.

QUESTION 2: Do environmental factors such as temperature and rainfall influence key antioxidant parameters in a matrix?

RESEARCH OUTCOMES: In Chapter 4 we are able to show that yes, environmental factors, and in particular rainfall, appear to have the greatest influence on antioxidant capacity and content in Australian wines. A pilot study of antioxidant capacity and content of Australian wines from 4 major growing regions was conducted, focusing on single variety wines, and vintages with the highest and lowest rainfall over a 10 year period. The 2008 and 2009 vintages had the most significant fluctuations, and were assessed by ORAC, CAA-RBC, total phenolic, total flavonoid and HPLC profiling techniques. Significant differences were seen between the *in vitro* ORAC and *ex vivo* CAA-RBC, where wine varieties with similar ORAC values had vastly difference CAA-RBC antioxidant activity and bioavailability. Finally, six bioactive compounds, namely 6-methylcourmarin, protocatechuic acid, vanillic acid, p-coumaric acid, rutin and chlorogenic acid, were identified as being most affected by environmental conditions and warrant further study. Interestingly, later in Chapter 6, chlorogenic acid was investigated for its homolytic BDEs and reported to have powerful antioxidant potential of the same level

as vitamin E, making it both potent and subject to fluctuation from environmental factors in the grape/wine matrix.

QUESTION 3: Do additives such as amino acids and copper nanoparticles influence the antioxidant capacity of matrices purported to be "superfoods"?

RESEARCH OUTCOMES: In Chapter 5 we are able to show that yes, amino acids and CuNPs exhibited reproducible extraordinary enhancements of antioxidant capacity in "superfood" matrices. The bilberry, coffee berry and apple concentrates were analysed via the ORAC assay. These matrices all exhibited strong antioxidant activity in the ORAC assay, and extracts of these matrices were supplemented with an amino acid, CuNPs, or a combination of both. The order of enhancement for both amino acids and amino acid/CuNPs was Tryptophan > Tyrosine > Methionine ≥ Histidine ≥ 4-Hydroxyproline in all three matrices, suggesting the enhancement effect is an inherent property of the additives and is essentially matrix independent. This order was also consistent with the order of calculated BDEs reflecting the relative inherent antioxidant potentials of the amino acids studied. Interestingly, even amino acids with no measurable ORAC value, namely 4-Hydroxyproline and Histidine, produced strong synergistic effects in the "superfood" matrices, giving rise to further study on previously overlooked amino acids. Finally, the presence of an "antioxidant zone" and a "substrate zone" as the molecular features involved in dramatically influencing antioxidant capacity measurements was hypothesized. Histidine-Cu(I) complex was used a model for DFT calculations, demonstrating how allowable protonation or equivalent structural/electronic change to an amino acid results in extraordinary labilization of the N-H bond, with the potential to enormously enhance antioxidant capacity.

QUESTION 4: Can common classes of constituents in a matrix be combined to investigate if synergies exist *in vitro* and *ex vivo*?

RESEARCH OUTCOMES: In Chapter 6 we are able to demonstrate that yes, synergies and antagonisms do exist between some classes of constituents commonly found in a food matrix, namely navel oranges and can be measured *in vitro* and *ex vivo*. Antioxidant activities of individual mixtures and combinations of classes of compounds were evaluated by ORAC and CAA-RBC assays respectively. Antagonisms/suppressions between sugars and vitamins, and between polyphenolics and flavonoids, were seen in the ORAC assay, however these same solutions produced antioxidant synergy in the CAA-RBC assay. Strong synergies were seen in the CAA-RBC assay, between polyphenolics and organic acids, where an auto-oxidation effect of organic acids was reversed and the antioxidant activity of the mixture greatly enhanced in combination with the polyphenol mixture. Synergies between polyphenols and sugars, vitamins, amino acids, benzoic acid preservative and CuNPs were all demonstrated. These findings support our earlier postulate of how interactions in the "substrate zone" at the molecular level can influence antioxidant capacity measurements by greatly lowering BDEs of O-H groups in the "antioxidant zone". Although overall ORAC and CAA-RBC data did not correlate, distinct clustering and several interesting outliers were noted. Cluster analysis revealed classes of constituents for which ORAC appears to be a reasonable predictor of CAA-RBC activity, with potential use for screening purposes. Finally, computational studies of Quercetin and Catechin demonstrated how antioxidants can be activated or deactivated upon perturbation of the substrate zone, and chlorogenic acid and caffeic acid were found to have BDEs similar to the vitamin E analogue Trolox, making them good candidates for future studies of antioxidant capacity and synergy.

QUESTION 5: Can factors at the molecular level be identified, via computational chemistry, which lead to the enhancement of antioxidant capacity?

RESEARCH OUTCOMES: In Chapters 5 and 6 we propose and explore the concept of "redox and substrate zones", and use computational chemistry to postulate a mechanism for antioxidant enhancement/synergy at the molecular level. DFT calculations demonstrate how homolytic BDEs for phenolic O-H groups can be influenced by interactions between the matrix and the substrate zone or an antioxidant and/or an amino acid. In the example of Histidine-Cu(I) complex, allowable protonation or equivalent structural/electronic change resulted in the lowest BDEs calculated for any of the molecules studied, far lower than other excellent antioxidants such as vitamin E. This presents a potential mechanism for the extraordinary antioxidant enhancements measured in the ORAC assay. Computational studies on the Quercetin molecule revealed that changes at the "substrate zone" resulted in the deactivation of one phenolic O-H group and activated another. In contrast, in the representative Catechin, compound, homolytic cleavage of the O-H bond with the lowest BDE activated four out of the six remaining O-H groups. These computational studies support our experimental observations of antioxidant activity, synergy and present mechanisms of factors that can influence antioxidant capacity measurements in food systems.

Summary of overall outcomes

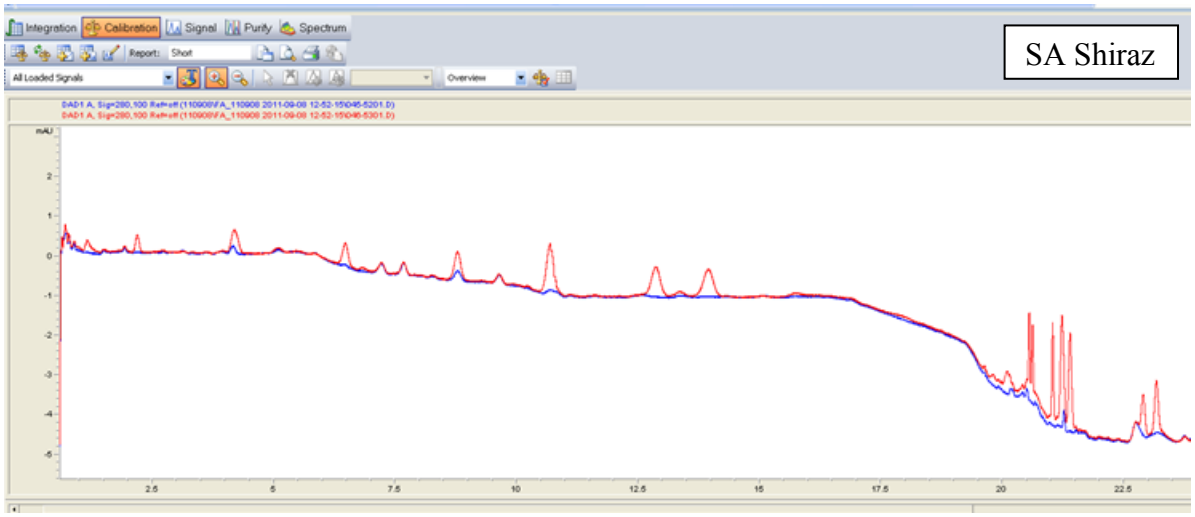
In summary, a number of factors were identified as influencing the ORAC and CAA-RBC antioxidant capacity measurements in food systems. Firstly, conversion of ORAC values to g T.E/kg or g T.E/L was recommended when using ORAC values on product labelling. Mass units are purported to be less misleading to consumers, not reporting very large and impressive-looking values when a simple conversion reveals most of them to be much more modest when viewed as a mass of vitamin E equivalent. Rainfall was identified as a factor influencing antioxidant activity of varieties of wine. Six antioxidants were identified as being susceptible to variation under changes in environmental conditions, for example during periods of extended drought or rainfall. CAA-RBC was able to demonstrate significant differences in bioavailability and variation of antioxidant capacity in wine samples that were not noted in the ORAC assay. In "superfood" matrices, addition of amino acids and CuNPs were identified as factors greatly influencing antioxidant capacity measurements. These additives exhibited inherent powerful synergies independent of the "superfood" matrix, suggesting common food constituents may interact at the substrate zone of amino acids, and postulating mechanisms independently and in complexation with Cu(I). Synergies were observed between classes of constituents in the absence of food matrices, particularly in the CAA-RBC assay. Bioactivity of synergistic groups was reported, supporting the postulation of substrate zone interaction at the molecular level influencing antioxidant activity *ex vivo*. Computational chemistry was used to postulate mechanisms for antioxidant synergy, activation and deactivation of phenolic O-H groups. The relationship between the antioxidant and substrate zones, even when remote at a molecular level, are critical influencing factors of antioxidant capacity measurements.

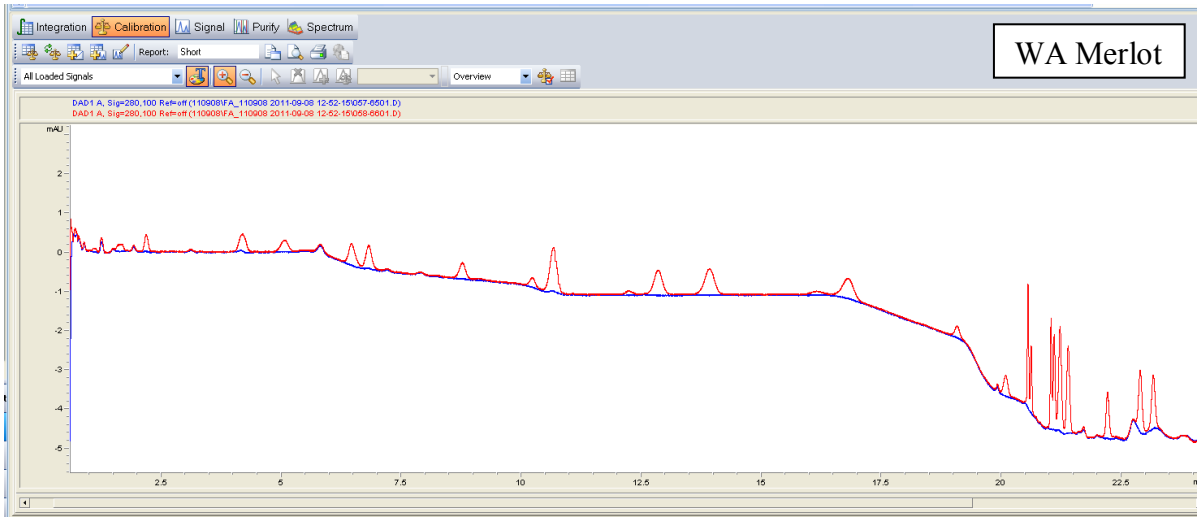
Future work

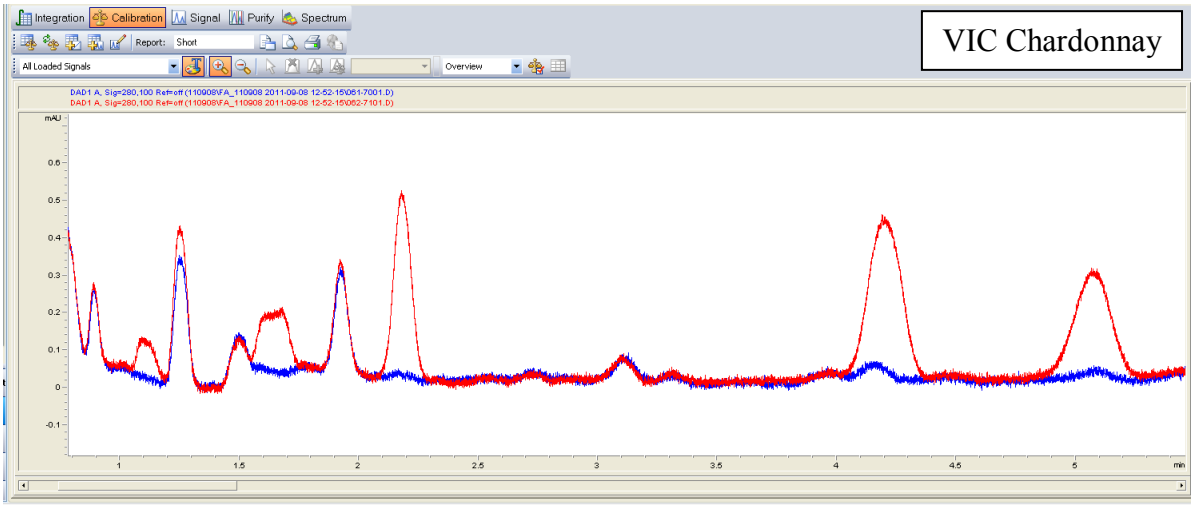
Although outside the scope of this thesis, the data obtained in Chapter 3 could be evaluated by a consumer survey. This would be useful in future work to obtain feedback on how meaningful this mode of reporting is to consumers and its potential to influence overall product selection. More research is also needed to investigate other factors influencing antioxidant capacity of grapes/wine produced under conditions of environmental stress. Of the compounds studied, six were identified as most influenced, and should be further studied to determine the mechanisms involved. Amino acids appear to greatly influence antioxidant capacity measurements, and more experimental trials are needed to elucidate trends for other amino acids which have not previously shown baseline antioxidant activity. Our results have demonstrated that the absence of baseline ORAC measurement is not a clear indicator of absence of antioxidant synergistic potential, as was seen for 4-Hydroxyproline and Histidine. CuNPs were another major contributing factor *in vitro*, and more work is needed to investigate if the proposed amino acid/Cu(I) complexation have *ex vivo/in vivo* activity. Finally, computational studies of chlorogenic acid and caffeic acid report excellent antioxidant potential comparable to that of vitamin E. These molecules should be investigated further experimentally.

Appendix A: Chromatographic Spectra and Conference presentations relevant to the scope of this thesis

Examples of Chromatographic RRLC Spectra of Bioactives in Wines Studied in Chapter 4







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Evaluation of ascorbic acid as a standard for the Oxygen Radical Absorbance Capacity (ORAC) Assay

Katherine Stockham[1,2], Rohani Paimin[2], John D Orbell[2], Paul Adorno[1], Saman Buddhadasa[1]

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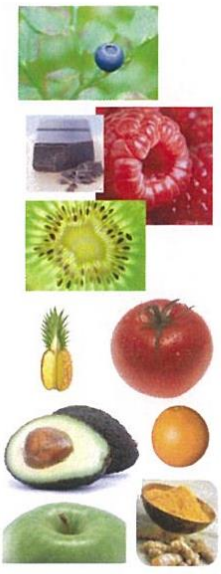
Introduction



- Oxygen Radical Absorbance Capacity assay
- Measure of antioxidant capacity of a sample against a specific radical species (peroxyl radical)
- Increasing in popularity
  - Health claims on product packaging
- Increasingly popular choice for antioxidant capacity measurements of food

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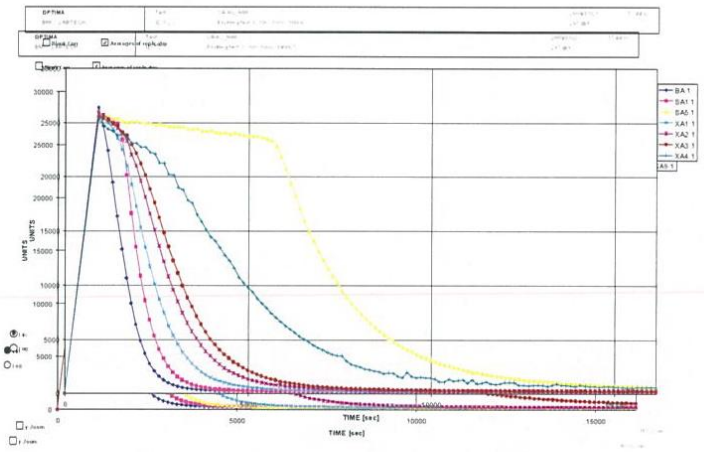
Basic Principle of Measurement

- Fluorescent Probe (Fluorescein)
- Peroxyl Radical Donor (AAPH)
- Radicals thermally generated (37°C)
- Attack probe to cause decay and loss of fluorescence
- Antioxidants present in sample prevent probe decay
- When antioxidant supply exhausted, radicals attack probe



1. *Fluorescence intensity measurements taken at regular intervals*
2. *Area under decay curves serves as measure of antioxidant capacity of sample against peroxyl radical*

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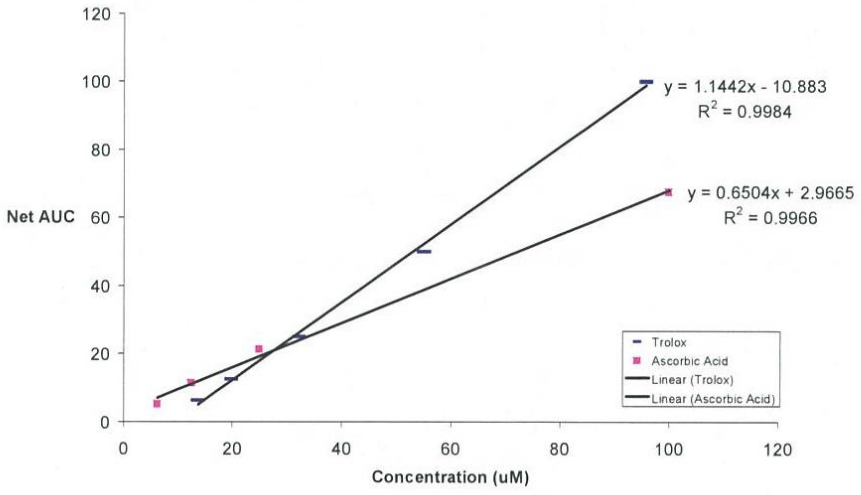
Standards Comparison



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Validation Summary

Validation parameters for both Trolox and ascorbic acid analytes over a calibration range of 6.25-100µM for both standards

Validation Parameters	Measurand	Replicates	Statistical Indicators
Linearity & Repeatability of Standards	Trolox	7	R^2 = 0.9996 ; $\%RSD_{(CL\ 3\sigma)}$ = 2.22
	ascorbic acid	7	R^2 = 0.9986 ; $\%RSD_{(CL\ 3\sigma)}$ = 2.32
Limit of Detection	Trolox	10	$_{(Net\ AUC)}$)13 ± 0.66 ; LOD = 2.0µM
	ascorbic acid	10	$_{(Net\ AUC)}$)12 ± 0.69 ; LOD = 2.0µM
Instrument repeatability	Phosphate buffer	30	$_{(AUC)}$) 21 ± 0.86 ; $\%RSD_{(CL\ 3\sigma)}$ = 0.04
Repeatability of Samples	Trolox	7	$MDL_{(\mu M)}$ = 1.4 ; $\%REC_{(CL\ 80\text{-}120\%)}$ = 110 ; $\%RSD_{(CL\ 3\sigma)}$ = 1.1
	ascorbic acid	7	$MDL_{(\mu M)}$ = 2.1 ; $\%REC_{(CL\ 80\text{-}120\%)}$ = 110 ; $\%RSD_{(CL\ 3\sigma)}$ = 1.6

($\bar{x}$) = mean, MDL = method detection limit, %REC = % recovery of 10µM spike of standard in natural mineral water matrix, %RSD = % relative standard deviation coefficient of variation, AUC = area under the curve, CL = control limit, LOD = limit of detection, R^2 correlation coefficient for linear regression plot

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Measurement Uncertainty

Measurement uncertainty estimation for ORAC values in the Trolox scale

Net AUC used to get result	Typical ORAC values over calibration range (µM T.E)	± Combined uncertainty	Error as %
35	27.815	4.2830	15%
45	38.273	4.2420	11%
75	69.647	4.5280	7%
95	90.564	4.9970	6%
100	95.793	5.1420	5%

(µM T.E = micro mol per liter of Trolox equivalents)

Measurement uncertainty estimation for ORAC values in the ascorbic acid scale.

Net AUC used to get result	Typical ORAC values over the calibration range (µM A.E)	± Combined uncertainty	Error as %
25	37.856	5.7610	15%
30	46.418	5.7850	12%
40	63.542	6.0090	9%
50	80.655	6.4460	8%
60	97.789	7.0550	7%

(µM A.E = micro mol per liter of ascorbic acid equivalents)

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Data Interpretation

ORAC Calculation

I $$AUC = \left(\left[\frac{f1}{f0} + \frac{f2}{f0} + \frac{f3}{f0} \ldots \frac{fi}{f0} \right] + 0.5 \right) \times CT$$

(f0 = initial fluorescence reading, fi = fluorescence reading at cycle i, and CT = cycle time in minutes)

II $$NetAUC(sample) = \left(\frac{AUC\,sample - AUC\,blank}{AUC\,Trolox - AUC\,blank} \right)$$

III $$ORAC/kg = \left[\frac{netAUC\,sample - c}{m} \right] \times DF$$

(where the equation, y = mx + c, is for a plot of netAUC vs concentration of standards (Trolox or Ascorbic acid) and DF = dilution factor of sample)

Dense liquids

$$\frac{x\mu M\ \text{equivalents}}{kg} \times \frac{1kg}{1000g} = \frac{x\mu M\ \text{equivalents}}{g}$$

To convert to xμM equivalents g to xμM equivalents ml, density factor is considered

IIIa $$\frac{x\mu M\ \text{equivalents}}{\left(\frac{g}{density\,(g/ml.)} \right)} = x\mu M\ \text{equivalents} \times \frac{density}{1ml.}$$

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Reporting in Mass Units

Conversion to mass units

Determine moles of ascorbic acid in the sample (based on the concentration in IV)

IV $$C = x\mu M = \left(\frac{x\mu M}{1 \times 10^{6} \mu M} \right) \times 1M = x \times 10^{-6} M$$

V $$n = C \times V$$

(where n = moles, C = concentration of ascorbic equivalents in molar units, V = volume in litres)

VI $$m = n \times MM$$

(where m = equivalent mass of Ascorbic acid,
n = moles of Ascorbic acid (V), MM = molar mass of Ascorbic acid)

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Results

Ten samples reported in ascorbic acid and Trolox equivalents. With the exception of dark chocolate, all other samples contained negligible fats. Consequently the ORAC hydrophilic results were taken to be the total antioxidant capacity (TAC) for the sample. TAC = ∑(ORAC(hydrophilic) + ORAC(lipophilic)). Results are shown in mass and molar units for comparison purposes.

Sample ID		$ORAC_{(TAC)}$ µmol T.E/g	ORAC g/kg T.E	$ORAC_{(TAC)}$ µmol A.E/g	ORAC g/kg A.E
Cranberry Juice		12.0	3.004	21.0	5.256
Orange Juice		15.0	3.754	25.0	6.257
Fresh Orange Juice		33.0	8.260	55.0	13.766
Ribena (ready to drink)		8.0	2.002	14.0	3.504
Fresh Lime Juice		9.0	2.253	17.0	4.255
Rooibos Tea		8.3	2.077	14.0	3.504
Black Tea		19.0	4.756	29.0	7.258
Apple Juice		6.4	1.602	9.6	2.403
Prune Juice		52.0	13.015	85.0	21.275
		$ORAC_{(TAC)}$ µmol T.E/g	ORAC g/kg T.E	$ORAC_{(TAC)}$ µmol A.E/g	ORAC g/kg A.E
Dark Chocolate (70%)	$ORAC_{(lipophilic)}$	3.8	0.951	15.0	3.754
	$ORAC_{(hydrophilic)}$	21.0	5.256	37.0	9.261
	$ORAC_{(TAC)}$	25.0	6.257	52.0	13.015

(TAC = total antioxidant capacity; T.E = Trolox equivalents; A.E = ascorbic acid equivalents) Results are taken as mean of n=7 replicates. Results are reported to 2 significant figures, and rounded up where necessary.

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Significance

- Results understandable to layperson
  - Added benefit of using Ascorbic acid units
    - Naturally occurring
    - Common in plant based foods
- Easier to communicate ORAC values in mass units
  - Molar units not commonly used in NIP labels

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Conclusion

- Ascorbic acid viable standard
  - Validated
    - Hydrophilic
    - Lipophilic
  - Comparable MU budget
- More effective communication of antioxidant capacity measurement
  - mass units vs. molar units
    - Solids
      - g/kg vs. µM T.E/kg (alternatively µM A.E/kg)
    - Liquids
      - g/L vs. µM T.E/L (alternatively µM A.E/L)

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National Measurement Institute
Port Melbourne

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Werribee

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Comparative studies on the antioxidant properties and bioactive content of wine from different growing regions and vintages

9th International Food Data Conference, Thailand

Katherine Stockham

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Introduction to study context

- A comparative study of
  - Australian wines from key growing regions
  - Different vintages
- To determine the effect of climate conditions on functional properties of wine
  - Antioxidant capacity (ORAC, CAA-RBC)
  - Bioactive Profile (HPLC-DAD)
  - Total phenolic and flavonoid content (Folin-Ciocalteu assay, $AlCl_3$ assay)

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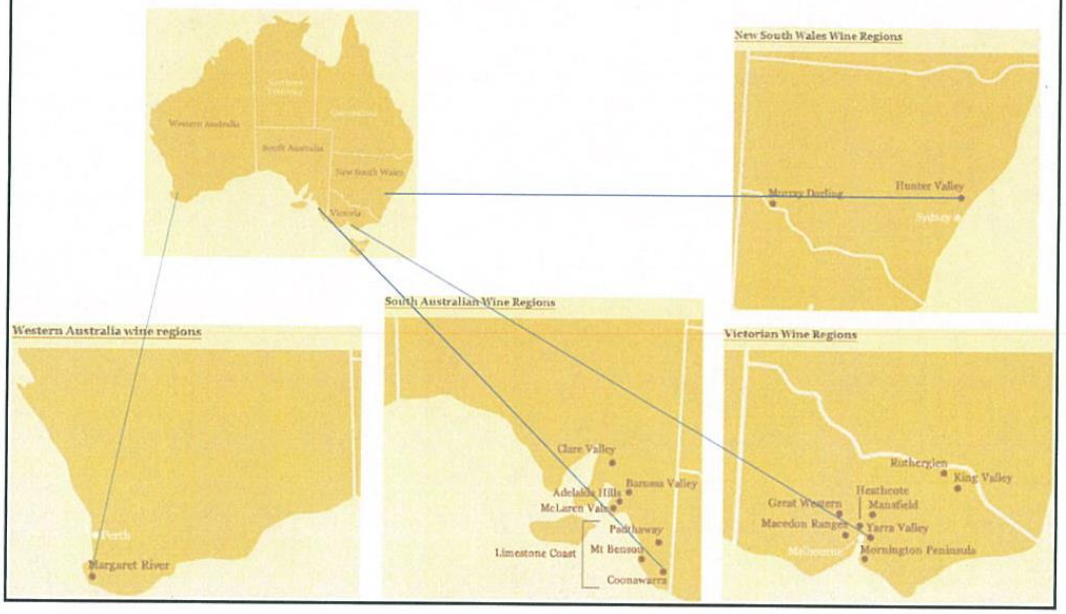
Wine regions in this study



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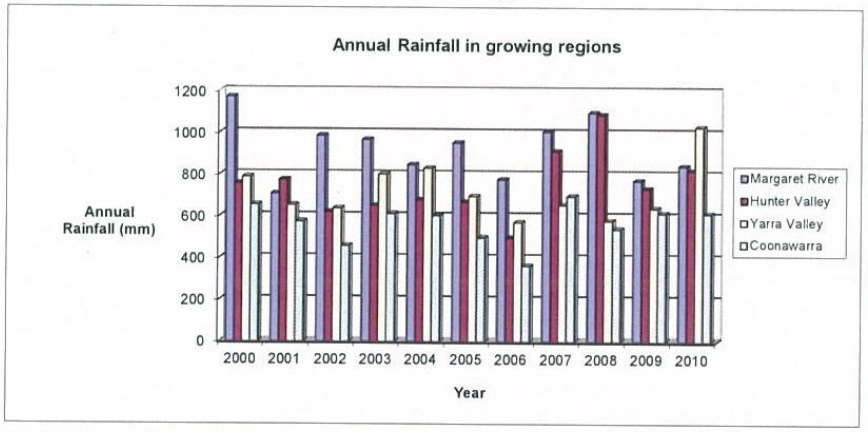
Rainfall 2000-10

- Greater fluctuation between regions in 2008, particularly during the main maturation phase
- 2009 overall representative of the decade average



	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010
Margaret River	1169.8	707.2	984.2	965.2	845.4	951	775.6	1005	1094.6	769.4	839.8
Hunter Valley	756.2	773.9	620.3	650	676.5	668	497.1	911.2	1085.6	731.3	818.3
Yarra Valley	787.9	653	637.2	799.2	829.2	694.4	571	654.4	579	638	1026.8
Coonawarra	655.8	574	458.8	611.3	603.5	497.9	362.2	695.7	538.5	614.9	612.4

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Wines

	2008			
	Shiraz	**Chardonnay**	**Cabernet Sauvignon**	**Merlot**
Yarra Valley	Brown Brothers	Sticks	Brown Brothers	Brown Brothers
Coonawarra	Wild Fox	Grant Burge	Wild Fox	Wild Fox
Hunter Valley	Barwang	McWilliams		
Margaret River	Select Parcel	Catching Thieves		

- Compare wines of same grape varieties from different regions
- 2 varieties across 4 regions
- Compare shiraz, cabernet sauvignon and merlot from the same wineries (same region)

	2009			
	Shiraz	**Cabernet Merlot**	**Cabernet Sauvignon**	**Pinot Noir**
Hunter Valley	Yellow Tail	Yellow Tail	Yellow Tail	Yellow Tail
Margaret River				Select Parcel
Yarra Valley				Sticks

- Compare wines from one region grown under the same conditions
- Compare wines from different regions but same grape variety

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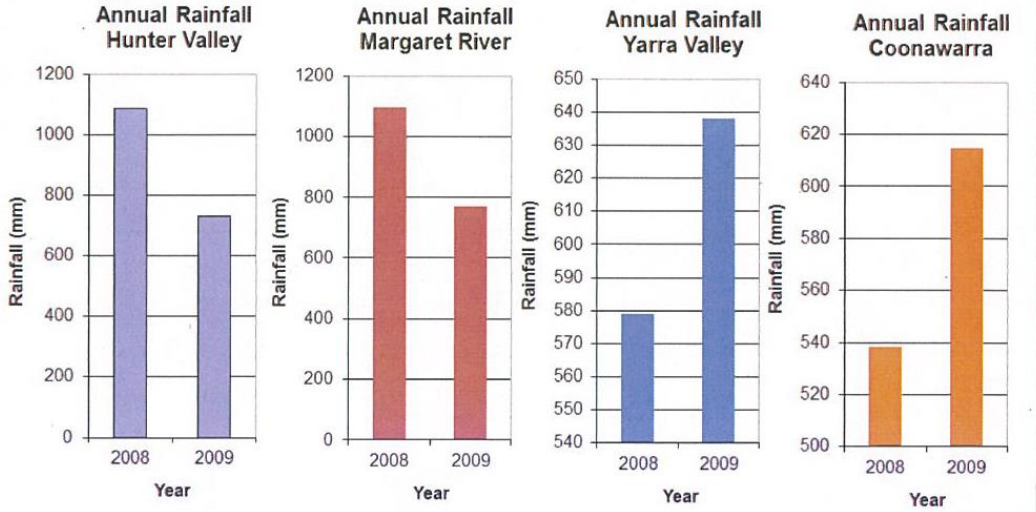
2008-9 Trends by region



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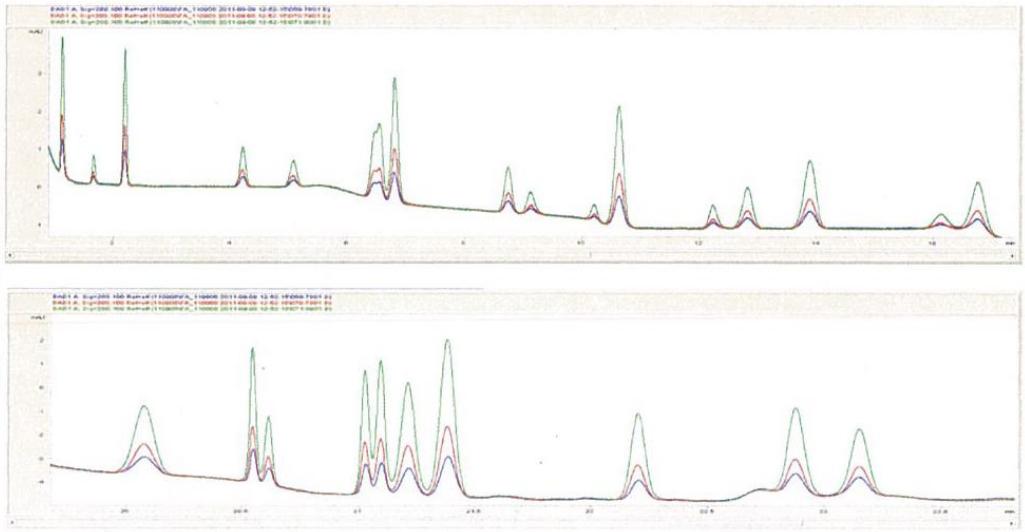
31 Bioactives studied



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Grape bioactives present in wines

		Caffeic acid	p-Coumaric acid	Transcinnamic acid	Quercetin
2008	VIC Merlot	x	x	x	x
	VIC Shiraz	x	x	x	x
	VIC Cab Sauv	x	x	x	x
	SA Merlot		x	x	x
	SA Shiraz		x	x	x
	SA Cab Sauv		x	x	x
	NSW Shiraz		x		x
	WA Shiraz		x	x	x
	WA Chardonnay		x	x	
	SA Chardonnay		x		
	VIC Chardonnay		x		
	NSW Chardonnay		x		
2009	NSW Cab Merlot		x	x	x
	NSW Shiraz		x		
	NSW Cab Sauv		x		
	NSW Pinot Noir		x		x
	WA Pinot Noir		x	x	
	VIC Pinot Noir		x		

- 31 Bioactives were profiled in 18 wines
- Summary of grape bioactives
- A number of bioactives can be attributed to oak barrels and/or wine corks, these were also profiled and will be published, however are not relatable to rainfall patterns/temperatures

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Summary of data

	2008			
	Shiraz	**Chardonnay**	**Cabernet Sauvignon**	**Merlot**
Yarra Valley	Brown Brothers	Sticks	Brown Brothers	Brown Brothers
Coonawarra	Wild Fox	Grant Burge	Wild Fox	Wild Fox
Hunter Valley	Barwang	McWilliams		
Margaret River	Select Parcel	Catching Thieves		

2008	**ORAC µM TE/L**	**CAA-RBC µM QE/L**	**Phenolics g/L GAE**	**Flavonoids g/L CE**	**Annual Rainfall**
NSW Shiraz	36400	6.62	2.91	1.71	1085.60
SA Shiraz	41900	5.44	2.92	1.86	538.50
VIC Shiraz	40600	7.65	3.53	1.65	579.00
WA Shiraz	49400	8.08	1.65	1.18	1094.60
2008	**µM TE/L**	**µM QE/L**	**g/L GAE**	**g/L CE**	**Annual Rainfall**
NSW Chardonnay	5100	-	0.44	0.34	1085.60
SA Chardonnay	5800	-	1.24	0.43	538.50
VIC Chardonnay	5800	-	1.24	0.43	579.00
WA Chardonnay	3800	-	1.17	0.38	1094.60
2008	**µM TE/L**	**µM QE/L**	**g/L GAE**	**g/L CE**	**Annual Rainfall**
SA Cabernet Sauvignon	30400	2.26	3.35	1.15	538.50
VIC Cab Sauvignon	31900	3.35	2.84	1.39	579.00
2008	**µM TE/L**	**µM QE/L**	**g/L GAE**	**g/L CE**	**Annual Rainfall**
SA Merlot	28000	4.14	1.07	1.38	538.50
VIC Merlot	32300	6.35	2.77	1.93	579.00

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Summary of data

	2009			
	Shiraz	**Cabernet Merlot**	**Cabernet Sauvignon**	**Pinot Noir**
Hunter Valley	Yellow Tail	Yellow Tail	Yellow Tail	Yellow Tail
Margaret River				Select Parcel
Yarra Valley				Sticks

2009	**ORAC µM TE/L**	**CAA-RBC µM QE/L**	**Phenolics g/L GAE**	**Flavonoids g/L CE**	**Annual Rainfall**
NSW Cab Merlot	32700	2.09	3.21	1.93	
NSW Cab Sauv	26300	2.67	2.68	1.89	731.3
NSW Shiraz	38700	6.22	2.96	3.06	
NSW Pinot Noir	31900	6.08	1.08	1.83	
VIC Pinot Noir	41500	3.13	3.32	2.61	638
WA Pinot Noir	45900	11.17	2.80	1.78	769.4

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Preliminary Findings 2008-9 Study

2008	ORAC µM TE/L	CAA-RBC µM QE/L	Phenolics g/L GAE	Flavonoids g/L CE	Annual Rainfall	ORAC g/L	%	CAA g/L	%	Phenolics g/L	%	Flavonoids g/L	%
NSW Shiraz	36400	6.62	2.91	1.71	1085.60	9.1106	0.9111	0.0020	0.0002	2.9110	0.2911	1.7100	0.1710
SA Shiraz	41900	5.44	2.92	1.86	538.50	10.4872	1.0487	0.0016	0.0002	2.9240	0.2924	1.8562	0.1856
VIC Shiraz	40600	7.65	3.53	1.65	579.00	10.1618	1.0162	0.0023	0.0002	3.5328	0.3533	1.6523	0.1652
WA Shiraz	49400	8.08	1.65	1.18	1094.60	12.3643	1.2364	0.0024	0.0002	1.6529	0.1653	1.1792	0.1179
2008													
NSW Chardonnay	5100	-	0.44	0.34	1085.60	1.2765	0.1276	-	-	0.4352	0.0435	0.3369	0.0337
SA Chardonnay	5800	-	1.24	0.43	538.50	1.4517	0.1452	-	-	1.2384	0.1238	0.4331	0.0433
VIC Chardonnay	5800	-	1.24	0.43	579.00	1.4517	0.1452	-	-	1.2384	0.1238	0.4331	0.0433
WA Chardonnay	3800	-	1.17	0.38	1094.60	0.9511	0.0951	-	-	1.1677	0.1168	0.3792	0.0379
2008													
SA Cav Sauv	30400	2.26	3.35	1.15	538.50	7.6088	0.7609	0.0007	0.0001	3.3510	0.3351	1.1485	0.1148
VIC Cab Sauv	31900	3.35	2.84	1.39	579.00	7.9843	0.7984	0.0010	0.0001	2.8429	0.2843	1.3869	0.1387
2008													
SA Merlot													
VIC Merlot	32300	6.35	2.77	1.93	579.00	8.0844	0.8084	0.0019	0.0002	2.7698	0.2770	1.9254	0.1925
2009													
NSW Cab Merlot	32700	2.09	3.21	1.93		8.1845	0.8184	0.0006	0.0001	3.2146	0.3215	1.9254	0.1925
NSW Cab Sauv	26300	2.67	2.68	1.89	731.3	6.5826	0.6583	0.0008	0.0001	2.6838	0.2684	1.8946	0.1895
NSW Shiraz	38700	6.22	2.96	3.06		9.6862	0.9686	0.0019	0.0002	2.9614	0.2961	3.0562	0.3056
NSW Pinot Noir	31900	6.08	1.08	1.83		7.9843	0.7984	0.0018	0.0002	1.0815	0.1081	1.8292	0.1829
VIC Pinot Noir	41500	3.13	3.32	2.61	638	10.3870	1.0387	0.0009	0.0001	3.3234	0.3323	2.6100	0.2610
WA Pinot Noir	45900	11.17	2.80	1.78	769.4	11.4883	1.1488	0.0034	0.0003	2.8039	0.2804	1.7754	0.1775

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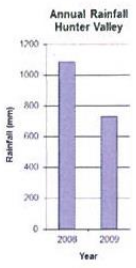
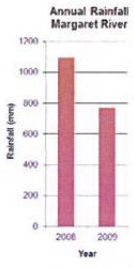
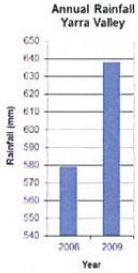
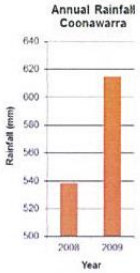
Summary of Findings

		Antioxidants In vitro	Antioxidants In vivo	Bioactives %	Phenolics %	Flavonoids %
Hunter Valley	Red	-	-	-	+	+
	White	+	N/D	-	-	-
Margaret River	Red	+	-	+	-	-
	White	-	N/D	+	+	+
Coonawarra	Red	+	-	-	-	+
	White	=	N/D	=	=	=
Yarra Valley	Red	-	+	+	+	-
	White	=	N/D	=	=	=









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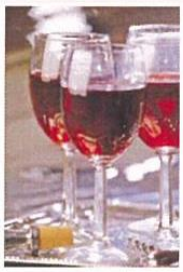
Conclusions

- In vitro and in vivo antioxidant capacity, total phenolic, total flavonoid and bioactive content of 18 wines across 4 regions and 2 vintages were investigated
- Red wines produced in WA (Margaret River) and NSW (Hunter Valley) have greater variation in antioxidant capacity
- Bioactive profiles and overall antioxidant capacity measurements appeared to be the most affected by climate conditions
- In the warmer and wetter regions, Margaret River red and white wines responded positively to the increased rainfall
- In the cooler and drier regions, only Yarra Valley red wines responded positively to the increased rainfall and both regions (Yarra Valley and Coonawarra) had comparable white wine responses – indicating that further studies on the effect of soil composition on bioactive content in wines is needed, in particular a study on red wines



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Thankyou

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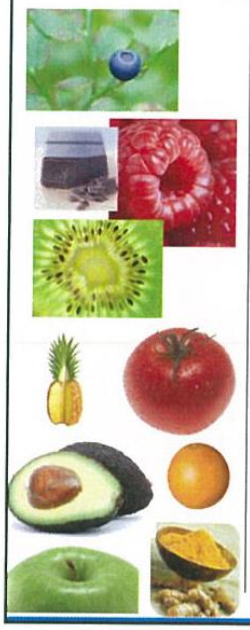

Investigation of the causes and extent of matrix effects on the Oxygen Radical Absorbance Capacity (ORAC) assay

Perspectives on matrix effects and approach to profiling antioxidant behaviour of sample matrices

A Preliminary Investigation

Katherine Stockham

B.Sc. Medical, Forensic & Analytical Chemistry
B.Sc. Honours (Chemical Sciences)
PhD Candidate

Supervisors:
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ORAC

- Oxygen Radical Absorbance Capacity assay
- Measure of antioxidant capacity of a sample against a specific radical species (peroxyl radical)
- Increasing in popularity
  - Health claims on product packaging
- Increasingly popular choice for antioxidant capacity measurements of food

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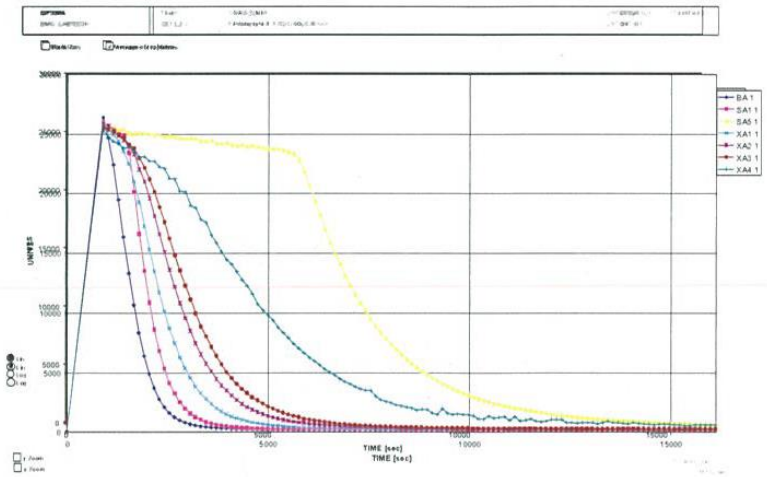
Basic Principle of Measurement

- Fluorescent Probe (Fluorescein)
- Peroxyl Radical Donor (AAPH)
- Radicals thermally generated (37°C)
- Attack probe to cause decay and loss of fluorescence
- Antioxidants present in sample prevent probe decay
- When antioxidant supply exhausted, radicals attack probe



1. *Fluorescence intensity measurements taken at regular intervals*
2. *Area under decay curves serves as measure of antioxidant capacity of sample against peroxyl radical*

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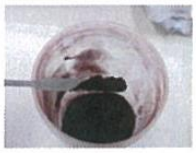
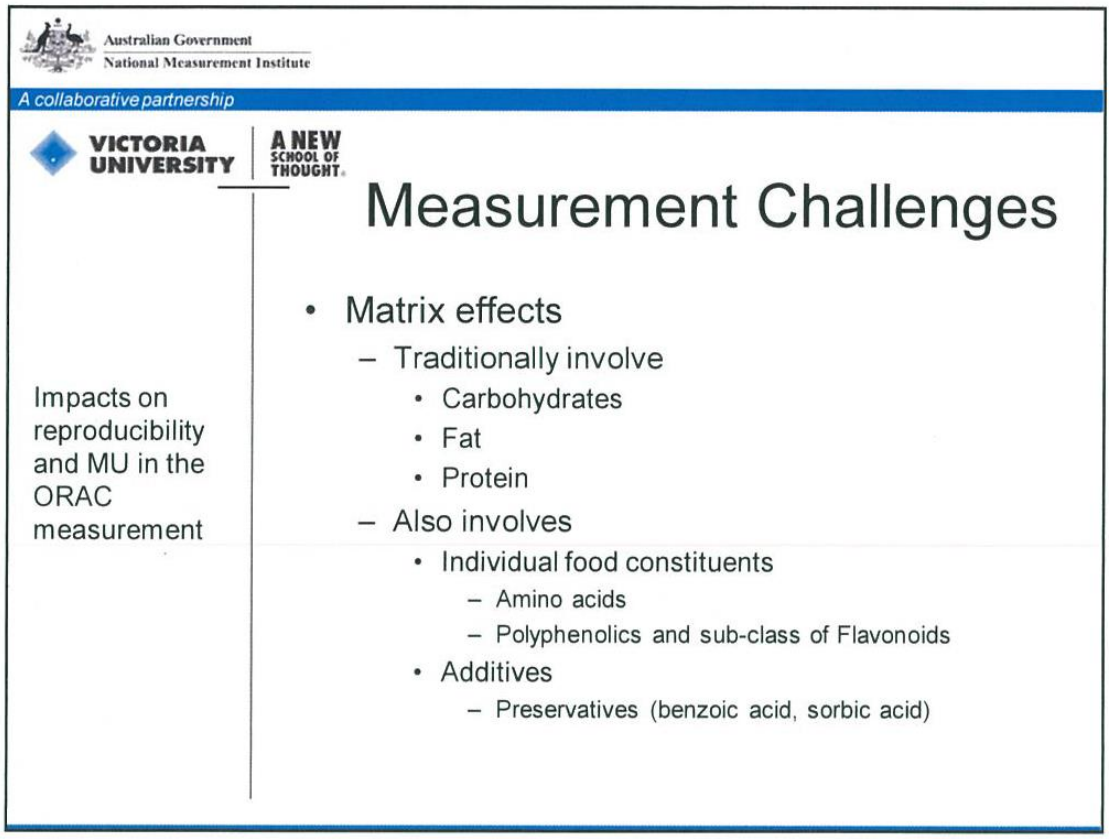
Novel Matrices



- Pharmaceutical samples
- Plant materials and extracts
- Health foods
- Supplements

Extraction Challenges

- Solubility/solvent choice
- Dilutions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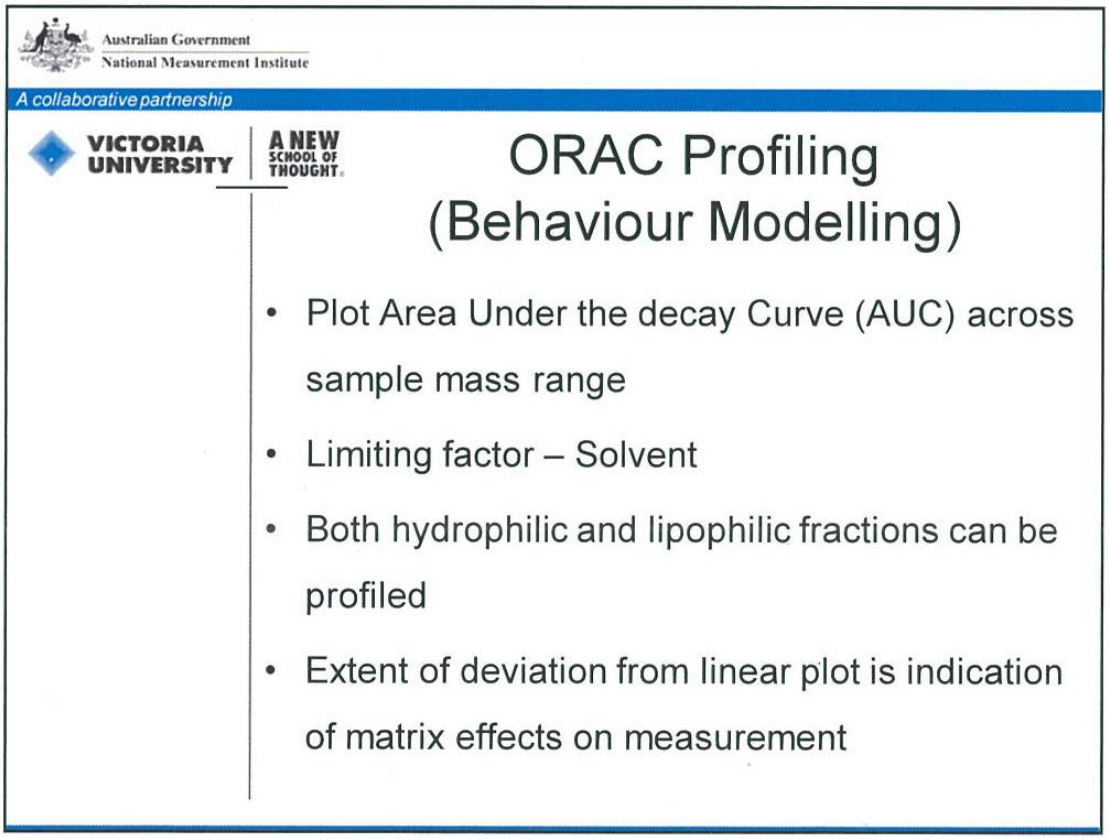
Measurement Challenges
Impacts on reproducibility and MU in the ORAC measurement
• Matrix effects
– Traditionally involve
• Carbohydrates
• Fat
• Protein
– Also involves
• Individual food constituents
– Amino acids
– Polyphenolics and sub-class of Flavonoids
• Additives
– Preservatives (benzoic acid, sorbic acid)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ORAC Profiling
(Behaviour Modelling)
• Plot Area Under the decay Curve (AUC) across sample mass range
• Limiting factor – Solvent
• Both hydrophilic and lipophilic fractions can be profiled
• Extent of deviation from linear plot is indication of matrix effects on measurement

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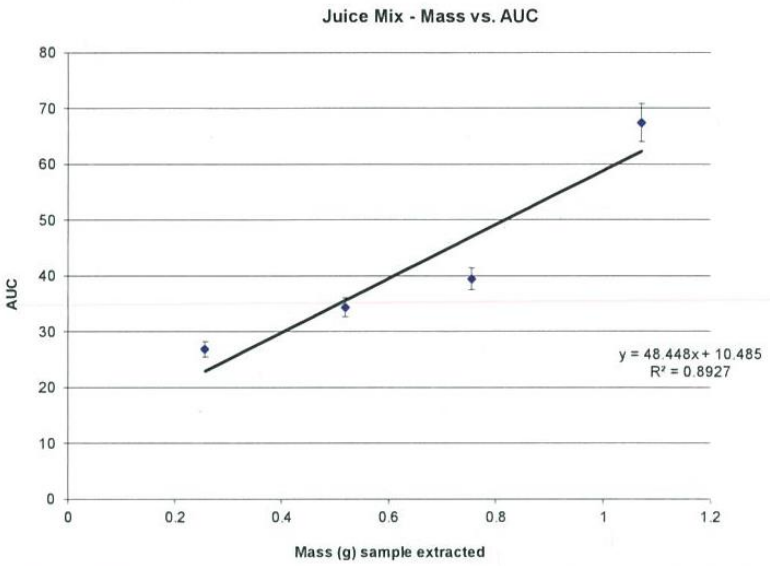
Current Research

- Investigation of components contributing to matrix effects
- Example of extent of matrix effects is shown



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Preliminary Results

- Inhibition
- Amplification

Trial Description	Histidine			
Sample (0.5g extracted)	*Juice Mix*	*Bilberry*	*Apple*	*Coffee Berry*
Amino Acid [10%] 20nmol/mL (uM T.E/kg)	ND	ND	ND	ND
Cu [10%] (uM T.E/kg)	243	243	243	243
Amino Acid & Cu [10% each] (uM T.E/kg)	98	98	98	98
Cu & Sample (uM T.E/kg)	149,962	1,715,753	28,395,708	2,389,390
Sample (uM T.E/kg)	38,780	264,857	8,864,387	418,306
Amino Acid & Sample (uM T.E/kg)	24,430	315,149	10,088,301	574,488
Amino Acid, Cu & Sample (uM T.E/kg)	201,535	2,379,477	34,914,366	3,061,463
% Inhibition of Sample / Amino Acid & Sample (uM T.E/kg)	37	N/A	N/A	N/A
% Amplification of Cu & Sample / Sample (uM T.E/kg)	387	648	320	571
% Amplification of Amino Acid & Sample / Sample (uM T.E/kg)	N/A	119	114	137
% Amplification of Amino Acid, Cu & Sample / Sample (uM T.E/kg)	520	898	394	732

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Preliminary Results

- Inhibition
- Amplification

Trial Description	4-Hydroxy-Proline			
Sample (0.5g extracted)	*Juice Mix*	*Bilberry*	*Apple*	*Coffee Berry*
Amino Acid [10%] 20nmol/mL (uM T.E/kg)	ND	ND	ND	ND
Cu [10%] (uM T.E/kg)	243	243	243	243
Amino Acid & Cu [10% each] (uM T.E/kg)	58	58	58	58
Cu & Sample (uM T.E/kg)	149,962	1,715,753	28,395,708	2,389,390
Sample (uM T.E/kg)	38,780	264,857	8,864,387	418,306
Amino Acid & Sample (uM T.E/kg)	15,824	233,628	9,293,479	421,303
Amino Acid, Cu & Sample (uM T.E/kg)	132,154	1,588,612	26,449,455	2,043,491
% Inhibition of Sample / Amino Acid & Sample (uM T.E/kg)	59	12	N/A	N/A
% Amplification of Cu & Sample / Sample (uM T.E/kg)	387	648	320	571
% Amplification of Amino Acid & Sample / Sample (uM T.E/kg)	N/A	N/A	105	101
% Amplification of Amino Acid, Cu & Sample / Sample (uM T.E/kg)	341	600	298	489

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Preliminary Results

- Inhibition
- Amplification

Trial Description	Methionine			
Sample (0.5g extracted)	*Juice Mix*	*Bilberry*	*Apple*	*Coffee Berry*
Amino Acid [10%] 20nmol/mL (uM T.E/kg)	45	45	45	45
Cu [10%] (uM T.E/kg)	243	243	243	243
Amino Acid & Cu [10% each] (uM T.E/kg)	126	126	126	126
Cu & Sample (uM T.E/kg)	149,962	1,715,753	28,395,708	2,389,390
Sample (uM T.E/kg)	38,780	264,857	8,864,387	418,306
Amino Acid & Sample (uM T.E/kg)	96,205	1,134,792	17,684,085	1,341,571
Amino Acid, Cu & Sample (uM T.E/kg)	255,599	2,445,362	35,872,543	3,115,397
% Inhibition of Sample / Amino Acid & Sample (uM T.E/kg)	N/A	N/A	N/A	N/A
% Amplification of Cu & Sample / Sample (uM T.E/kg)	387	648	320	571
% Amplification of Amino Acid & Sample / Sample (uM T.E/kg)	248	428	199	321
% Amplification of Amino Acid, Cu & Sample / Sample (uM T.E/kg)	659	923	405	745

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Preliminary Results

- Inhibition
- Amplification

Trial Description	Tyrosine			
Sample (0.5g extracted)	Juice Mix	Bilberry	Apple	Coffee Berry
Amino Acid [10%] 20nmol/mL (uM T.E/kg)	70	70	70	70
Cu [10%] (uM T.E/kg)	243	243	243	243
Amino Acid & Cu [10% each] (uM T.E/kg)	257	257	257	257
Cu & Sample (uM T.E/kg)	149,962	1,715,753	28,395,708	2,389,390
Sample (uM T.E/kg)	38,780	264,857	8,864,387	418,306
Amino Acid & Sample (uM T.E/kg)	156,695	1,574,771	23,976,777	1,819,651
Amino Acid, Cu & Sample (uM T.E/kg)	519,118	5,470,758	63,477,102	5,855,499
% Inhibition of Sample / Amino Acid & Sample (uM T.E/kg)	N/A	N/A	N/A	N/A
% Amplification of Cu & Sample / Sample (uM T.E/kg)	387	648	320	571
% Amplification of Amino Acid & Sample / Sample (uM T.E/kg)	404	595	270	435
% Amplification of Amino Acid, Cu & Sample / Sample (uM T.E/kg)	1,339	2,066	716	1,400

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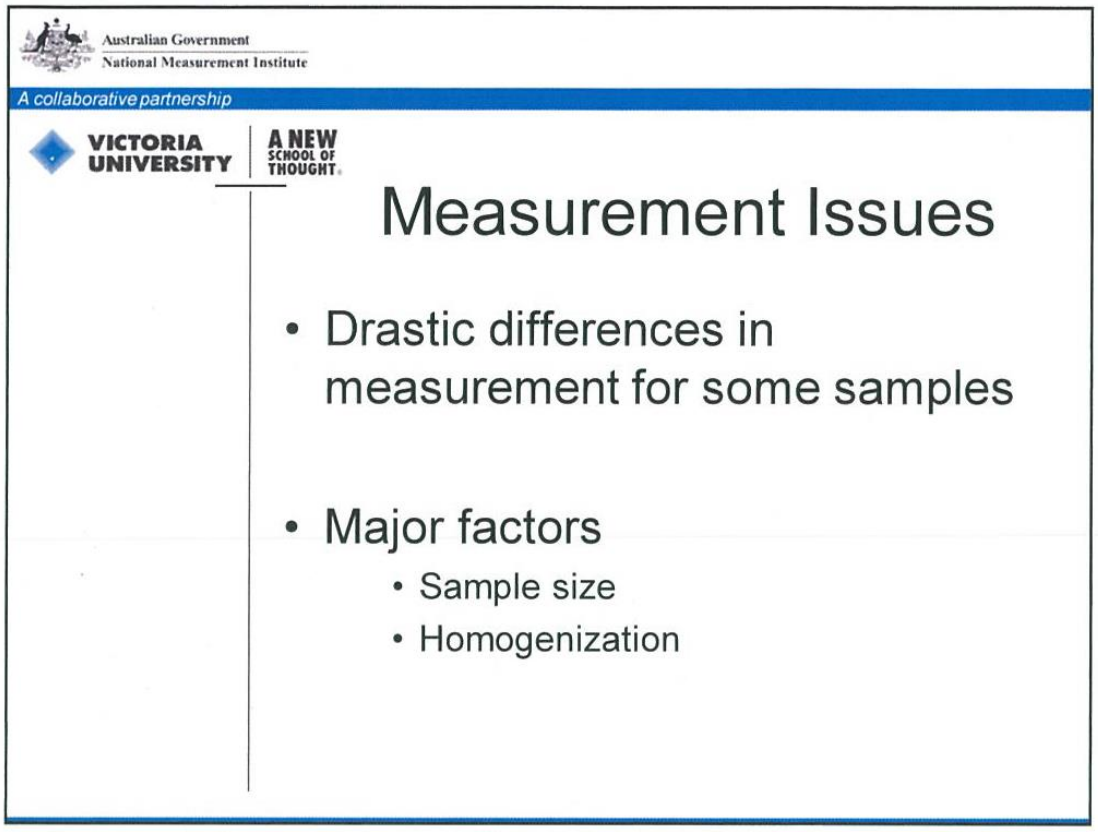
Preliminary Results

- Inhibition
- Amplification

Trial Description	Tryptophan			
Sample (0.5g extracted)	Juice Mix	Bilberry	Apple	Coffee Berry
Amino Acid [10%] 20nmol/mL (uM T.E/kg)	177	177	177	177
Cu [10%] (uM T.E/kg)	243	243	243	243
Amino Acid & Cu [10% each] (uM T.E/kg)	343	343	343	343
Cu & Sample (uM T.E/kg)	149,962	1,715,753	28,395,708	2,389,390
Sample (uM T.E/kg)	38,780	264,857	8,864,387	418,306
Amino Acid & Sample (uM T.E/kg)	342,823	4,017,275	44,164,507	4,390,860
Amino Acid, Cu & Sample (uM T.E/kg)	623,799	6,681,445	70,377,710	7,415,129
% Inhibition of Sample / Amino Acid & Sample (uM T.E/kg)	N/A	N/A	N/A	N/A
% Amplification of Cu & Sample / Sample (uM T.E/kg)	387	648	320	571
% Amplification of Amino Acid & Sample / Sample (uM T.E/kg)	884	1,517	498	1,050
% Amplification of Amino Acid, Cu & Sample / Sample (uM T.E/kg)	1,609	2,523	794	1,773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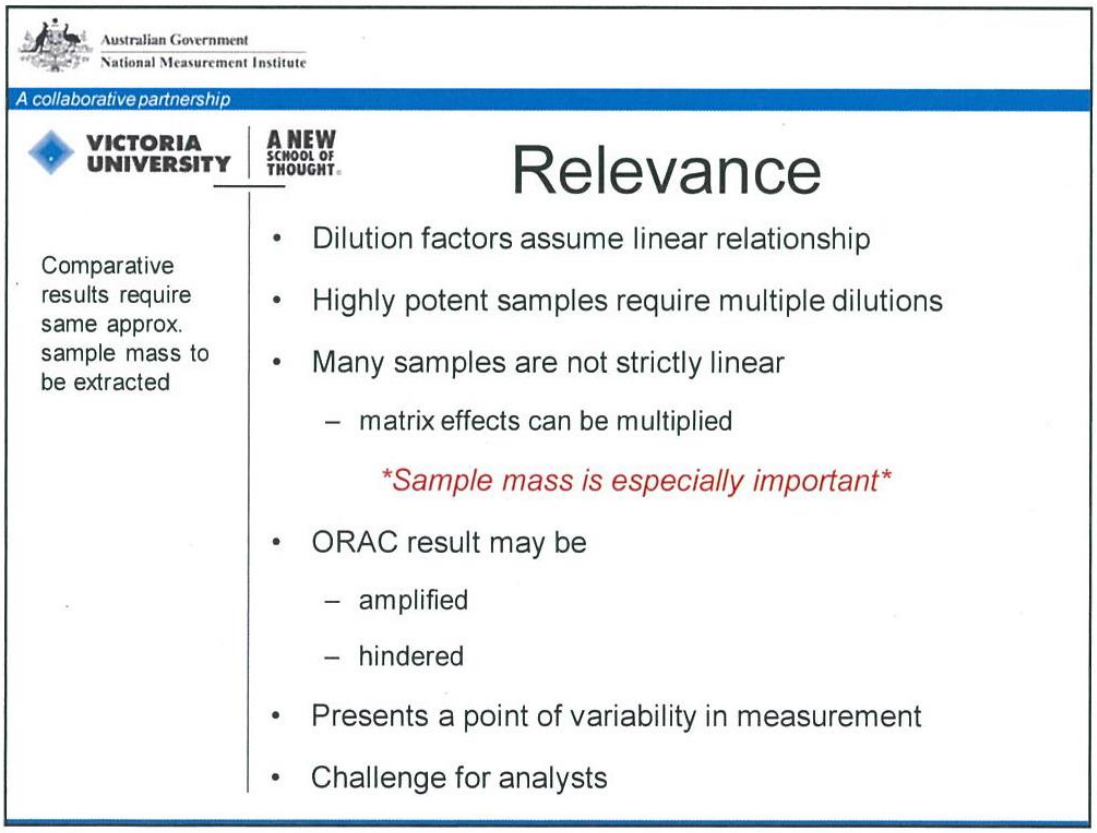
Measurement Issues
• Drastic differences in measurement for some samples
• Major factors
• Sample size
• Homogenization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Relevance
Comparative results require same approx. sample mass to be extracted
• Dilution factors assume linear relationship
• Highly potent samples require multiple dilutions
• Many samples are not strictly linear
– matrix effects can be multiplied
Sample mass is especially important
• ORAC result may be
– amplified
– hindered
• Presents a point of variability in measurement
• Challenge for analysts

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Preliminary Investigation

- Identify key groups responsible for antioxidant capacity
  - Amino acids
  - Preservatives
  - Metals (chelation)
  - Phenolic compounds
  - Vitamins
- Prepare mock matrix
  - Systematic additions
  - Identify areas of high variability
  - Replicate analysis
  - Quantify matrix effects of each compound
- Concurrent trials with real matrices

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Food Constituents of Interest

- Specialized plant products
  - Concentrates
  - Powders
  - Cereals
  - Health products
    - Herbal extracts
    - Plant oils
- Tea (multiple varieties)
- Fruit based juices

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Future Directions

- Cellular based assay (liver cancer cells)
- Comparison of antioxidant capacity assay *in vitro*
- Preferred measure of matrix effects
  - Accounting for cellular uptake and metabolism
  - Negates matrix effects
  - Comparison of Total Antioxidant Capacity (TAC) ORAC with Cellular Antioxidant Activity (CAA)

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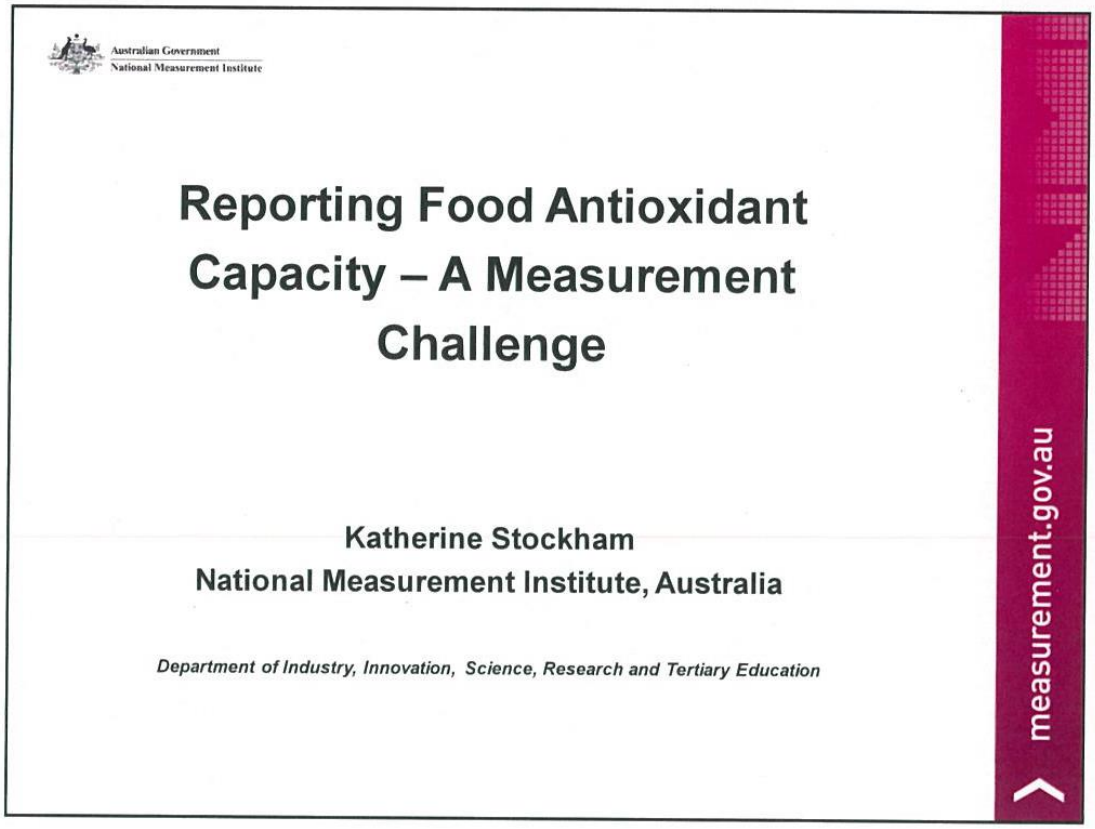
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Reporting Food Antioxidant Capacity – A Measurement Challenge
Katherine Stockham
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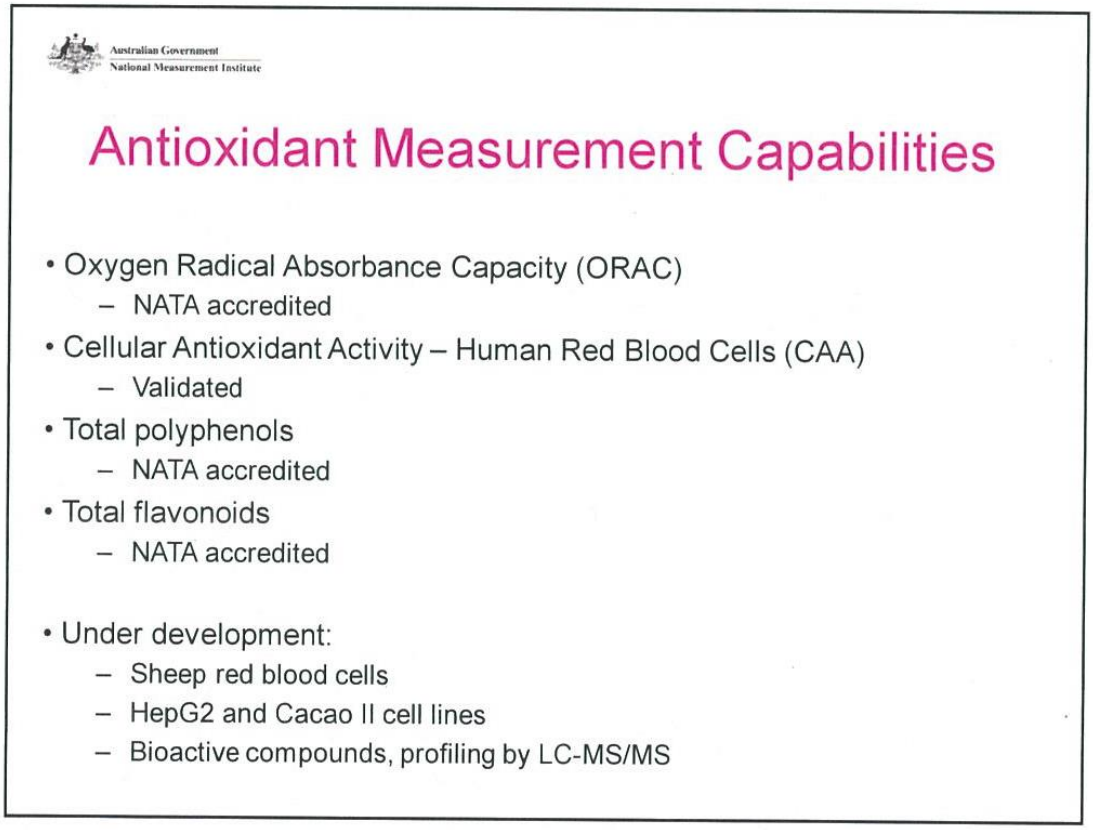
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Antioxidant Measurement Capabilities
• Oxygen Radical Absorbance Capacity (ORAC)
– NATA accredited
• Cellular Antioxidant Activity – Human Red Blood Cells (CAA)
– Validated
• Total polyphenols
– NATA accredited
• Total flavonoids
– NATA accredited
• Under development:
– Sheep red blood cells
– HepG2 and Cacao II cell lines
– Bioactive compounds, profiling by LC-MS/MS

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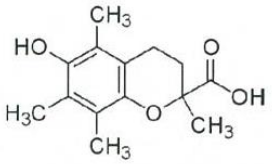
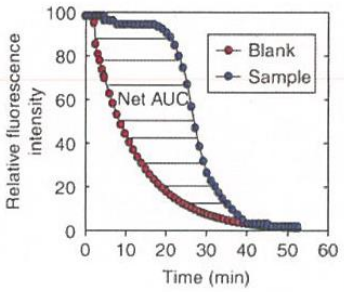
ORAC

- Fluorescence intensity measurement
- Fluorescein probe
- AAPH radical generator
- Vitamin E analogue standard (Trolox)



[Trolox (VEA)]

- Kinetic measurement
  - Area under the curve (AUC)
  - Blank corrected (Net AUC)
  - Compared to Net AUC of Trolox over calibration range
- Hydrophilic and lipophilic fractions
- Total antioxidant capacity (TAC) reported



[Prior *et al.* (2003)]

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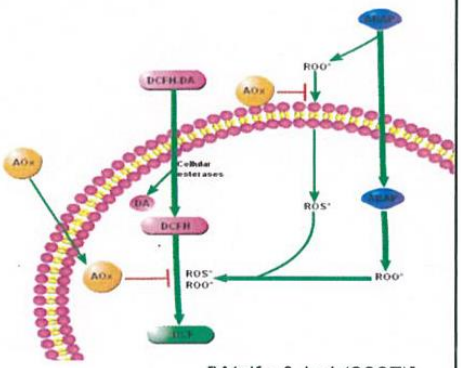
CAA – Red Blood Cells

- Fluorescence intensity measurement
- Fluorescein probe
- AAPH radical generator
- Quercetin standard



[Wolfe & Lui (2007)]

- Kinetic measurement
  - Area above the curve (AAC)
  - Blank corrected (Net AAC)
  - Compared to Net AAC of Quercetin over calibration range
- Hydrophilic (PBS based) extract
- Cellular antioxidant activity (CAA) reported

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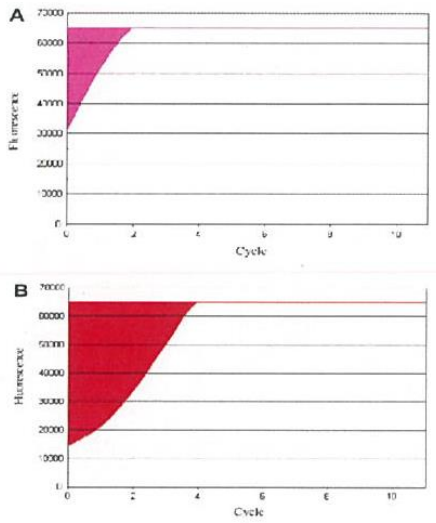
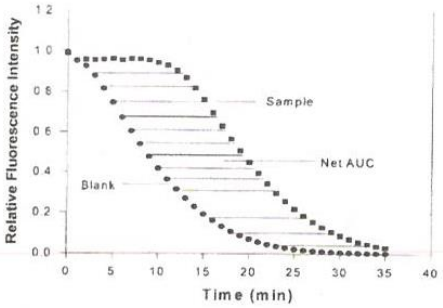
Quantitation

CAA – RBC (AAC) vs. ORAC (AUC)





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The Australian Scenario

- Industry interest in antioxidants – focus on total antioxidant capacity
- Widely requested assay is ORAC
- Used in product marketing and can be found on food labels in Australia

Industry Focus on:
- Product development
- Marketing
- New foods
- Neutraceuticals

NMIA Focus on:
- Consistency
- Interpretation
  - Realistic and reproducible

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ORAC

- Most commonly requested antioxidant capacity assay at NMIA
- Challenging matrices
- Minor differences in processing – large difference in ORAC value

- Special investigations for product development
  - Pine needle extracts (capsules and fractions during processing stages)
  - Wine (standard and alcohol removed wines)
  - Eggs (effect of feeding programmes)
  - Fruit preparations (dehydrated and homogenised) eg:
    - Blueberry
    - Apple
    - Bilberry
    - Goji berry
    - Coffee berry

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Sample Preparation

- Preparation varies, dependant on
  - client or industry request (routine vs. developmental/research focus)

- Homogenisation just prior to extraction and analysis

- Liquid nitrogen
  - "sticky" foods
    - Berries, muesli bars and sweets, grains

- Pre-freezing
  - chocolate bars
  - eggs

- Preparation and extraction date and time recorded (consistency)

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ORAC Investigation Activities

Pathways:

- Industry generated
  - Wine (standard vs alcohol removed)
  - Processed fruit
  - Fresh fruit (seasonal variation program)
  - Plant extracts for neutraceutical companies
  - Eggs (animal feeding programs)

- Internal initiatives
  - Chemical classes responsible for
    - Amplification or Inhibition (causes of measurement bias)
  - Matrix effects and reproducibility
  - Behaviour modelling
    - Relationship between sample mass, multiplier and dilution factor

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ORAC Investigation Activities – Industry generated

- Wine (standard vs alcohol removed)
  - Processing, lowered ORAC by 80-90%
  - Reproducibility was very good for alcohol removed wines
- Processed fruit (dried and homogenised)
  - Observations and comparisons with cellular antioxidant activity (CAA) assay
- Fresh fruit (seasonal variation program)
  - Varietal observations
  - Reproducibility (10-20%)
- Plant extracts for neutraceutical companies
  - Solubility, additives
  - Highly variable results, poor reproducibility
  - Plant waxes and interferences
  - Suitability of ORAC for these antioxidant capacity assessments
- Eggs (animal feeding programs)
  - Control feeding group vs specialised feed

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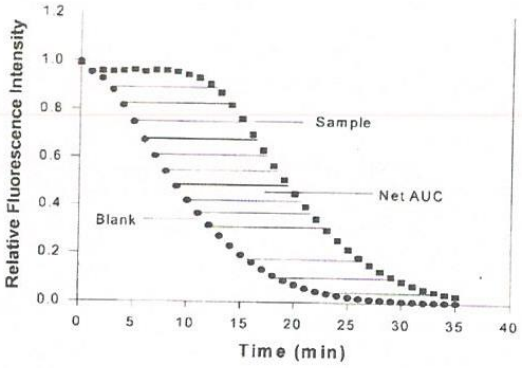
ORAC Investigation Activities

- Chemical classes responsible for
  - Amplification or Inhibition (causes of measurement bias)
  - Synergisms or antagonistic relationships

- Organic acids
- Amino acids
- Vitamins
- Sugars
- Preservatives
- Polyphenolics
- Flavonoid sub-groups
- Copper colloid solution



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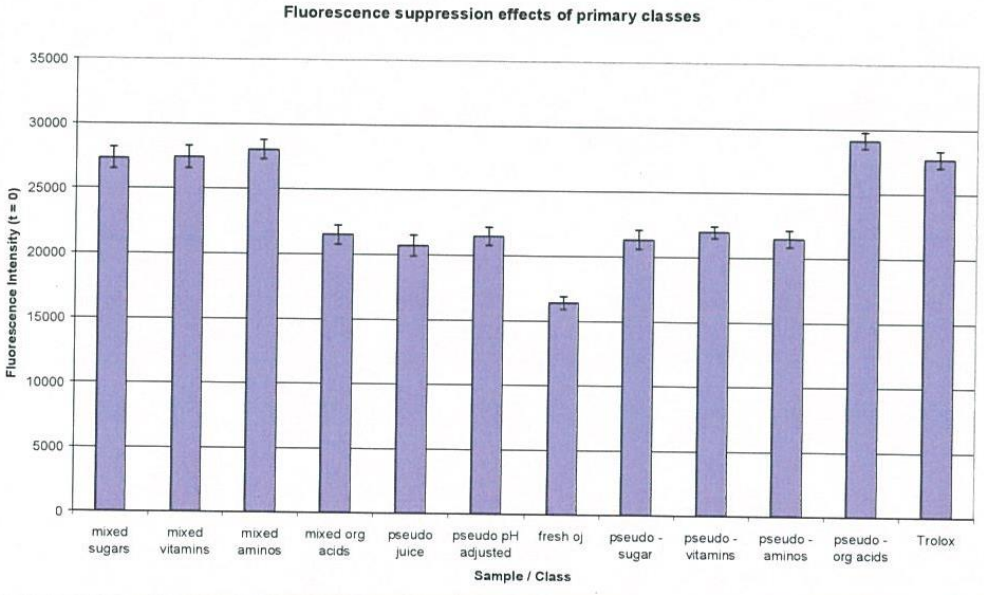
ORAC - Signal Suppression



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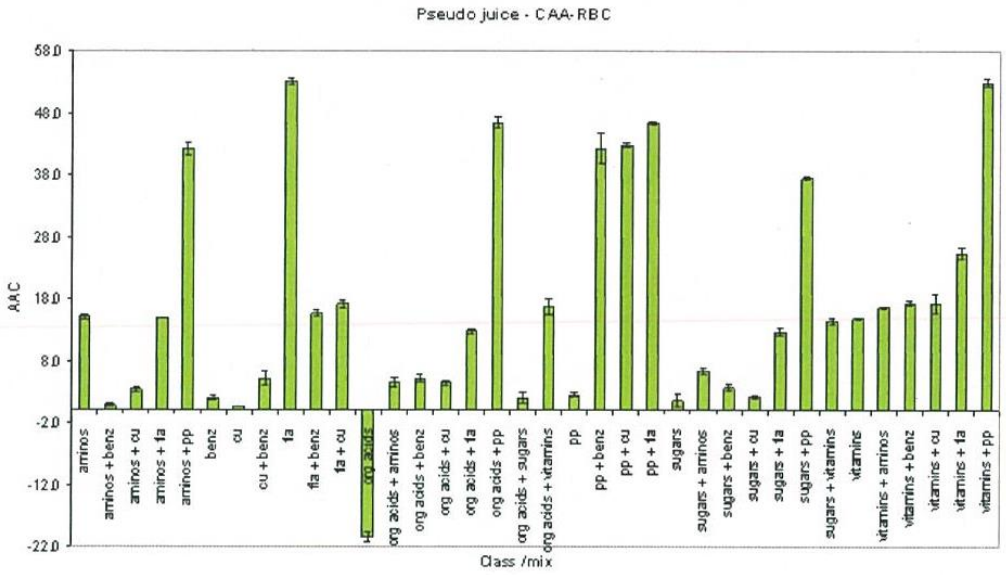
CAA – Synergism and Antagonism



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Matrix Effects

- Organic acids (neg bias, antagonistic)
- Amino acids (synergistic)
- Vitamins (synergistic)
- Sugars (antagonistic)
- Preservatives (antagonistic)
- Polyphenolics (synergistic)
- Flavonoid sub-groups (synergistic)
- Copper colloid solution (antagonistic)

- Strong synergisms between a number of classes and the polyphenolics group
- Similar trends seen in real food systems

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ORAC Investigation Activities

- Real foods – systematic study of 8 classes of compounds
  - Foods classified according to composition and level of processing
    - Strawberry
    - Apple
    - Tea
    - Cocoa
- Comparisons with CAA in human red blood cells
  - Modified extract, PBS based, hydrophilic antioxidants only
  - Same extract used for both ORAC and CAA assays
- Observations on the affect of these classes of compounds between the two assays
- Behaviour modelling
  - Relationship between sample mass, multiplier and dilution factor

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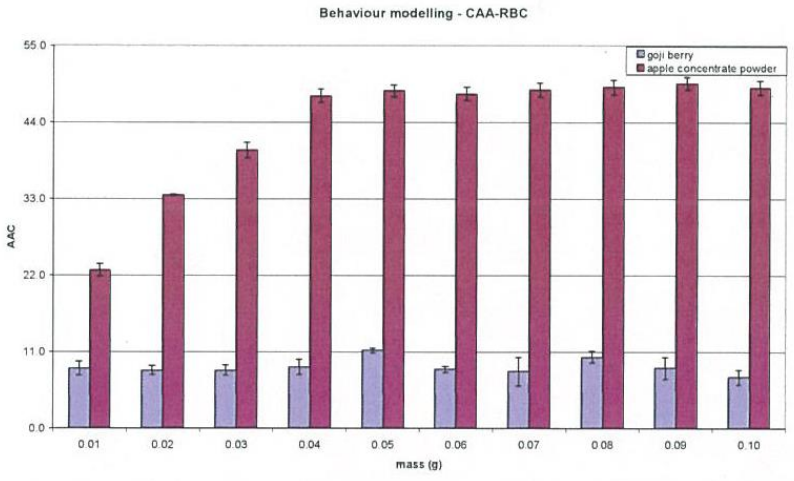
Behaviour modelling

- Relationship between sample mass, multiplier and dilution factor
- Able to determine suitable sample mass and dilution factor
- Understanding of how antioxidant activity of a food sample performs as a function of mass



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Future Directions

- Functional foods growing area
  - Phytosterols
  - Bioactives
  - Fatty acids
  - Antioxidants
- Phytochemical profile linked to variety, climate and agricultural practices
  - eg: Strawberries (flavour, colour, phytochemical and vitamin C content)
- Responsible generation and use of data
- Understanding of the factors influencing "total antioxidant capacity"
- Expand research and method development into new cell lines
  - intestinal cell line
  - absorption
  - metabolites
    - compounds responsible for antioxidant activity biologically
- Promote responsible and informed reporting of antioxidant capacity in foods

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