VALIDATION OF TWO BIO-ANALYTICAL ASSAYS FOR THE MEASUREMENT OF HYDROPHILIC ANTIOXIDANT CAPACITY IN SEVERAL FOOD AND BEVERAGE COMMODITIES IN ACCORDANCE WITH ISO 17025 REGULATORY GUIDELINES

By

OLIVIA LESHIA PARBHUNATH

Thesis submitted in fulfilment of the requirements for the degree

Master of Technology: Biomedical Technology

in the
Faculty of Health and Wellness Sciences

at the
CAPE PENINSULA UNIVERSITY OF TECHNOLOGY

Supervisor: Prof Jeanine L Marnewick
Co-supervisors: Dr Glenda Davison
Mr Fanie Rautenbach

Bellville
March 2013

PREFACE

This thesis is submitted in fulfilment of the requirements for the degree Master of Technology in the discipline of Biomedical Technology. Chapter 1 provides a brief background to the research project and states the projected aims and objectives. A literature review in chapter 2 discusses key concepts related to the project, provides a rationale for why the project was performed and supplies an aid to interpret results obtained. Chapters 3 and 4 are two articles which will be submitted for publication and investigates the two main aims of the research project. These two chapters contain separate introduction, methods and materials, results and discussions. A general discussion summarising the integrated results of the thesis follows in chapter 5. A general conclusion and future recommendations concludes the thesis. As the thesis is written in an article-based format, each chapter has separate numbering systems and references according to the relevant journal requirements.

DECLARATION

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

Signed **Date**

ABSTRACT

The accurate and consistent measurement of antioxidants is crucial to evaluating their biological role in the prevention and delay of cancer and other pathological conditions. Hence, the performance of the analytical method utilized should be evaluated for acceptable levels of accuracy, precision and other performance parameters according to internationally accepted standards. Additionally, the measure and influence of existing errors should be evaluated and the method optimized to reduce such errors.

In furtherance of this vital aim, this research project sought out to optimize and validate two bio-analytical assays for the measurement of total antioxidant capacity and L-ascorbic acid (L-AA), respectively in food commodities. The validation procedure was performed in accordance with ISO 17025 international standard.

The first study in this thesis evaluated, optimized and validated the hydrophilic oxygen radical absorbance capacity (H-$ORAC_{FL}$) assay using fluorescein for total antioxidant capacity in various food and beverage products. The assay demonstrated good results with regard to accuracy, precision, linearity, specificity, limits of detection (LOD) and quantification (LOQ) and robustness. The extraction solvent (60% ethanol) recovered excellent antioxidant yields for most samples tested. The optimization of the method in terms of temperature and sample usage on the micro-plate significantly ($p<0.05$) reduced errors and subsequently improved precision substantially.

The second study evaluated and optimized the ultra-violet high performance liquid chromatography (UV-HPLC) assay for L-ascorbic acid (L-AA) measurement in various food and beverage products. The assay was successfully validated in terms of accuracy, precision, linearity, specificity, LOD, LOQ, robustness and system suitability. All validation experiments demonstrated excellent recoveries (99 to 103%), consistently good linearity within the calibration concentration range ($R^2 = 0.999$) and the repeatable low coefficient of variations (COVs) (<5%) were indicative of good precision. The assay was robust and specific, with a high level of sensitivity demonstrated for all samples tested.

Further studies such as evaluating the method performance parameters of the manually performed $ORAC_{FL}$ assay for measuring lipophilic antioxidants, are recommended. Additionally, the validation approach may be applicable to other antioxidant capacity assays, as well as samples other than food commodities.

ACKNOWLEDGEMENTS

I thank

- My Lord and Savior Jesus Christ. For the word of God states, “With men this is impossible, but with God all things are possible” (KJV, Matthew 19:26).

- My supervisor Prof Jeanine Marnewick and co-supervisor, Dr Glenda Davison, for their guidance and support.

- My co-supervisor, Mr Fanie Rautenbach for his excellent guidance, patience and willingness to always share his knowledge with me.

- Berenice Lidwine Alinde who is a good friend and colleague, for all her valuable suggestions and advice during the final stages of writing up this thesis.

- Dr Farzana Rahiman who is a good friend and was always willing to share her knowledge with me.

- CPUT University Research Funding (URF) for financial assistance.

- My husband and eternal love, Abraham for his continuing and unfailing love and support as well as his time and effort invested in this thesis. This work has only been possible because of his steady and unwavering encouragement, urging me on in the face of all my doubts and fears. To him I also dedicate this thesis.

- My daughters, Tashiana and Ashriya for the joy and optimism they spread around. They are the most precious things in my life and I dedicate this thesis to them.

- My parents, Les and Priscilla Dalais for their continual and unwavering love, encouragement and support. The value and importance of education was one gift given by both of them that has greatly impacted my life.

- My sister, Tatum and brother, Cameron for their continued love, support and prayers.

DEDICATION

For

My husband, Abraham

My daughters, Tashiana and Ashriya

ABBREVIATIONS

AAPH:	2,2‘-Azobis (2–amidinopropane) dihydrochloride
ALA:	Alpha lipoic acid
ANOVA:	Analysis of variance
AOAC:	Association of Official Analytical Chemists
AOC:	Antioxidant capacity
AQA:	Analytical quality assurance
AUC:	Area under curve
AWA:	Acetone/distilled water/acetic acid
CAC:	Codex Alimentarius Commission
CAT:	Catalase
CDC:	Centers for Disease Control and Prevention
CDER:	Centre for Drug Evaluation and Research
CFR:	Code of federal regulations
CGMP:	Current good manufacturing practice
CITAC:	Cooperation of International Traceability in Analytical Chemistry
CoQ10:	Co-enzyme Q10
COV:	Coefficient of variation
CRMs:	Certified reference materials
DF:	Dilution factor
DHA:	Dehydroascorbic acid
DKG:	Diketogluconic acid
DMPD:	N,N-dimethyl-p-phenylenediamine
DNA:	Deoxyribonucleic acid
DQ:	Design qualification
EPR:	Electron pair resonance
ESR:	Electron spin resonance
FCR:	Folin Ciocalteu reagent
FDA:	Food and Drug Administration
FRAP:	Ferric ion reducing antioxidant power
GAE:	Gallic acid equivalent
GLP:	Good laboratory practice
GPX:	Glutathione peroxidase
HAT:	Hydrogen atom transfer
$H\text{-}ORAC_{FL:}$	Hydrophilic-oxygen radical absorbance capacity (Fluorescein)

ICH:	International Conference on Harmonisation
IQ:	Installation qualification
ISO/ IEC:	International Organization for Standardization/International Electrotechnical Commission
ISO:	International Standardization Organization
IUPAC:	International Union of Pure and Applied Chemistry
IU:	International units
L-AA:	L-Ascorbic acid
LOD:	Limit of detection
LOLR:	Limit of linear response
LOQ:	Limit of quantification
MPA:	Meta-phosphoric acid
NDA:	National Drug Administration
·OH:	Hydroxyl
OQ:	Operational qualification
OSRC:	Oxidative stress research centre
PAA:	Peak absorbance area
PQ:	Performance qualification
QC:	Quality control
REC:	Recovery
RO:	Alkoxyl
ROO:	Peroxyl
ROS:	Reactive oxygen species
RSD:	Relative standard deviation
RT:	Room temperature
RTM:	Resolution test mixture
SANAS:	South African National Accreditation Service
SD:	Standard deviation
SET:	Single electron transfer
SOD:	Superoxide dismutase
SOP:	Standard operating procedure
SSTs:	System suitability tests
STD:	Standard
TAC:	Total antioxidant capacity
TE:	Trolox equivalent
TEAC:	Trolox equivalent antioxidant capacity
TRAP:	Total radical trapping antioxidant parameter

USDA:	United States department of agriculture
USFDA:	United States Food and Drug Administration
USP:	United States Pharmacopoeia
UV:	Ultra-violet
UV-HPLC:	High performance liquid chromatography with ultra-violet detection
VMP:	Validation master plan
WFSO:	World Food Safety Organization
WHO:	World Health Organization

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER 2: Literature Review

CHAPTER 3: Validation of a manual oxygen radical absorbance capacity (H-ORAC$_{FL}$) assay for the measurement of total antioxidant capacity (TAC) in food and beverage products

CHAPTER 4: Optimization and validation of a Reverse-Phase High Performance Liquid Chromatography (RP-HPLC) method with ultra-violet (UV) detection for the measurement of L-ascorbic acid in food and beverage products

Appendices

Appendix C

Appendix D

LIST OF FIGURES

CHAPTER 2: Literature Review

CHAPTER 3: Validation of a manual oxygen radical absorbance capacity (H-$ORAC_{FL}$) assay for the measurement of total antioxidant capacity (TAC) in food and beverage products

CHAPTER 4: Optimization and validation of a Reverse-Phase High Performance Liquid Chromatography (RP-HPLC) assay with ultra-violet (UV) detection for the measurement of L-ascorbic acid in food and beverage products

LIST OF EQUATIONS

LIST OF APPENDICES

CHAPTER ONE: BACKGROUND

1.1 Background

Oxidative stress resulting from an imbalance in redox status between oxidants and antioxidants in favour of the former, has been implicated as a major factor in the pathogenesis of numerous human diseases (Palmieri and Sblendorio, 2007). There is a wealth of scientific evidence verifying the association between increased consumption of products containing antioxidants such as L-ascorbic acid, vitamin E and polyphenols to name but a few, with a decreased incidence of many chronic and degenerative diseases as well as premature ageing (Pham-Huy *et al.*, 2008; Zhao, 2005). As a result, exogenous antioxidants have received a wealth of attention in the past few years owing to their putative role in managing and treating various chronic illnesses, as well as the immense revenue it has produced for manufacturers (Hasler, 1998). For a long time manufacturers have labelled their products with specific "health claims" related to exogenous antioxidants to endorse health promoting properties associated with consumption of such products (Verbeke, 2006). A leading market researcher demonstrated the rapid escalation of antioxidant-containing products from only 37 in 2006 to 135 launches in 2008 in Europe alone (Daniells, 2009).

In view of the plethora of antioxidant health claims appearing on food and beverage product labels, various national and international organizations as well as most governments, have found it necessary to implement specific control and regulatory measures to this rapidly burgeoning market, coercing food and beverage manufacturers to substantiate such health claims (Asp, 2005). Among these, the Food and Drug Administration (FDA), World Health Organization (WHO), United States Department of Agriculture (USDA), Centers for Disease Control and Prevention (CDC), the Codex Alimentarius Commission (CAC) and the World Food Safety Organization (WFSO) are major players involved in the implementation and maintenance of such regulatory systems that dictate a detailed framework for production, handling, testing, marketing, labelling and certification of food and beverage products (Schmelzer, 2012).

The recently amended South African labelling legislature [Regulation 146/2010 as part of the Foodstuffs, Cosmetics, and Disinfectants Act (Act 54 of 1972)] is targeted at keeping up with global trends in protecting the consumer from "misleading" advertising and unverified disease-associated health claims that could influence the consumer's purchasing choices (De Villiers, 2009). Much of the regulations instituted into the new labelling legislature are

derived from regulatory systems established by the Food and Agriculture Organization (FAO) and WHO (Center for Science in the Public Interest, 1998). The legislature has placed immense pressure on South African food manufacturers, compelling them to substantiate any specific claims made on a product by a reputable South African National Accreditation Service (SANAS) accredited laboratory (Food and beverage reporter, 2010; Hu, 2010; Johannes, 2010). One aspect of these challenges lies within utilizing laboratories whose processes are accredited against international and national regulatory standards. Various regulatory bodies, some of which include the Cooperation of International Traceability in Analytical Chemistry (CITAC), International Union of Pure & Applied Chemistry (IUPAC), International Standardization Organization (ISO), Association of Official Analytical Chemists (AOAC), US Food and Drug Administration (USFDA) and the International Conference on Harmonisation (ICH) emphasize the need for a sound accredited quality control system in laboratories to justify the quality and reliability of such products.

The Oxidative Stress Research Centre (OSRC) at Cape Peninsula University of Technology (CPUT) in Bellville (South Africa) currently provides an analytical service to major companies in the food and beverage industries whereby active components such as L-ascorbic acid and a wide variety of antioxidants such as vitamin E, polyphenols, carotenoids, etc. are analyzed. Among the many assays performed by the laboratory, the oxygen radical absorbance capacity using fluorescein ($ORAC_{FL}$) and high performance liquid chromatography (HPLC) assays for total antioxidant capacity (TAC) and L-ascorbic acid analysis respectively, are by far the most requested assays by these industries. To date and to our knowledge there is no validated $ORAC_{FL}$ assay in South Africa.

The rising interest in antioxidants has resulted in the apparent rise in $ORAC_{FL}$ and HPLC assay requests at the OSRC by the local and national food and beverage industries. As a result, the OSRC is attempting to obtain SANAS accreditation. This would be in compliance with both the International Organization for Standardization/International Electrotechnical Commission (ISO/IEC) 17025 standard as well as national government legislation.

1.2 Aims and objectives of the project

Since ISO/IEC 17025 accreditation is about establishing and maintaining quality as well as demonstrating competency, the validation of these assays, being an indispensible part of the accreditation process is positively warranted.

The primary aims of this research project are:

- to validate a spectro-fluorometric $ORAC_{FL}$ method for the measurement of antioxidant capacity in various food and beverage matrices.
- to validate an HPLC method with for the measurement of vitamin C concentration in various food and beverage matrices.

These will be accomplished by investigating the following method performance parameters (International Conference on Harmonisation, 1996; International Conference on Harmonisation, 2005; International Organization for Standardization/International Electrotechnical Commission, 2005):

- Accuracy
- Precision
- Linearity
- Range
- Limit of detection
- Limit of quantification
- Sample stability
- Selectivity/specificity
- Robustness
- System suitability

Results from the performance parameters will provide documented evidence that the methods fulfil the requirements for their intended use. Additionally, results will ensure compliance to regulatory quality standards, namely ISO/IEC 17025:2005.

Further to this, the OSRC also provide quality training for postgraduate students, who also utilize both the $ORAC_{FL}$ and HPLC assays in their scientific research. Validation of these assays will not only add credibility to their results, but also provide them the opportunity to compete in the international science arena as peer-reviewed publication of postgraduate research studies is essential.

1.3 References

Asp, N. 2005. Rationale and scientific support for health claims on foods. *South African Journal of Clinical Nutrition,* 18**:**98-101.

Center for Science in the Public Interest. 1998. *Food labeling for the 21st century: a global agenda for action. Report by the Center for Science in the Public Interest*. http://www.cspinet.org/reports/labelrept.pdf [10 August 2012].

Daniells, S. 2009. *The growth brand of 'antioxidant'*. http://www.nutraingredients.com/Industry/The-growth-of-brand-antioxidant [12 May 2012].

De Villiers, K. 2009. *Label laws get teeth*. www.ipsa.org.za/downloads_new.php?do=download&id=432 [12 May 2012].

Food and beverage reporter. 2010. *Health claims amendment likely to 2011 labelling regulations*. http://www.fbreporter.com/index.php?option=com_content&view=article&id=24517:health-claims-amendment-likely-to-2011-labelling-regulations-&catid=1002:news-update-5-november-2010 [12 May 2012].

Hasler, C. M. 1998. *Functional foods: their role in disease prevention and health promotion*. http://www.nutriwatch.org/04Foods/ff.html [15 November 2012].

Hu, C. 2010. *Natural dilemma over new labelling legislation*. http://www.foodreview.co.za/beverage-review/188-natural-dilemma-over-new-labelling-legislation [12 May 2012].

International Conference on Harmonisation. 1996. *Guidance for Industry Q2B Validation of Analytical Procedures: Methodology*. http://www.fda.gov/downloads/Regulator%20yInformation/Guidances/UCM128049.pdf [15 September 2012].

International Conference on Harmonisation. 2005. *Validation of Analytical Procedures: Text and Methodology Q2(R1) Part I and II*. http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf [11 August 2012].

International Organization for Standardization/International Electrotechnical Commission. 2005. *ISO/IEC 17025: 2005, General requirements for the competence of testing and calibration laboratories* http://bbs.labsky.com/uploads/2005-8/2005-08-24_205709.pdf [11 October 2012].

Johannes, J. 2010. *How secure is your brand and trade name?* http://www.foodreview.co.za/food-labelling-and-legislation/207-how-secure-is-your-brand-and-trade-name [12 May 2012].

Palmieri, B. & Sblendorio, V. 2007. Oxidative stress detection: what for? *European Review for Medical and Pharmacological Sciences,* 11**:**27-54.

Pham-Huy, L. A., He, H. & Pham-Huy, C. 2008. Free radicals, antioxidants in disease and health. *International Journal of Biomedical Sciences,* 4**:**89-96.

Schmelzer, C. D. 2012. *Regulatory agencies*. http://www.diet.com/g/regulatory-agencies [12 May 2012].

Verbeke, W. 2006. Functional foods: Consumer willingness to compromise on taste for health? *Food Quality and Preference,* 17**:**126-131.

Zhao, B. 2005. Natural antioxidants for neurodegenerative diseases. *Molecular neurobiology,* 31**:**283-293.

CHAPTER TWO: LITERATURE REVIEW

2.1 Oxidative Stress and antioxidants

2.1.1 Reactive oxygen species and oxidative stress

Molecular oxygen is an indispensable component to the optimal functioning of all metabolic processes within the body. The sequential reduction of oxygen to water by cells for the generation of aerobic energy, results in the formation of various reactive intermediates (Sies, 1997). They are collectively known as “reactive oxygen species” (ROS) because they are highly reactive radicals containing one or more unpaired electrons in the outer orbital and may be classified into two groups (Powers *et al.*, 1999).

The first group consists of radical compounds which include the nitric oxide radical, the superoxide radical, the hydroxyl radical, the peroxyl radical, the alkoxyl radical and singlet oxygen. The second group consists of non-radical compounds which include hypochlorous acid, hydrogen peroxide, organic peroxides, aldehydes and ozone (Kohen and Nyska, 2002). Their action is not damaging per se, as they are required for optimal functioning of metabolic processes, muscular contraction and immune defence (Konig *et al.*, 2001). Aside from their generation through endogenous mechanisms, exogenous factors such as radiation, pollution, certain drugs, cigarette smoke, as well as pathological states, promote their formation (Halliwell and Gutteridge, 1985).

To neutralize the deleterious effects of ROS and maintain redox balance, the body possesses antioxidant defence mechanisms which are illustrated in Figure 2.1. Of all the existing mechanisms, the antioxidant defence system which includes endogenous antioxidant enzymes, constitutes the most potent defence against ROS (Kohen and Nyska, 2002). It is when this redox balance is tipped in favour of oxidants, that oxidative stress occurs, with damaging effects to important cell structures, such as oxidative DNA damage, amino acid oxidation in proteins and polyunsaturated fatty acid oxidation, as illustrated in Figure 2.2 (Guzik *et al.*, 2003; Halliwell, 1994; Pignatelli *et al.*, 1998).

Studies have shown that oxidative damage to various cellular components results in loss of cellular function which may play a significant role in the ageing process and the pathogenesis of many chronic diseases (Berlett and Stadtman, 1997; Halliwell and Aruoma, 1991). There is accumulating evidence supporting the role of oxidative stress in many cardiovascular diseases (Fearon and Faux, 2009; Griendling and FitzGerald, 2003;

Madamanchi *et al.*, 2005; Mueller *et al.*, 2005), cancers (Noda and Wakasugi, 2001; Valko *et al.*, 2006), neurological diseases (Schapira, 2008), and pulmonary diseases (Rahman and Adcock, 2006).

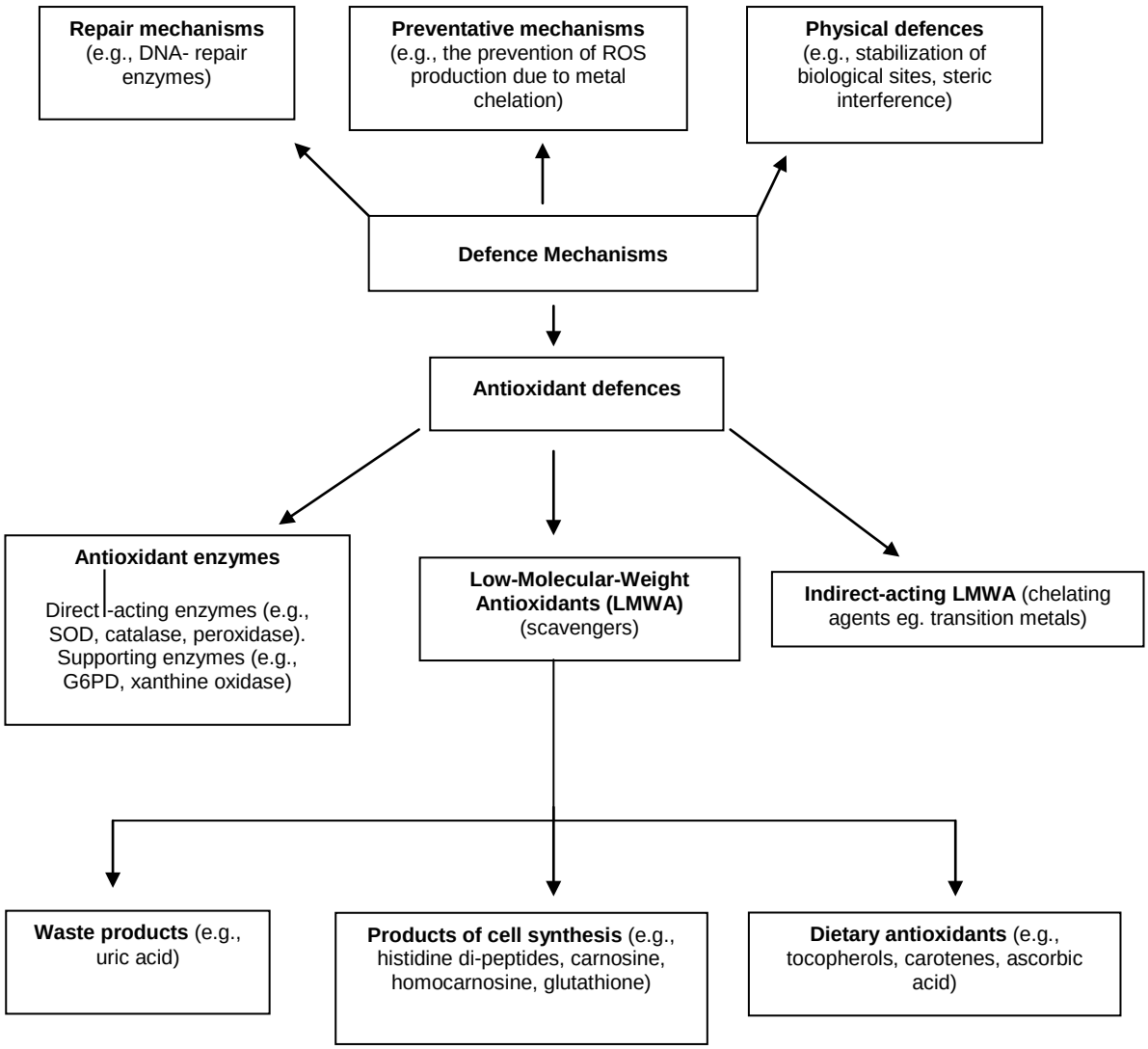


Figure 2.1 Antioxidant defence systems *in vivo* (adapted from Kohen and Nyska, 2002)

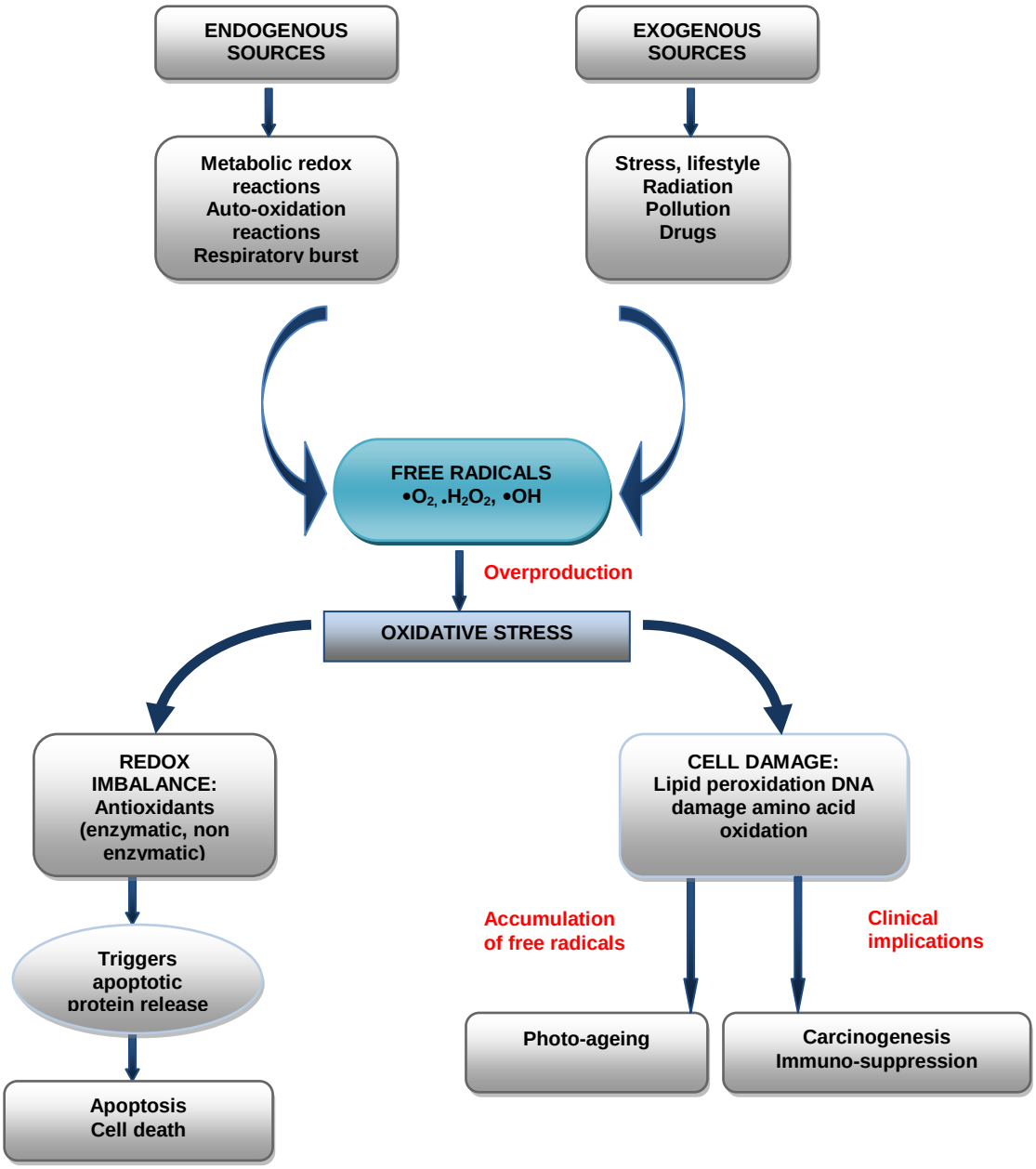


Figure 2.2 Implication of free radicals in oxidative stress (adapted from Chen *et al*, 2005)

2.1.2 Effects of oxidative damage

2.1.2.1 Fatty acid oxidation

Three steps are known to induce fatty acid oxidation: autoxidation, photo-oxidation and enzyme activation. Auto-oxidation is a three step process involving the sequences: initiation, propagation and termination (Catala, 2009). Hydrogen abstraction by radicals, some of which include hydroxyl (·OH), alkoxyl (·RO) and peroxyl (·ROO) of polyunsaturated fats in cells, are the basis for the initiation of a chain reaction effect in which more radicals are produced, contributing to the pathogenesis of many diseases as well as premature ageing (Yagi, 1982). Additionally, reports have reported the effects of lipid peroxidation may be linked to modifications in cellular membrane function and structure, contributing to loss of cell functionality (Halliwell and Gutteridge, 1985), some of which include reduced lipid fluidity (Rice-Evans and Burdon, 1993) and modification to protein structures (Nigam and Schewe, 2000). An important biomarker of lipid peroxidation is malondialdehyde (MDA) which has been associated with the pathogenesis of certain diseases, in particular atherosclerosis and diabetes (Kesavulu *et al.*, 2001), cancer (Cirak *et al.*, 2003) and Alzheimer's disease (Bourdel-Marchasson *et al.*, 2001).

2.1.2.2 DNA damage

At least two mechanisms have been reported for the implication of free radicals in DNA damage. One mechanism is through the intonation of gene expression. Certain free radicals have been shown to activate protein kinase and poly pathways and subsequently interfere with signal transduction pathways. Consequently, this may promote inappropriate gene expression for the advancement and propagation of cancer (Cerutti and Trump, 1991). In the second mechanism, the development of cancer promoted by free radicals may be attributed to their ability to induce changes in DNA such as transmutations and chromosomal reshuffling (Guyton and Kensler, 1993). As a result, this could prevent DNA replication, or produce incorrect replication, contributing to the initiation of carcinogenesis (Bohr *et al.*, 1995).

2.1.2.3 Oxidative damage to proteins

ROS are damaging to proteins through a variety of oxidative mechanisms some of which include oxidation of the protein backbone, production of protein-protein cross-linkages, amino acid side chain oxidation and protein fragmentation. As a result, these induce

modifications in the structure and subsequently decrease the polarity of the surface. The hydrophobic surface is recognised by proteases which aid in digesting the structurally altered surface. Should the protein molecules not be digested by proteases, further structural and chemical changes occur, resulting in the generation of a non-polar pigment called lipofuscin. This pigment accumulates within senescent cells, contributing significantly to the ageing process (Sitte, 2003).

2.1.3 Antioxidants

To combat these detrimental effects induced by free radicals, the body possesses a complex antioxidant system which is divided into enzymatic and non-enzymatic antioxidants (Finaud *et al.*, 2006). Endogenous enzymatic antioxidants include superoxide dismutase (SOD), alpha lipoic acid (ALA), co-enzyme Q10 (CoQ10), catalase (CAT), and glutathione peroxidase (GPX). The defence of SOD against free radicals in response to exercise has been well established in human and animal studies (Jenkins, 1988; Ji, 1999; Sen, 1995). Non-enzymatic antioxidants may be endogenous or obtained through the diet and include vitamin E, vitamin C, flavonoids, carotenoids, polyphenols, uric acid and bilirubin. Of significant importance are vitamin C, vitamin E and glutathione. The key roles of some endogenous antioxidants are summarized in Table 2.1.

There is sufficient evidence suggesting an amplified antioxidant response is generated in chronic exercise. Ohno *et al.* demonstrated enhanced catalase and glutathione reductase activity in subjects after ten weeks of training (Ohno *et al.*, 1988) . In another study, blood lipid peroxidation was significantly reduced in a subject in response to long-term training (Yagi, 1992). However with ageing, the diminishing ability of the body to produce endogenous antioxidants due to the effect of increased free radical generation, is believed to contribute to premature ageing and the pathogenesis of many chronic illnesses (Reiter, 1995).

2.1.3.1 Exogenous antioxidants

Stress, poor lifestyle and ageing are all factors that consume the body's restricted source of endogenous antioxidants. (Pham-Huy *et al.*, 2008). The health benefits associated with exogenous antioxidants supplementation has spiked a definite awareness among food and beverage, nutraceutical companies as well as consumers in general. Substantial global evidence suggests a positive association between increased consumption of fruit and vegetables, and a reduced risk of cardiovascular disease, certain types of cancers, and

possibly ageing (Ames *et al.*, 1993; Block *et al.*, 1992). Exogenous antioxidants provided by dietary sources include hydrophilic antioxidants such as vitamin C (L-ascorbic acid) and a large portion of polyphenols, and lipophilic anitioxidants namely, vitamin E (tocopherols and tocotrienols) and carotenoids, to name but a few (Moure *et al.*, 2001).

2.1.3.2 Endogenous antioxidants

Table 2.1: Summary of biological roles of endogenous antioxidants (adapted from Yuan and Kitts, 1997)**.**

	Antioxidants	Biological Role	Cellular location
Endogenous enzymatic antioxidants	Superoxide dismustase (SOD)	Converts reactive free superoxide radicals to hydrogen peroxide (H_2O_2).	Mitochondria, cytosol
	Catalase (CAT)	Together with GPx, converts H_2O_2 into H_2O and O_2.	Cytosol, cellular peroxisomes, mitochondria of the heart
	Glutathione peroxidase (GPx)	Removal of organic peroxides and reduces H_2O_2 to H_2O.	Mitochondria and cytosol
Endogenous non-enzymatic antioxidants	Ubiquinone	Prevents lipid peroxidation in lipid structures of cells. Plays a role in recycling vitamin E.	Mitochondria, nucleus, cytosol, endoplasmic reticulum of cells of heart, liver and kidney.
	Alpha-Lipoic acid	Effective in recycling vitamin C. Serves as a substitute for glutathione.	Endogenous thiol
	Glutathione	Scavenges hydroxyl, carbon centred radicals. Plays an important role in recycling vitamin E.	Non-protein thiol. Found in the cells of most organs i.e lens of the eye, liver, kidney, lungs, heart.

Maintaining the redox balance between oxidants and antioxidants is crucial in maintaining optimal functioning body systems. Therefore, supplementation with exogenous antioxidants through the diet remains an integral factor to the antioxidant system's ability to reduce the effects of oxidative stress (Andre *et al.*, 2010; Biehler and Bohn, 2010; Ratnam *et al.*, 2006). However, there is evidence to suggest that the intake of high concentrations of exogenous antioxidants can be detrimental and upset the redox balance thus contributing to oxidative stress (Galati and O'Brien, 2004; Valko *et al.*, 2004).

In one study, the protective properties of the flavanoid quercetin, were demonstrated in low doses, whilst at high concentrations it was found to lower cell survival (Robaszkiewicz *et al.*, 2007). In some other studies, high doses of flavonoids such as myricetin and quercetagetin, contributed to free radical formation by autoxidation, and high concentrations of quercetin generated radicals by redox cycling (Gaspar *et al.*, 1994; Hodnick *et al.*, 1986; Metodiewaa *et al.*, 1999). In light of the controversy surrounding the effect that concentration, type and matrix of exogenous antioxidants has on its ability to scavenge free radicals, there is sufficient consistent evidence demonstrating a positive association between the consumption of plant food (fruit and vegetables) and improved longevity and delay in pathological conditions, in contrast to that available for consumption of antioxidant supplementation (Temple, 2000).

Antioxidants derived from the diet work synergistically with endogenous antioxidants in the eradication of free radicals. A decrease in any one or more antioxidants remains a major contributor to the pathogenesis of many chronic and debilitating illnesses. Each antioxidant is idiosyncratic in terms of its structure and function (Donaldson, 2004; Willcox *et al.*, 2004).

2.1.3.2a Vitamin E

Vitamin E is a lipophilic vitamin and exhibits potent antioxidant activity. Of its eight stereo-isomers, only alpha-tocopherol is biologically active in humans (Nguyen *et al.*, 2006). Due to its fat solubility, it confers protection to the cell membrane from free radicals by preventing lipid peroxidation. The health benefits of vitamin E as an antioxidant have been advocated in the prevention of some diseases such as gastrointestinal cancers, breast and prostate cancers, certain cardiovascular diseases, arthritis and some neurological disorders (Gaziano *et al.*, 2009; Karlson *et al.*, 2008; Lee *et al.*, 2005; Mgekn *et al.*, 2008). Vitamin E contributes to the prevention of atherosclerosis by reducing the production of thromboxane which leads to platelet aggregation, a process associated with the pathogenesis of atherosclerosis (Packer, 1991).

Conversely, the prolonged use of vitamin E as a supplement in high doses of 400IU or greater, have been shown to increase the risk of death (all-cause mortality). Therefore, caution must be exercised when supplementing with high concentrations of vitamin E as its safety has not been sufficiently established in human trials (Miller III *et al.*, 2005). Foods that are rich in vitamin E include eggs, fruit, meat, nuts, cereals, wholegrain, vegetable oil, germ and wheat oil (Willcox *et al.*, 2004).

2.1.3.2b Carotenoids

Carotenoids are natural pigments produced by various plants and microorganisms, which give fruit and vegetables their yellow pigmentation. There are about fifty carotenoids present in a standard diet, which exhibit antioxidant activity. Of these lycopene and beta-corotene have received much attention and investigation for their antioxidant activity in combating oxidative stress and subsequently preventing chronic disease particularly cancer (Liu, 2004).

The exact mechanism by which carotenoids confers such protection is still not well understood, however several possibilities have been reported in the literature: 1) some carotenoids, in particular beta-carotene, exhibit provitamin A activity (converts to vitamin A) which is required for vision. In certain cancers, such as cancers of the stomach and bronchus, decreased levels of beta-carotene and vitamin A were reported (Machlin, 1995). 2) Certain carotenoids can modulate the enzyme actions of lipoxygenases and subsequently regulate the activity of its end products (proinflammatory and immunomodulatory molecules) (Bendich, 1993). The potent antioxidant behaviour of carotenoids is attributed to its ability to quench singlet oxygen, (eg. lycopene) and trap peroxyl radicals particularly beta-carotene (Sies and Stahl, 1995). Lycopene has demonstrated a protective role in reducing the incidence of prostate cancer (Dahan *et al.*, 2008).

Dietary sources rich in carotenoids include vegetables (squash, spinach, carrots, tomatoes), fruits, grains and oil (Donaldson, 2004; Willcox *et al.*, 2004).

2.1.3.2c Flavonoids

Flavonoids are polyphenols found in most plants. They may be classified into flavanols, flavanones, flavones, isoflavones, catechins, anthocyanins, proanthocyanins (Sandhar *et al.*, 2011). Flavonoids are also referred to as “nutraceuticals” which are defined “as a food or parts of food that provide medical or health benefits, including the prevention and treatment of disease” (Lin and Weng, 2006). The health benefits of flavonoids lie within their powerful antioxidant behaviour (Miller, 1996). Consumption of foods containing high amounts of

flavonoids has demonstrated protection against retinal diseases induced by oxidative stress (Hanneken *et al.*, 2006). Their putative role in lowering cardiovascular mortality incidence in Mediterranean populations where the consumption of red wine is high, has been well established (Formica and Regelson, 1995). Recently, other biological effects which include anti-inflammatory, anti-carcinogenic and antiviral properties are being widely explored for their application in preventing many chronic diseases (Middleton Jr, 1998).

Dietary sources of flavonoids and their beneficial health effects are summarised in Table 2.2. Green tea is a rich source of flavonoids especially catechin and quercetin and has demonstrated potent inhibition of lipid peroxidation induced by free radicals (Tijburg *et al.*, 1997). In one study, both green and black tea demonstrated strong antioxidant activity by preventing peroxidation, with green tea exhibiting appreciably more potent activity than black tea (Serafini *et al.*, 1996). In another recent study Marnewick and co-workers showed a positive correlation between increased rooibos herbal tea intake and a decreased risk for cardiovascular disease (Marnewick *et al.*, 2011). The photo-protection of the main polyphenolic compound, epigallocatechin-3-gallate (EGCG) found in green tea, is currently being explored for its use in the treatment and prevention of UV-B light induced skin disorders such as melanoma and non-melanoma skin cancers as well as photoageing (Katiyar, 2003). Furthermore, the protective effect of rooibos against liver cell damage and fibrosis in rats indicates that the antioxidant and scavenging abilities of rooibos may possibly offer the same protection in patients with hepatopathies (Uličná *et al.*, 2003).

Table 2.2: Classification of flavonoids and their antioxidant potential in reducing the risk or preventing disease ([a] adapted from the USDA Database for the Flavonoid Content of Selected Foods, 2003)

Flavonoids	Dietary sources[a]	Examples[a]	Beneficial effects	References
Flavones	Cereals, herbs, parsley, celery, citrus peel.	Luteolin	Prevention of atherosclerosis, neurodegenerative disorders e.g., Alzheimers disease, specific cancers .	(Cermak, 2008; Fotsis *et al.*, 1997; Rezai-Zadeh *et al.*, 2008)
Flavonols	Tea, red wine, apple, berries, tomatoes, lettuce, onion, kale, broccolli	Quercetin, kaempferol, myricetin	Reduced risk of cancer, stroke, coronary heart disease.	(Crozier *et al.*, 2000; Hertog and Hollman, 1996)
Flavanones	Citrus fruit, fruit juices.	Hesperitin, naringenin, erioditYol	Decreased risk of cardiovascular disease, anti-inflammatory and anticarcinogenicproperties, decreased risk of ishaemic stroke.	(Morand *et al.*, 2011; Yao *et al.*, 2004)
Isoflavones	Soy beans, soy products.	Daidzein, genistein, glycetein.	Implicated in the prevention of osteoporosis, hereditary chronic nose bleed syndrome, autoimmune disorders.	(Barnes, 1998)
Flavanols	Tea, chocolate, red wine, apples and berries.	Gallocatechin, catechin, epicatechin, epigalocatechin	Decreased incidence of lung cancer, coronary heart disease, asthma.	(Boyer and Liu, 2004; Yao *et al.*, 2004)
Anthocyanins	Dark coloured fruit (black current, plum, cranberries, red current) and flowers.	Cyanidin, delphindin, malvidin, pelargonidin, peonidin.	Decreased incidence of cardiovascular disease, premature ageing, inflammatory responses.	(Sandhar *et al.*, 2011; Zafra-Stone *et al.*, 2007)

2.1.3.2d Vitamin C (L-ascorbic acid)

Vitamin C is a hydrophilic vitamin and is fundamental for the biosynthesis of carnitine, collagen, neurotransmitters and certain hormones (Li and Schellhorn, 2007; Rebouche, 1991). Its application in the treatment and prevention of cancer is derived from its pro-oxidant activity which is exhibited at high concentrations of vitamin C (Chen *et al.*, 2005). This property is conducive to the generation of hydrogen peroxide, thereby providing a probable mechanism for the anti-carcinogenic effect of L-AA (Blot *et al.*, 1993).

The therapeutic effects of vitamin C has evidently warranted its use in the treatment of terminal cancer patients (Yeom *et al.*, 2007). The administration of high dose intravenous L-AA in end stage cancer patients reduced the rate of tumour expansion appreciably, enhancing their quality of life, where symptoms such as fatigue and pain were significantly reduced (Padayatty *et al.*, 2006). The antioxidant behaviour of vitamin C is derived from its ability to reduce unstable free oxygen radicals to stable ascorbate radicals. This phenomenon is possibly linked to L-AAs protective roles, which include prevention of DNA-induced damage by oxidation (Lutsenko *et al.*, 2002; Sweetman *et al.*, 1997), prevention of lipid peroxidation (Kimura *et al.*, 1992) and protection of proteins by repairing oxidative stress-induced damaged amino acids (Cadenas *et al.*, 1998; Hony and Butler, 1984).

Another bioactive mechanism that L-AA may use to induce an anti-carcinogenic effect is in its ability to stimulate the apoptotic sequence in DNA-mutated cells. Oxidants such as UV radiation, certain chemicals can induce mutagenic changes in DNA, thereby modulating gene expression in signal transduction pathways (Allen and Tresini, 2000). Low pharmacological levels of L-AA has been revealed to modulate gene expression by stimulating the expression of the p73 gene and MH1, subsequently predisposing the cell to the apoptotic process, particularly in the presence of oxidants (Catani *et al.*, 2002). In another study, L-AA together with its lipophilic by-products, were able to induce apoptosis, halt the cell cycle and assuage cellular propagation in human glioblastoma and pancreatic tumour cells by decreasing the expression of insulin-like growth factor-1 receptor (Naidu *et al.*, 2003; Naidu *et al.*, 2001). Evidently, the ability of L-AA to modulate gene expression and affect certain cell signalling pathways, hold great potential for its use in treating and preventing various types of cancers.

Vitamin C can inhibit protein oxidation by decreasing the radical initiators and prevent radical propagation. This is especially evident in protein oxidation of the lens in the eye contributing to the development of cataracts (Stadtman, 1992; Stadtman and Berlett, 1998). Vitamin C may contribute to a decreased risk in the pathogenesis of cataracts by behaving as a substrate for certain radical products in protein oxidation (Baynes, 2001).

Vitamin C's functionality is not restricted to sustaining human health and treating disease, but has also established a niche in the food and cosmetics industries. Its application in the cosmetics industry has been well established due to its potent reducing ability (Allemann and Baumann, 2008). Its protection of the skin against exogenous oxidants such as radiation, chemicals, pollutants makes it a valuable ingredient in the formulation of skin care products (Rona *et al.*, 2004).

The use of vitamin C as a preservative in food and beverage products to prevent oxidation and subsequently prolong food and beverage shelf life, is well established within the food and beverage industries (Aruoma, 1994). A unique chemical reaction of vitamin C advocating its use as a preservative involves vitamin C donating two electrons to the relevant radical species which could include superoxide, hydroxyl, peroxyl radicals; sulphur, nitrogen or oxygen radicals. Subsequently, vitamin C is a reducing agent and in donating its electrons, it precludes the oxidation of other compounds, however, becoming oxidized itself in this reaction (Padayatty *et al.*, 2003). When vitamin C loses its first electron to another harmful radical, it becomes a free fairly stable un-reactive radical. This property is what deems vitamin C a good antioxidant as it reduces a highly deleterious free radical and generates a potentially harmless free radical. This action is referred to as free radical scavenging or quenching (Bielski *et al.*, 1975).

2.2 Oxidative stress methodologies

Several methodologies are available in the literature for the determination and measurement of oxidative stress (Cao and Prior, 1998; Llesuy *et al.*, 2001). Figure 2.3 illustrates the three different approaches to detect and measure oxidative stress. The direct measurement of radicals using electron spin (pair) resonance (ESR, EPR) and spin trapping methods involves the detection of radicals that are stable or where stability is induced after reacting with another molecule (Rice-Evans *et al.*, 1991). However many radicals are extremely unstable and thus this method of detection may not always prove reliable.

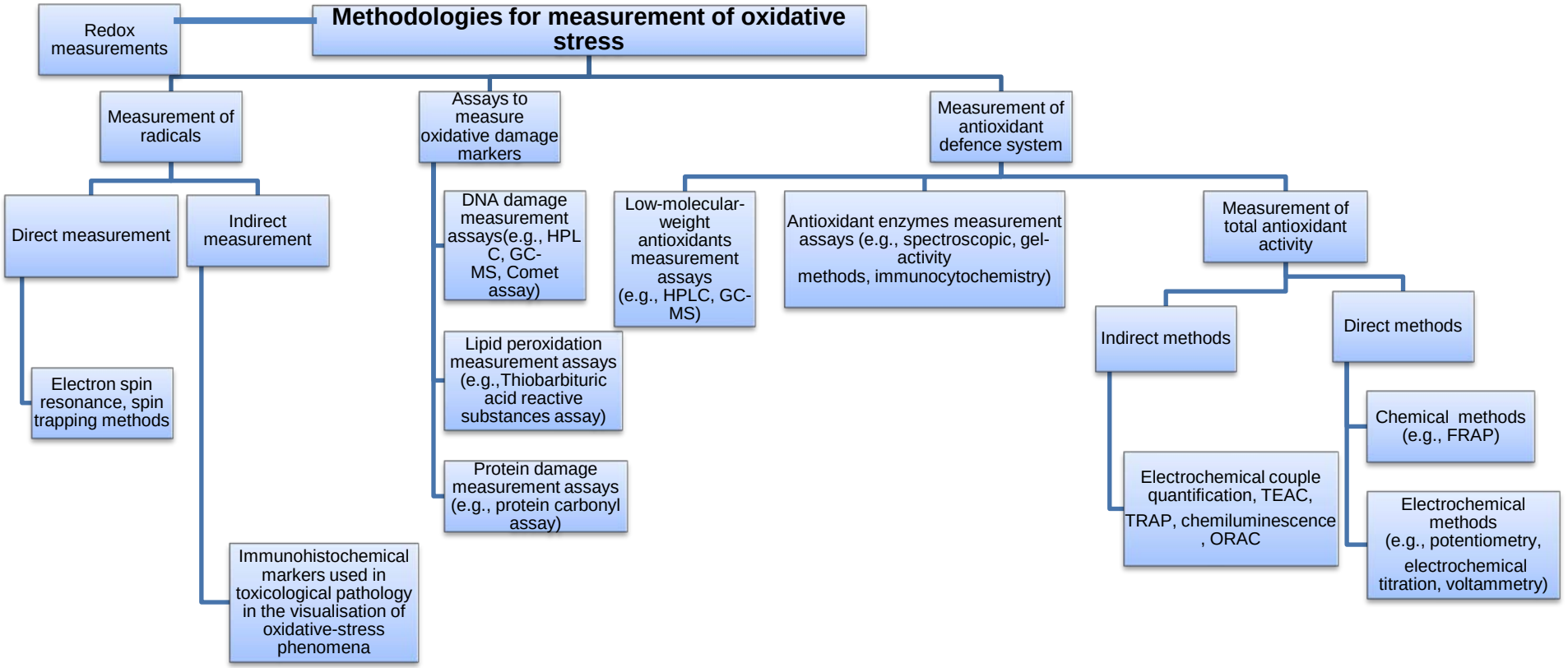


Figure 2.3 Methodologies for oxidative stress measurement (adapted from Kohen and Nyska, 2002). Abbreviations: FRAP, Ferric reducing antioxidant power; GC-MS, Gas chromatography-mass spectrometry; HPLC, High performance liquid chromatography; ORAC, Oxygen radical absorbance capacity; TEAC, Trolox equivalent antioxidant capacity; TRAP, total reactive antioxidant potential.

Another approach is the measurement of oxidative damage by measuring end-products formed as a result of ROS interaction with macromolecules of the cell such as DNA, lipids and proteins (Esterbauer, 1996; Fairbairn *et al.*, 1995; Levine *et al.*, 2000). This approach is preferable as end-products are easily quantifiable owing to their stability.

In recent years total antioxidant capacity (TAC) assays have gained popularity among the food and nutraceutical sectors. These assays measure the antioxidant capacity of a sample to inhibit ROS-induced oxidative damage, by "scavenging" free radicals (Prior and Cao, 1999). The protective roles of low molecular-weight antioxidants such as L-AA and glutathione are well established (Bendich *et al.*, 1986; Padayatty *et al.*, 2003; Sai *et al.*, 1992). Several antioxidant methodologies exist for their measurement, however HPLC determination and measurement are a commonly used method owing to its sensitivity and ability to measure low molecular weight antioxidants (LMWA) accurately at low limits (Vovk *et al.*, 2005). For the purpose of the study, the review will focus solely on the $ORAC_{FL}$ assay for TAC and HPLC for L-AA measurement.

2.2.1 Antioxidant assays

In general, there are two mechanisms by which antioxidants can "scavenge radicals". One involves the ability of the antioxidant to quench free radicals by hydrogen donation and is referred to as the hydrogen atom transfer (HAT) mechanism; the other, the single electron transfer (SET) mechanism involves the ability of a potential antioxidant to transfer one electron to reduce any compound, which could include metals, carbonyls and radicals (Prior *et al.*, 2005). The reduction reaction in the SET based method results in a colour change which is directly proportional to the antioxidant concentration in the sample. The end point of the reaction is attained when the colour change ceases to occur. The change in absorbance is plotted against the concentration of antioxidants in the sample and a linear curve is obtained. The slope of the curve represents the antioxidant's reducing ability and may be expressed as trolox equivalent (TE) or gallic acid equivalent (GAE) (Huang *et al.*, 2005).

Assays utilizing the SET mechanism include the Total Phenols assay by Folin Ciocalteu reagent (FCR), Trolox equivalent antioxidant capacity (TEAC) assay, the ferric ion reducing antioxidant power (FRAP) assay, the N,N-dimethyl-p-phenylenediamine (DMPD) assay, and the Cu(II) reduction capacity assay (Huang *et al.*, 2005) The HAT-based methods generally employs the use of (1) a synthetic free radical initiator which reacts rapidly with oxygen thereby generating a continuous flow of peroxyl radicals, (2) an oxidizable substrate such as a molecular probe for monitoring the reaction progress, (3) an antioxidant which reacts with

peroxyl radicals thereby preventing them from reacting with the oxidizable molecular probe, hence controlling the degree of oxidation and (4) and reaction kinetic parameters (Huang *et al.*, 2005; Karadag *et al.*, 2009).

HAT mechanisms are solvent and pH independent and the reaction usually proceeds quite rapidly, and is usually concluded within seconds to minutes (Prior *et al.*, 2005). Their quantification is determined from kinetic curves (Huang *et al.*, 2005). HAT-based methods that have received much attention for their applicability in determining total antioxidant capacity are the ORAC and total radical trapping antioxidant parameter (TRAP) assays. Due to the complex nature of food and beverages matrices, it can be an arduous task to measure the antioxidant activity of each antioxidant compound present separately. Therefore, the need for a rapid, standardized antioxidant assay to measure antioxidant capacity would be ideal. However, one of the biggest obstacles in standardizing an antioxidant capacity (AOC) assay is the scarcity of fully validated antioxidant assays available (Huang *et al.*, 2005).

Prior and co-workers (2005) highlighted certain key features that a "standardized" AOC assay should comprise of which include (1) the ability of the assay to measure chemical reactions occurring in potential applications, (2) makes use of a biologically relevant radical source, (3) is simple, (4) employs the use of an assay with a defined endpoint as well as a chemical mechanism, (5) easily obtainable instrumentation, (6) good within-in run and day-to-day precision, (7) adaptable for the determination of both hydrophilic and lipophilic antioxidants and the utilization of various radical sources, and (8) adaptable to high throughput analysis (Prior *et al*., 2005). Of all the assays available, the $ORAC_{FL}$ assay has surfaced as the "gold standard" for measuring the peroxyl radical scavenging capacity of a sample due to its inter-laboratory validation as well as the acknowledgement it has received within the food industry (Huang *et al.*, 2005).

2.2.1.1 $ORAC_{FL}$ assay

In the past few years, the requests for antioxidant assays have increased in frequency at the OSRC (Bellville, Cape Town). This occurrence is not unanticipated seeing that:

- International and national regulation is rapidly assuming a significant precedence in the food manufacturing sector.
- Consumers are becoming more health conscious and aware of "antioxidant" containing products.

The ORAC assay was developed by Cao *et al.* (1993) on the basis of the original work of Glazer (Cao *et al*, 1993; Glazer, 1990). A further improved $ORAC_{FL}$ assay developed by Ou *et al.*, and subsequently modified by Davalos *et al.*, has rapidly become the most widely accepted method to measure TAC in food, beverage and biological samples (Ou *et al*., 2001; Davalos *et al*., 2004)

- ***Principle of $ORAC_{FL}$ assay:***

The method is based on the inhibition of oxidation of a fluorescent probe induced by peroxyl radicals generated by thermal decomposition of 2,2'-Azobis (2–amidinopropane) dihydrochlo-ride (AAPH) (Ghiselli *et al.*, 1995; Glazer, 1990). The reaction is performed in 75 mM phosphate buffer at pH 7.4 and employs the biological relevant radical source AAPH. The reaction mixture consisting of fluorescein as the oxidizable substrate, antioxidant (either trolox standard solution or an antioxidant containing sample) and AAPH are all contained in wells of a microplate maintained at a temperature of 37^{o}C. The antioxidant binds the peroxyl radical source, AAPH, resulting in a delay in fluorescence decay. Fluorescence measurements are standardized to the blank (no antioxidant present) curve (Wu *et al.*, 2004). The area under the fluorescence decay curve (AUC) is calculated as in Equation 2.1.

where f_0:initial fluorescence reading at time 0 mins

f_i: is the fluorescence reading at time *i.*

$$AUC = 0.5 + \frac{f1}{f0} + \cdots \frac{fi}{f0} + \frac{f7}{f4} + \cdots + \frac{f34}{f0} + 0.5\left(\frac{f35}{f0}\right) \quad \textbf{Equation 2.1}$$

The net AUC of a sample is calculated by subtracting the AUC of the blank from the AUC of the relevant sample: Net AUC= [AUC_{AOX} – AUC_{BL}] as illustrated in Figure 2.4. $ORAC_{FL}$ results are calculated using a regression equation between trolox concentration in µM and the net area under the curve. Final $ORAC_{FL}$ values are expressed as micromole trolox equivalents per litre and per gram for liquid and solid samples, respectively (Huang *et al.*, 2002a).

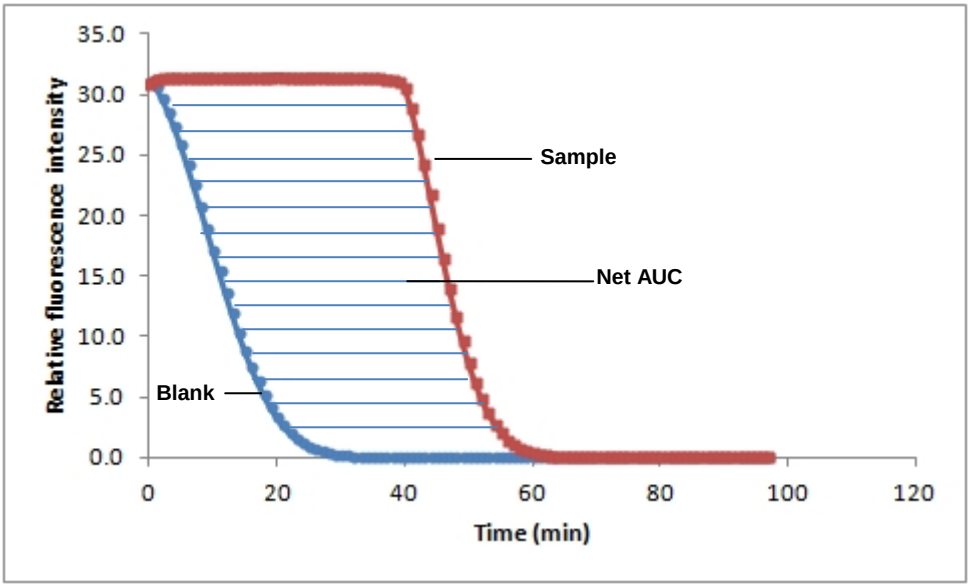


Figure 2.4: Fluorescence decay curve of $ORAC_{FL}$ assay

The $ORAC_{FL}$ assay is unique in that it can directly measure the hydrophilic chain-breaking antioxidant capacity against the peroxyl radical, and combines both the time and degree of inhibition into a single quantity (Ou *et al.*, 2001). Another advantage is that the assay is performed at conditions similar to that *in vivo* i.e. pH 7.4, a temperature of 37^{o}C and utilizes a biological relevant radical (Bisby *et al*., 2008). Furthermore similar assay conditions and standards are used for both the hydrophilic (H) and lipophilic (L) $ORAC_{FL}$ assay, such that the two values can be added together to record a TAC for a sample (Prior *et al.*, 2003).

Even though the Food and Drug Administration (FDA) currently does not have any regulations compelling standardization within the nutraceutical and food supplementation sector, other pressing factors already discussed in this review allude to the necessity and importance for validating the $ORAC_{FL}$ assay (Ou *et al.*, 2001).

2.2.1.2 Vitamin C (L-Ascorbic acid) UV-HPLC

L-ascorbic acid (L-AA) is the biological isomer of vitamin C, and the form that is predominantly measured and the form that will be measured for the current study. For the purposes of this thesis, the abbreviation L-AA will be used to refer to L-ascorbic acid. L-AA may be converted to dehydroascorbic acid (DHA) (Sandhar *et al.*, 2011) by oxidative reactions generated by conditions such as increased pH, increased temperature, light exposure, oxygen exposure as well as reactivity with certain metals and enzymes. This oxidation reaction is reversible with DHA reduced to L-AA, provided the DHA is not incidently further oxidized to diketogluconic acid (DKG), as this oxidation is non-reversible (Deutsch, 2000).

The Association of Analytical Chemists (AOAC) provides several methodologies for the determination of L-AA in vitamin supplements as well as in juices, however some of these methods are outdated and have encountered certain obstacles. These include the inability to measure L-AA in some complex matrices, the oxidation of certain naturally occurring substances in fruit and certain methods requiring a derivatization step (Van de Velde *et al.*, 2011). Examples of these methodologies include spectrophotometry, spectrofluorimetry, electrochemistry and chromatography (Calokerinos and Hadjiioannou, 1983; Liu *et al.*, 1982; Sánchez-Mata *et al.*, 2000). Putative factors such as improved specificity and sensitivity, ease of use operation, as well as an omitted derivatization step has favoured the use of UV-HPLC as a popular and preferred method for the measurement of L-AA (Novakova *et al.*, 2008) .

- *Principle of HPLC:*

In general, HPLC employs the use of high pressure (approximately 400 bar) to pump sample components in a liquid mobile phase through a column containing the stationary phase [contains porous particles, the surface of which has a chemically bonded phase; commonly used is a C18 column (250x4.6 mm, with 5-μm particles)] to which sample components will adsorb to. The degree of adsorption is influenced by the unique chemical and physical reactivity with the stationary phase as it travels through the length of the column, and the stationary and mobile phase composition. Upon elution of the sample components at the end of the column, they are detected and measured by a detector which provides an output to a computer, which subsequently displays results in a liquid chromatogram. Figure 2.5 illustrates the main components of an HPLC system. The retention time, which is the amount

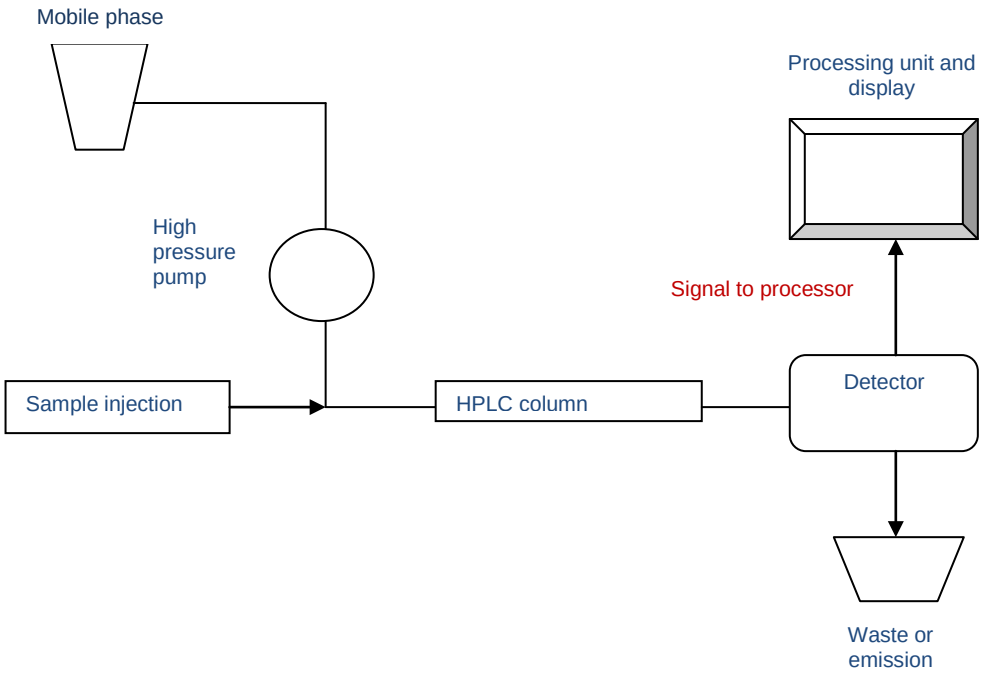


Figure 2.5 Schematic flow diagram of the components of an HPLC system

of time a component takes to elute from the column upon injection, is the most important indicator for sample component identification. Usually the height or area of the peak is proportional to the component concentration (Talamona, 2005).

The uv-HPLC assay utilized by the OSRC at CPUT for the measurement of L-AA is a simple isocratic reverse-phase HPLC method (Odriozola-Serrano *et al.*, 2007). The assay is performed at 25 °C and a single mobile phase is used to separate the L-AA component from the other components in the sample, which is subsequently detected by a diode array UV detector set at a wavelength of 245 nm. Identification of L-AA is performed by comparing the retention time of the sample peak with that of the L-AA standard peak. The results are then calculated using the linear regression equation (Equation 2.2) between L-AA concentration and the peak absorbance area:

$$y = mx + c \quad \text{.....Equation 2.2}$$

Where: y is the analyte response
m is the slope of linear plot
x is the analyte concentration

c is the intercept

2.3 Accreditation

The rapid trend in developing and implementing quality assurance procedures and processes in many industries and companies, especially the food and beverage manufacturing sector, warrants the need for laboratories, many of which are in collaboration with or an integral component of food and beverage companies, to operate a quality assurance system in conformity to the ISO/IEC 17025 standard. The ISO/IEC 17025 is an international standard which provides a detailed framework of standards and guidelines and subsequently stipulates the general requirements for the competence of testing and calibration in laboratories which include management and technical requirements (Vlachos *et al.*, 2002).

The conforming of laboratories to the specified criteria defined within the framework of the ISO/IEC 17025 standard, and the formal recognition of this by an authoritative body, is the process known as accreditation. The ISO definition of accreditation is as follows: “a procedure by which an authoritative body gives formal recognition that a body or person is competent to carry out specific tasks” (Kenny, 2001). This process recognizes a laboratory’s technical capability and is usually specific for tests of the systems, products, components, or materials for which the laboratory claims proficiency**.** Subsequently, accreditation provides an indication for potential clients to recognise and select reliable analytical, measurement and calibration services for the analysis of their products (Gough and Reynolds, 2000).

2.3.1 South African National Accreditation System (SANAS)

The induction of the South African National Accreditation System (SANAS) in 1996 in South Africa initiated the free trade of products to and from South Africa, without any economic trade barriers, a pivotal factor contributing to efficient global trading and increased economic revenue (South African National Accreditation System, 2008a).

The SANAS is recognised by the South African government as the only National Accreditation Organization which provides formal recognition to laboratories which are deemed competent to perform specific tasks (South African National Accreditation System,

2008b). The achievement of SANAS accreditation is rapidly becoming a sought after phenomenon in South Africa as can be seen in Figure 2.6.

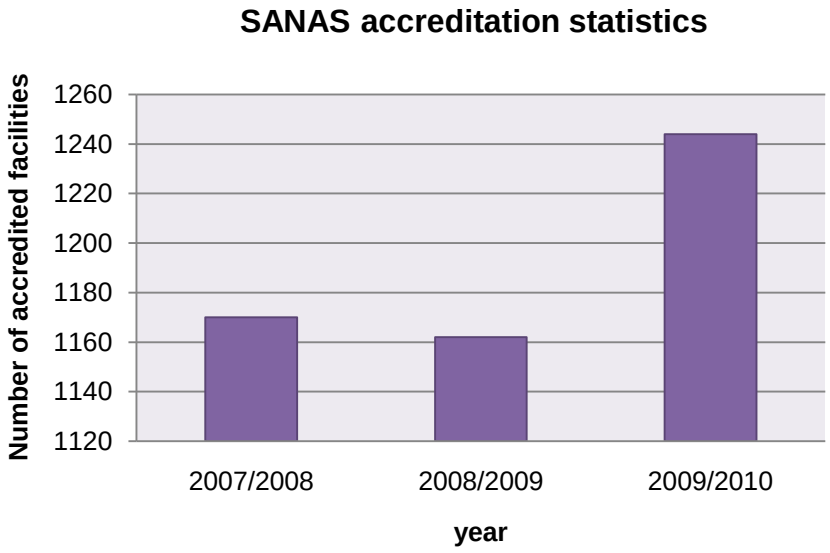


Figure 2.6 Number of facilities accredited by SANAS between 2007 and 2010 (adapted from SANAS annual report 2009/2010).

2.3.2 Validation

Validation of analytical methods in accordance with regulatory compliance and most international standard organizations namely, EURACHEM, ICH and ISO, is encouraged and becoming necessary in analytical laboratories. In order to determine and evaluate this quality, assays are subjected to a series of method-performance experiments to establish whether the results of such experiments meet the requirements for its intended use. This process, better known as validation, forms the basis of analytical quality assurance (AQA), and is mandatory and a prerequisite for obtaining ISO/IEC 17025 accreditation (Taverniers *et al.*, 2004).

ISO defines validation as “confirmation by examination and provision of objective evidence that the particular requirements of a specified intended use are fulfilled” (Taverniers *et al.*, 2004). Therefore validation is an integral component of quality assurance that is used to demonstrate and verify that a particular assay is in fact measuring what it is intended to measure (Wood, 1999). The Handbook for Quality Assurance of Metrological Measurements defines method validation as “documenting the quality of an analytical procedure, by establishing adequate requirements for performance criteria, such as accuracy, precision, detection limit, etc. and by measuring the values of these criteria” (Taylor and Oppermann, 1986).

The EURACHEM Guide (The Fitness for Purpose of Analytical Methods) stipulates three requirements that all validation processes must comply with, irrespective of the nature of the analytical method (Songara and Prakashkumar, 2011):

1) The entire analytical method must be validated. Pre-treatment of the sample, sample component extraction, pre-dilution or concentration of samples are all components of the method that must not be overlooked.

2) The entire range of concentrations for which the method demonstrates good accuracy and precision must be validated.

3) A wide range of matrices must be tested during the validation procedure to detect the possible effects on the sample analyte measurement.

The Handbook of Chemometrics and Qualimetrics describes two types of method performance criteria: primary and secondary. The primary group consists of performance characteristics such as precision, accuracy, trueness and detection limit, while the secondary group includes those that could influence the primary performance characteristics

and include linearity, range, quantification limit, selectivity and sensitivity (Massart *et al.*, 1997). The decision to perform an internal method validation versus an inter-laboratory validation lies within certain requirements of the laboratory such as the operational requirements, feasibility of assay and availability of financial and material resources.

Other factors that may warrant the performance of an internal validation instead of an inter-laboratory validation of an analytical method is that the method may be utilized mainly for research purposes, the method may be imported from another source so the reliability of such a method must be confirmed by a validation, or where there exists a lack of interest by other laboratories to take part in a inter-laboratory validation process (AOAC, 2002). Internal method validation is the most basic level of validation and is useful for accessing precision over an extended period of time (García-Campaña *et al.*, 2000).

Several method validation protocols have been described from various international regulatory bodies such as the Code of Federal Regulations (CFR), United States Pharmacopoeia (USP) Convention, FDA, Current Good Manufacturing Practice (CGMP), National Drug Administration (NDA), American Health Association and the Internal Conference on Harmonisation (Jenke, 1996). Moreover, some other institutions such as International Union of Pure and Applied Chemistry (IUPAC), Cooperation on International Traceability in Analytical Chemistry (CITAC/EURACHEM) and importantly ISO/IEC provide a comprehensive series of guidelines in this respect (International Organization for Standardization/International Electrotechnical Commission, 2005; Thompson *et al.*, 2002). According to Wood, should the method for which the validation is required be one that has been adopted from published scientific literature with given characteristics, the laboratory may only be required to verify the method with limited validation performance characteristics such as repeatability and reproducibility (Wood, 1999).

A validation protocol does not need to adhere to any specific format, and may be adapted to the requirements of the analytical method's purpose and functionality, the client's requirements, and in compliance to the relevant regulatory governance. The distinction between the type of method measurement also dictates the relevant method performance parameters that should be included in the validation study, at the least. As in the current study, the hydrophilic $ORAC_{FL}$ and UV-HPLC assays are both quantitative measurement assays. Subsequently, typical method performance parameters against which these methods would be validated are accuracy, precision, specificity, detection limit, quantification limit, linearity, range, and ruggedness.

The first and foremost step in initiating a full validation procedure is to develop a validation plan detailing the step-by-step procedure that the study would follow. The validation plan presumes that the relevant instrument has been decided on already, and the method has been developed (Huber, n.d).

2.3.3 Validation master plan

A validation master plan (VMP) defining and detailing the processes to be developed and implemented to perform the validation, should include the steps outlined below. Figure 2.7 further illustrates the sequence of processes involved in performing a full method validation.

- The application, purpose and scope of the assays must be clearly defined.
- The validation approach and the relevant method performance characteristics against which the method will be tested must be defined.
- The acceptance criteria for the method performance characteristics must be established.
- Pre-validation criteria and experiments must be defined.
- Pre-validation experiments must be performed.
- Method performance experiments must be performed.
- Results to be evaluated and acceptance criteria must be adjusted if necessary.
- A standard operating procedure (SOP) must be developed for executing the method in the routine laboratory.
- Analytical quality control checks and system suitability tests must be developed for executing routinely in the laboratory.
- A detailed validation report including all results and data must be prepared.
- The validation report should be reviewed, approved and archived.

Validation criteria relevant to the scope of the method should also be determined in the initial stages of the validation procedure such as described by Huber:

- Analytes that must be detected by the method.
- The expected concentration range should be defined.
- The sample matrices that will be used.
- Specific legal and regulatory stipulations that the method must comply with.
- Whether information should be qualitative or quantitative.

- All the relevant equipment that would be utilized during the validation procedure should be described and their performances should be substantially scientifically verified.
- The competencies of the analysts that would participate in the validation procedure should be verified.

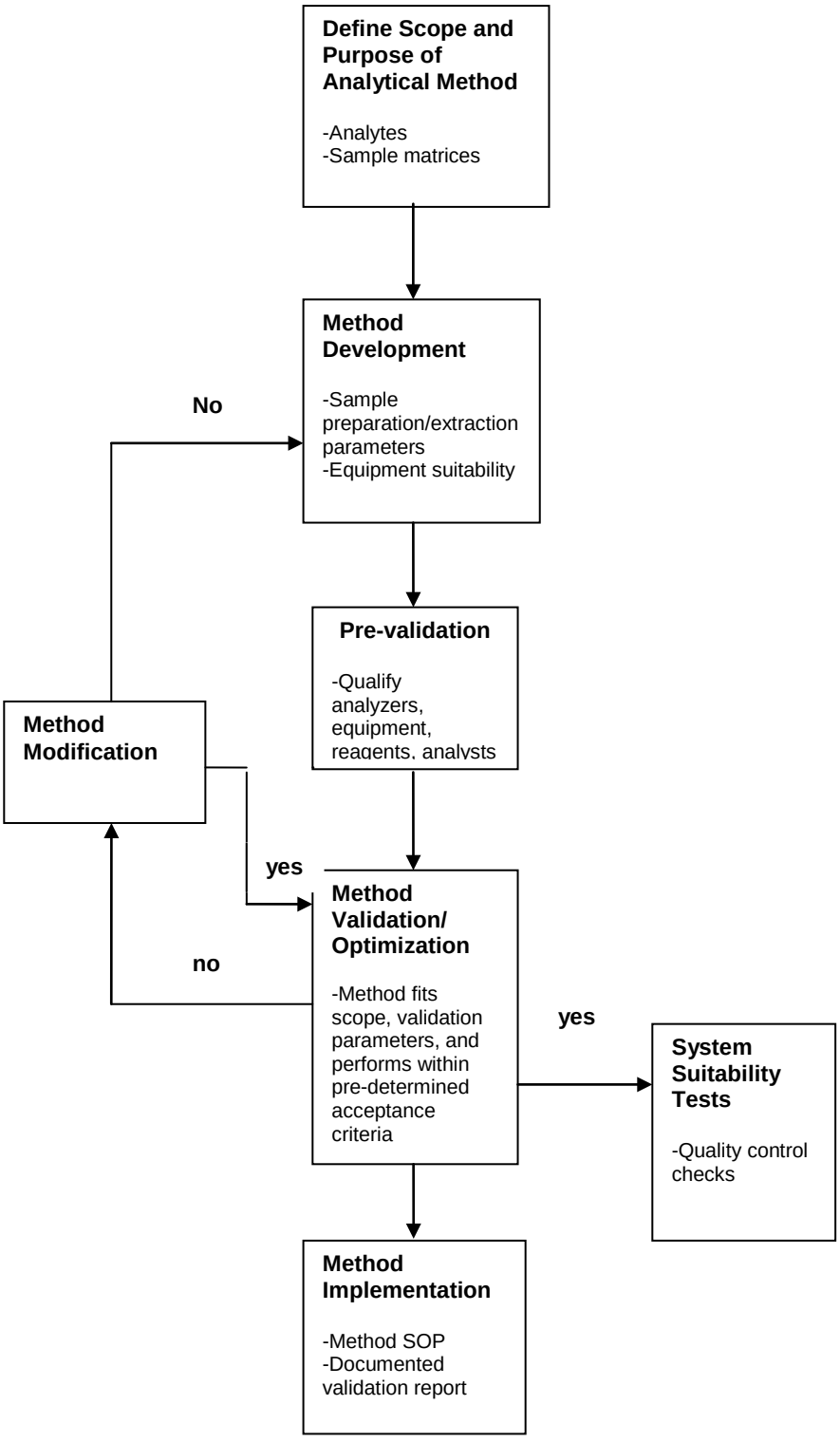


Figure 2.7. Method Validation Strategy

2.4 Pre-validation elements

When performing a method validation it is to be assumed (as described by Roper, 2001) that:

- The equipment on which the testing is carried out is broadly suited to the application.
- The samples used in the analysis are known to be sufficiently stable.
- There are no major changes in the laboratory environment eg. humidity, temperature.
- Analysts who are performing the assay are competent.
- Any equipment that will be utilized in the preparation of samples for the assays are functioning optimally.

These conditions must be verified by evaluating the performance specifications of the analyzers, equipment, reagents and standards utilized in a validation study. Subsequently, it is mandatory in every analytical method validation to carry out all relevant pre-validation experiments. This procedure encompasses the evaluation and verification of all the pre-analytical variables that could significantly impinge on the assay. It is imperative that every action executed, problems encountered, recourse taken as well as all accompanying data generated thereof during the pre-validation stage, be documented (Songara and Prakashkumar, 2011). The following components must be evaluated as a prerequisite to the validation procedure.

2.4.1 Analytical analyzer qualification

It is essential that the analyzers utilized in the test procedure are effectively tested, calibrated and maintained. The validation of the analyzers usually commences at the vendor's site where every component and the applicable software is intensely validated in terms of its development, design and functionality in compliance with guidelines provided by various regulatory standards and agencies namely, Good Laboratory Practice (GLP), cGMP, ISO/IEC 17025 and the FDA (International Conference on Harmonisation, 2000; International Organization for Standardization/International Electrotechnical Commission, 2005; Food and Drug Administration, 2011). During this stage which is also regarded as the qualification stage, four integral qualification processes which consist of a design qualification (DQ), installation qualification, operational qualification (OQ), and performance qualification (PQ) are performed between the manufacturer's site and the laboratory site as illustrated in Table 2.3 (Winter, 2006).

Table 2.3 Instrument qualification process (adapted from Winter, 2006).

Vendor's Site	Laboratory site	Laboratory site
Structural and software validation – DQ Compliance with ISO/IEC 17025, FDA, cGMP.	Functional validation and qualification- IQ, OQ	Calibration, maintenance and system suitability tests – OQ, PQ
Vendor responsibility	Before use, vendor assistance	Laboratory's responsibility

Abbreviations: cGMP, current Good Manufacturing Practices; DQ, design qualification; FDA, Food and Drug Administration; ISO/IEC, International organization for standardization/international electrotechnical commission; IQ, installation qualification, OQ, operational qualification; PQ, performance qualification.

The IQ verifies that the analyzer is received as per design specifications and the installation is performed correctly. The OP verifies that the all the modules of the analyzer are operating in compliance with the predetermined requirements for accuracy, linearity and precision. This procedure may be in the form of running self diagnostic tests for the various components or more specific tests to validate certain critical parameters eg. detector wavelength accuracy, injector or flow-rate precision in an analyzer used for HPLC assays (Shabir, 2003).

The purpose of the PQ is to confirm system performance and is usually conducted at the laboratory site where it is subjected to the conditions under which the testing method would be run. Because these tests are performed at the laboratory site where conditions may differ substantially to that of the vendor's site, the acceptance criteria for PQ tests tend to be significantly less stringent than those defined for the OQ. In the case of HPLC the PQ utilizes a method with a well resolved chemically stable analyte mixture known as the resolution test mixture (RTM) (Hartmann *et al.*, 2008) that is used to demonstrate injector precision and pump flow stability (Grisanti and Zachowski, 2002). Most companies utilize an RTM consisting of caffeine, uracil, theophylline and 8-chlorotheophylline which should demonstrate consistent peak heights for all components at a specific wavelength. Similarly, varying concentrations of caffeine solutions are used to demonstrate the linearity of the detector response within the linear range of HPLC detectors. Other parameters tested and verified as part of the PQ for an HPLC analyzer are detector wavelength accuracy, column oven temperature and auto-sampler temperature accuracy (Crowther *et al.*, 2008).

An added benefit of performing the PQ at the laboratory site, is that competent laboratory staff could implement a PQ system with assistance from the relevant vendor and execute PQ tests on a routine basis. Calibration, maintenance and system suitability tests could be incorporated into the PQ standard operating procedure (Huber, 2009).

2.4.2 Qualification of analytical solutions and samples

To provide reliable results, chemicals, reagents, standards and quality control samples must be checked, evaluated and documented against the following criteria where applicable, prior to commencing the validation study (Huber, 2009):

- they should be available in sufficient quantities
- they must be accurately identified
- they must be verified for stability
- they should be verified for exact composition and purity

Most stock solutions and reagents utilized in bio-analytical assays are prepared from commercially available pure chemical compounds. Major international companies such as Sigma-Aldrich and Merck provide adequate documentation verifying the purity, chemical characterization, integrity, and expiration of their products in accordance with ISO guidelines (Sigma Aldrich, 2012; Merck Millipore, 2012). This documentation must always be available for laboratories whose methods utilize such materials and are undergoing validation.

The stability of stock solutions, which are generally prepared from commercially purchased products such as aqueous buffers and organic solvents, must be evaluated during the validation procedure as their stability cannot be matched to the commercially available products from which they are made up. The stability of stock solutions should be verified by comparing results of assays run by utilizing standards made up from a freshly prepared stock solution to the results generated from an assay run in which standards were made up from a stored stock solution (Nowatzke and Woolf, 2007).

Calibration standards may be made up from spiking stock solutions with certified reference material, provided that the standards are evaluated separately for stability, as the manufacturer's expiration dates on reference materials are not applicable to these standards (Viswanathan *et al.*, 2007). Prepared stock solutions and calibration standards must be assessed for both long-term and short-term stability taking into consideration their storage requirements. Samples must also be tested for long-term stability (between the time a sample is received in the laboratory till the time it is analyzed), short-term stability (time the

sample spends on the bench top during before it is analyzed), and freeze-thaw stability (three freeze-thaw cycles) to assess whether any analyte degradation has occurred (Food and Drug Administration, 2001)

2.4.3 Qualification of equipment

The state of equipment used during sample preparation and sample analysis has substantial influence on the final result generated. Subsequently the assessment of all relevant equipment such as pipettes, analytical balances, pH meter, thermometers, glassware, centrifuges and fridges is crucial in the pre-validation stage. Procedures such as calibration, maintenance, services (where applicable), and cleaning must be performed frequently (once a month) on all equipment (International Organization for Standardization/International Electrotechnical Commission, 2005).

The key functions of calibrating equipment are to evaluate their measurement capability and subsequently affirm the comparability of the results produced with those attained by other laboratories; to validate analytical methods and used to implement quality control. With consistent use the functioning of equipment may alter over time due to changes in the laboratory environment such as temperature, humidity changes or due to normal wear and tear of mechanical or electronic components. Some of these changes may not be apparent initially and could potentiate erroneous results. Replacement of mechanical parts in equipment could also contribute to changes in results (United Nations Office on Drugs and Crime, 2009).

According to section 5.5.2 of ISO/IEC 17025:2005, “Calibration programmes shall be established for key quantities or values of the instruments where these properties have a significant effect on the results. Before being placed into service, equipment (including that used for sampling) shall be calibrated or checked to establish that it meets the laboratory´s specifications requirements and complies with relevant standard specifications. It shall be checked and/or calibrated before use” (International Organization for Standardization/International Electrotechnical Commission, 2005). It is therefore crucial that a laboratory implement a comprehensive system in which regular preventative maintenance and calibration procedures are stipulated and adhered to. In doing so, equipment and instrument problems are minimized, and the regular incidences of replacing mechanical parts and utilizing vendor support are prevented subsequently saving the laboratory unnecessary financial costs.

2.5 Validation method performance parameters

The ISO/IEC 17025 guide details validation parameters by which analytical assays should be validated (International Organization for Standardization/International Electrotechnical Commission, 2005). Typical parameters that are recommended and should be evaluated are selectivity/specificity, accuracy, precision (repeatability, intermediate precision), limit of detection (LOD) or detection limit, limit of quantification (LOQ) or quantification limit, linearity and linear range.

2.5.1 Precision

"The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple samples of the same homogenous sample under prescribed conditions" (Chan, 2008; International Conference on Harmonisation, 2005). It has been established that determining the precision of an analytical procedure will reveal the degree of random error caused by factors that vary in the operation of the method (Westgard, 2008). Precision can be determined at three levels as mentioned below.

2.5.1.1 Repeatability/Intra Assay Precision

Repeatability or intra-assay precision refers to the closeness of agreement between the results of multiple measurements of the same homogenous sample under the same operating conditions (the method, the standard samples, the equipment, the laboratory and the analyst) over a short interval of time (Chan, 2008; International Conference on Harmonisation, 2005).

Part II of the ICH Harmonized Tripartite Guideline for Validation of Analytical Methods suggests that repeatability should be determined by performing a minimum of nine determinations covering the specified range (eg. three repetitions performed at three different concentration range) (International Conference on Harmonisation, 2005).

2.5.1.2 Intermediate precision

Intermediate precision refers to the closeness of agreement between the results of multiple measurements of the same homogenous sample under varying operating conditions and

expresses within-laboratory variations such as day to day variation, analyst variation, equipment variation, variation of standards and reagents. The main purpose of intermediate precision is to ensure that the analytical method will continue to generate consistent results once the validation procedure is over. Intermediate precision may also be determined by performing a minimum of nine determinations spanning the specified range (International Conference on Harmonisation, 2005).

2.5.1.3 Reproducibility

The reproducibility measures the precision among different laboratories. The main purpose of reproducibility is to ensure that the analytical method will produce the same results in different laboratories and is especially important if the method is to be used in other laboratories. The following are factors that could affect the method's reproducibility (Association Of Analytical Chemists International, 2007; International Organization for Standardization/International Electrotechnical Commission, 2005):

- variation in environmental conditions
- analysts at varying levels of competence
- variation in reagent and analyzer conditions e.g. mobile phase, pH, flow rate
- equipment from different suppliers
- materials and reagents of varying quality from different batches and suppliers

For the purpose of this study the reproducibility will not be determined for the following reasons 1) neither of the methods will be used in another laboratory, 2) to our knowledge no other validated H-ORAC$_{FL}$ assay currently exists in South Africa with which the reproducibility may be determined.

The precision is expressed as standard deviation (s) as shown in Equation 2.3.

$$s = \sqrt{\frac{1}{N-1}\sum_{i=1}^{N}(x_i - \bar{x})^2} \quad \textbf{Equation 2.3}$$

where
$\bar{x}$: mean
x_i: observed value
N: number of observations

The relative standard deviation (see equation 2.4) is a more reliable expression of precision and is expressed as:

$$\%RSD = \left({}^{s}/_{\bar{x}}\right) \times 100 \quad \text{.....Equation 2.4}$$

where:
s: standard deviation
$\bar{x}$: mean

The precision for food samples can vary between 2 and 20%, however this is still largely dependent on analytical factors such as sample matrix, analyte concentration and analyzer performance (Huber, n.d). The precision should not be greater than 15% of the coefficient of variation (Uličná *et al.*) for all concentration levels tested, except at the LOQ where precision is acceptable at less than 20% of the coefficient of variation (COV) (Bansal and DeStefano, 2007).

2.5.2 Accuracy

Accuracy measures the degree to which the determined value of an analyte in a sample corresponds to the true value (International Conference on Harmonisation, 2005). The accuracy of an analytical method may be determined by 1) comparing the results produced against another validated reference method provided that the uncertainty of the reference method is known; 2) using certified reference materials (CRMs) and 3) performing recovery studies (Huber, n.d). The FDA/CDER recommends a minimum of three concentrations spanning the expected concentration range to be tested. The mean value obtained should be within 15% of the true value except at the limit of detection where it should be within 20% (Bansal and DeStefano, 2007).

Recovery studies may be used if there are no reference materials available and may be determined by spiking and recovery. A sample is analyzed in both its original state and after the addition (spiking) of a known mass/concentration of the analyte to the test sample. The difference between the two results expressed as a percentage is known as the percentage recovery (Thompson *et al.*, 2002). Recovery methods include the spiked-placebo recovery and standard addition methods.

2.5.2.1 Spiked–placebo recovery method

In this method known concentrations of the analyte of interest are added to formulation blanks, spanning three concentration levels of the expected analytical range and assayed in triplicate. The measured result is compared to the expected result, which is expressed as a percentage recovery (Australian Pesticides and Veterinary Medicines Authority, 2004).

2.5.2.2 Standard addition method

Known concentrations (spanning the expected concentration range) of standards (containing the analyte/s of interest) are added to samples which have already been analyzed both in its original state and after addition of standards. The results obtained will be compared to the expected result and expressed as percentage recovery (Australian Pesticides and Veterinary Medicines Authority, 2004).

For accuracy determinations the recovery depends largely on the sample matrix, the sample extraction process and the amount of analyte present. Hence it is recommended that recovery studies should be performed on different sample matrices, and at different levels of concentration for each matrix type as is performed with the standard addition method (Taverniers *et al.*, 2004).

2.5.3 Linearity

The linearity of an analytical method is its ability to produce results that are directly proportional to the concentration of analytes in the sample within a given range (Chan, 2008; International Conference on Harmonisation, 2005).

Evaluating linearity is crucial to the validation procedure as it can contribute significantly to errors in the final analytical result, should it be ignored. Assessing and establishing linearity is performed initially by utilizing calibration standards consisting of the same matrix as those of the potential samples. The ICH recommends using a minimum of five concentrations spanning the expected concentration range for determining linearity (International Conference on Harmonisation, 2005).

Several factors must be taken into consideration when evaluating and establishing linearity. The measurements should be performed in triplicate in a single series and then randomly distributed about the measuring range. If the method is expected to detect the analyte of interest at low concentrations, additional calibration standards in this range must be included.

Linearity should be demonstrated and evaluated by visual inspection of a plot of the response as a function of analyte concentration or content. If a linear relationship is evident, then the test results should be assessed by appropriate statistical methods such as a regression line by the least squares method. The correlation coefficient, y-intercept, slope of the regression line, residual sum of squares, as well as a plot of the data must be demonstrated (International Conference on Harmonisation, 2005).

The plot must be inspected for any outliers. The cause for the deviated value should be ascertained before the decision whether to reject or replace an outlier is executed. If the problem cannot be resolved and the laboratory has sufficiently identified a cause for an outlier then it may be rejected, provided there are sufficient linear calibration points in the measuring range (De Souza and Junqueira, 2005).
In establishing linearity for the proposed method, the FDA recommends that each standard used be prepared individually as opposed to preparing them from serial dilutions as it is easier to identify any errors that may be present. In addition, the calibration curve should exclude the zero or blank calibration point (Shah, 2007). Normally an acceptable calibration curve with a linear calibration equation is one that should have an intercept not significantly different from zero. The FDA recommends a linear correlation coefficient (r^2) of ≥ 0.95, however Causey *et al* commonly agree it should be ≥ 0.99 (Causey *et al.*, 1990).

2.5.4 Range

The range of an analytical method is the interval between the upper and lower concentration of an analyte in the sample for which it has been established that the analytical method produces an acceptable level of accuracy, precision and linearity (Australian Pesticides and Veterinary Medicines Authority, 2004; International Conference on Harmonisation, 2005). The range is usually determined during linearity studies and is normally expressed in the same units as the test results. The ICH recommends testing several concentration levels across the specified concentration range in triplicate demonstrating at each point acceptable accuracy and precision (International Conference on Harmonisation, 1996; International Conference on Harmonisation, 2005).

2.5.5 Limit of detection (LOD)

There are several terms used for the limit of detection. Eurachem accepts ‘detection limit’, whilst ISO prefers ‘minimum detectable concentration’ and IUPAC prefers ‘minimum

detectable (true) value' (Taverniers *et al.*, 2004). Irrespective of the term use, the definition is accepted as the same by all regulatory organizations: the limit of detection of an analytical procedure is the lowest concentration of an analyte in a sample that can be detected but not necessarily quantified as an exact value under the stated experimental conditions(Australian Pesticides and Veterinary Medicines Authority, 2004; International Conference on Harmonisation, 1996; International Conference on Harmonisation, 2005). There are several different methods available to determine detection limit and these are mentioned below.

2.5.5.1 Visual evaluation

This method may be utilized for both non-instrumental and instrumental methods. The detection limit is established by analyzing a sample series of known concentration and determining the lowest level/concentration at which the analyte of interest is detected. (International Conference on Harmonisation, 1996; International Conference on Harmonisation, 2005)

2.5.5.2 Signal to Noise method

This method is applicable to chromatographic analyses as a result of the baseline noise exhibited. The response produced from samples containing low concentrations of analyte is compared to those of blank samples and the lowest concentration at which the analyte can be detected is established as the LOD. The ICH considers a signal that corresponds to signal-to-noise ratio of 2:1 or 3:1 as acceptable. Other sources recommends analyzing a blank sample between six to ten times and determining the standard deviation of these responses (Australian Pesticides and Veterinary Medicines Authority, 2004; Huber, 2007).

2.5.5.3 Standard Deviation of the Response and the Slope

The limit of detection (LOD) may be estimated from the standard deviation of the response and the slope of the calibration curve (see Equation 2.5).

$$LOD = [3.3 \times (\sigma)]/S \quad \text{.....Equation 2.5}$$

where:
σ:standard deviation of the response
S:the slope of the calibration curve

The slope of the calibration curve may be determined from the analyte's calibration curve, while sigma may be determined from the standard deviation calculated from the responses elicited by a series of blank samples (International Conference on Harmonisation, 1996).

2.5.6 Limit of quantification (LOQ)

The limit of quantification is the lowest concentration of an analyte in a sample that can be quantified with acceptable accuracy and precision under the prescribed experimental conditions (Australian Pesticides and Veterinary Medicines Authority, 2004; International Conference on Harmonisation, 1996; International Conference on Harmonisation, 2005). There exist several methods for the determination of LOQ, however the choice of method is reliant on whether the proposed method for which it will be used is instrument or non-instrument based. As with the LOD, the LOQ utilizes the same methodologies which include visual evaluation, signal to noise method and standard deviation of the response and slope.

2.5.6.1 Visual evaluation

The detection limit is established by analyzing a sample series of known concentration and determining the lowest level/concentration at which the analyte of interest is quantifiable with reliable accuracy and precision (International Conference on Harmonisation, 1996; International Conference on Harmonisation, 2005).

2.5.6.2 Signal to Noise method

The response produced from samples containing low concentrations of analyte is compared to those of blank samples and the lowest concentration at which the analyte can be quantified is established as the LOQ. Acceptable signal-to-noise ratio is 10:1 (Australian Pesticides and Veterinary Medicines Authority, 2004; Huber, 2007).

2.5.6.3 Standard Deviation of the Response and the Slope

Similarly to the LOD, the quantification limit may be estimated from the standard deviation of the response and the slope of the calibration curve and is expressed in Equation 2.6.

$$LOQ = [10.0 \times (\sigma)]/S \quad \text{.....Equation 2.6}$$

where:

σ:standard deviation of the response
S:the slope of the calibration curve

The slope of the calibration curve may be determined from the analyte's calibration curve. Sigma may be determined from the standard deviation calculated from the responses elicited by a series of blank samples (International Conference on Harmonisation, 1996).

2.5.7 Selectivity/specificity

There has been much debate over the suitability of using the terms 'specificity' or 'selectivity' to describe an analytical method. Vessman has provided a review on the use of both these terms by major scientific regulatory institutions. He also differentiated between selectivity and specificity (Vessman, 1996). Selectivity refers to the ability of an analytical method to detect and measure a number of analytes of interest in the presence of other endogenous or exogenous substances in the sample matrix under the stated conditions of the method. Specificity refers to the ability of an analytical method to detect and measure only a single analyte. Few analytical methods are specific, whilst most methods exhibit selectivity.

The IUPAC favours the use of 'selectivity' to describe analytical methods (Aboul-Enein, 2000). The ICH definition for specificity is more applicable to pharmaceutical products containing active drug components and formulations as well as impurities. Therefore, it is apparent that validating an analytical method for selectivity is more appropriate for the measurement of antioxidants as in this study. The selectivity may be determined by assaying several blank and sample matrices which contain the analyte of interest and then adding potential interfering substances to these samples and subsequently comparing the responses to identify the presence of interferences. In HPLC, the chromatogram can be examined for differences in peak purity and retention times (Huber, n.d; Mitic *et al.*, 2011).

2.5.8 Robustness

The ICH describes robustness as the ability of an analytical method to remain unaffected by small but deliberate changes in method parameters thereby giving a good indication of its reliability during normal operational conditions (Australian Pesticides and Veterinary Medicines Authority, 2004; International Conference on Harmonisation, 1996; International

Conference on Harmonisation, 2005). The FDA does not include robustness as a validation parameter in its guidelines. However it is essential to test for robustness of an analytical method if it is to be used on a long-term basis, because deterioration of solutions, equipment and analyzer components as well as environmental factors are inevitable. The ICH guidelines recommend testing for robustness during the method development stage as any inconsistencies can be addressed and the method modified if need be (International Conference on Harmonisation, 1996). Typical changes for standard tests (as described by Chan, 2008 and Huber, 2007) could include:

- variation in analytical solution stability
- variation in solvent extraction composition
- changing membrane filters
- variation in sample and standard dilutions
- variation in mixing and centrifugation times
- variation in sample and standard stability

Typical changes that could occur in a chromatographic assay could include:

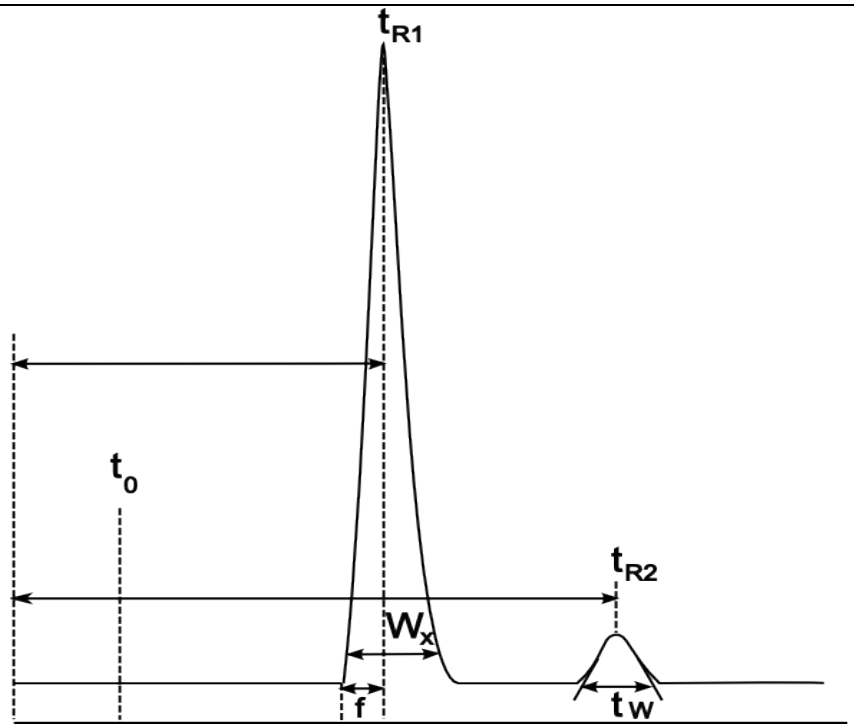
- variation in pH of mobile phases
- utilizing columns from different suppliers or batches
- composition of mobile phases (isocratic or gradient elution)
- variation in temperature
- variation in flow rate

These factors should not only be evaluated during the method validation procedure, but also incorporated in the system suitability testing that should be carried out consistently under normal operating conditions (International Conference on Harmonisation, 1996; International Conference on Harmonisation, 2005).

2.5.9 System suitability tests (SST's)

According to the CDER/FDA "Guidance for Industry" guidelines, system suitability tests are "based on the concept that the equipment, electronics, analytical operations, and samples to be analyzed constitute an integral system that can be evaluated as a whole" (International Conference on Harmonisation, 1996). The testing components that should be incorporated into a system suitability procedure are dependent on the analytical method it will be used for. Ideally, it is recommended that system suitability testing be performed prior to initiating sample analysis, however in cases where the turn-around-time is of great significance, SST's may be performed during or after sample analysis. The USP general chapter 1058

have specific requirements for implementing SSTs for chromatographic analyses (Dong *et al.*, 2001). The Centre for Drug Evaluation and Research/ Food Drug Administration (CDER/FDA) defines specific system suitability parameters against which a chromatographic analytical system should be tested against as illustrated in Figure 2.8 (Food and Drug Administration/ Center for Drug Evaluation and Research, 1994). These parameters and their respective acceptance criteria are summarized in Table 2.4.



Where:

W_x= width of the peak determined at either 5% (0.05) or 10% (0.10) from the baseline of the peak height

f = distance between peak maximum and peak front at W,

t_0 = elution time of the void volume or non-retained components

t_R = retention time of the analyte

t_w = peak width measured at baseline of the extrapolated straight sides to baseline

Figure 2.8: Typical system suitability parameters to test for in a chromatographical system (adapted from FDA/CDER Reviewer Guidance: Validation of Chromatographic Methods, US Food and Drug Administration, 1994)

Table 2.4 System suitability parameters in a chromatographic system (adapted from FDA/CDER: Reviewer Guidance, 1994)

Parameter	Description	Limit
Capacity factor (k´)	**$k' = (t_R - t_0)/t_0$** Measure of the elution time of the non-retained peak.	**>2**
Injection precision (RSD)	Indicates the performance of the HPLC which includes the plumbing, column, and environmental conditions at the time at which the samples are analyzed.	**≤ 1% for n ≥ 5**
Resolution (Rs)	**$R_s = (t_{R2} - t_{R1})/(^1/_2)(t_{w1} + t_{w2})$** Resolution is a measure of how well two peaks are separated. This is essential for reliable quantification.	**>2**
Number of theoretical plates (N)	**$N = 16(t_R - t_w)^2$** Theoretical plate number is a measure of column efficiency, that is, how many plates can be located per unit run-time of the chromatogram.	**>2000**

2.6 Validation issues

2.6.1 $ORAC_{FL}$ assay

A search in the literature reveals validation of the $ORAC_{FL}$ assay is in fact lacking in many areas of the assay. Huang and co workers developed and validated an ORAC assay for lipophilic antioxidants (Huang *et al.*, 2002a). Ou *et al* developed and validated an $ORAC_{FL}$ assay using fluorescein as the fluorescent probe (Ou *et al.*, 2001). Common method performance parameters that were used to validate these assays included accuracy, precision, limit of detection, limit of quantification, and robustness. Other validation parameters such as selectivity, linearity, range, implementation of routine quality control and pre-validation parameters such as optimizing extraction procedures, qualification of equipment, materials and analyzers have been minimally addressed. Prior and co-workers reported obstacles hindering validation of the $ORAC_{FL}$ assay (Prior *et al.*, 2005). These include the inability to optimize the robustness of the assay, failure to develop and implement a clearly defined SOP, failure to establish a correct measuring range, poorly trained staff, incorrect extraction and sampling procedures, incorrect storage of sample and reagents.

One aspect highlighted in the literature, is the feasibility and stability of fluorescein as the oxidizable substrate. However fluorescein tends to be pH sensitive and below pH7, its efficacy is significantly reduced (Ou *et al.*, 2001). Another report demonstrated good results with the use of an automated liquid handling system attached to a fluorescence microplate reader (Huang *et al.*, 2002b). However, in most research facilities, manual pipetting is still employed, therefore frequent calibration and maintenance are essential for the generation of reliable results. The heating of phosphate buffer to $37^{o}C$ prior to adding to AAPH, and the importance of maintaining the micro-plate at this temperature was observed to reduce the COV by about 50% compared to no prior incubation at $37^{o}C$ (Prior *et al.*, 2003). The issue of inconsistencies observed in the external wells of the micro-plate has also been documented (Lussignoli *et al.*, 1999). All these validation issues will be addressed and evaluated in the current study. Prior *et al* reported fundamental requirements and key performance parameters such as analytical range, recovery, repeatability and reproducibility that should be included in both standardizing and validating antioxidant capacity assays (Prior *et al.*, 2005). The first part of this study will focus on the investigation of these parameters as part of the validation process.

2.6.2 UV-HPLC assay

The extensive use of HPLC assays coupled with detailed validation studies in the pharmaceutical and nutraceutical industries is apparent. Most UV-HPLC methods utilized for the measurement of vitamin C have been validated for beverages such as fruit juices, wine and beer (Odriozola-Serrano *et al.*, 2007). The validation of UV-HPLC assay for vitamin C in foods such as fruit and vegetables is minimal. There is ample international regulatory guidelines, detailing the validation of HPLC methods for drugs and drug products (Center for Drug Evaluation and Research Reviewer Guidance' Validation of Chromatographic Methods, 1994).

The scope of the method usually dictates the necessary parameters that should be included in the validation, and it may be decided upon by the laboratory itself (Huber, n.d). Much of the error in results generated by a chromatographic method is derived from poor qualification and maintenance of the instrument utilized. Another major issue is that validating an HPLC system can be very time-consuming and take up valuable sample analysis time (Beinert, 1994). Subsequently, it is acceptable to select a few relevant parameters, applicable to the scope of the method to verify the correct and reliable operation of the entire HPLC system (Gilroy and Dolan, 2004).

The various modules of an HPLC system are subject to daily variations such as temperature, reagents, chemicals, analysts and subsequently must be frequently inspected for optimal functioning. Hence, system suitability testing is of great significance in maintaining and verifying the performance of a chromatographic system. The incorporation of specific tests into a system suitability protocol can help identify specific problems during routine analysis of samples. The use of blanks can detect problems of carryover, contamination, pump pressure and flow rate (Dolan, 2001). Calibration standards can indicate problems of retention times, peak shapes and are eventual indicators of column and mobile phase problems, some of which commonly are changes in column temperature, degradation of the column and changes in mobile phase composition. Evaluation of these components provides information regarding the precision, specificity and column stability of the HPLC system (Dong *et al.*, 2001). The second half of this research study will focus on some of these components as part of the validation of the HPLC assay for vitamin C.

2.7 References

Aboul-Enein, H. Y. 2000. Selectivity versus specificity in chromatographic analytical methods. *Accreditation and Quality Assurance: Journal for Quality, Comparability and Reliability in Chemical Measurement,* 5**:**180-181.

Allemann, I. B. & Baumann, L. 2008. Antioxidants used in skin care formulations. *Skin Therapy Letter,* 13**:**5-9.

Allen, R. & Tresini, M. 2000. Oxidative stress and gene regulation. *Free radical biology and medicine,* 28**:**463-499.

Ames, B. N., Shigenaga, M. K. & Hagen, T. M. 1993. Oxidants, Antioxidants, and the Degenerative Diseases of Aging. *Proceedings of the National Academy of Sciences of the United States of America,* 90**:**7915-7922.

Andre, C. M., Larondelle, Y. & Evers, D. 2010. Dietary antioxidants and oxidative stress from a human and plant perspective: A review. *Current Nutrition & Food Science,* 6**:**2-12.

Aruoma, O. 1994. Nutrition and health aspects of free radicals and antioxidants. *Food and chemical toxicology,* 32**:**671-683.

Association Of Analytical Chemists International. 2007. *How to Meet ISO 17025 Requirements for Method Verification*.
http://www.aoac.org/accreditation/alacc_guide_2008.pdf [10 November 2012].

Association of Official Analytical Chemists. 2002. *AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals*
http://www.aoac.org/Official_Methods/slv_guidelines.pdf [7 October 2012].

Australian Pesticides and Veterinary Medicines Authority. 2004. *Guidelines for the Validation of Analytical Methods for Active Constituent, Agricultural and Veterinary Chemical Products*.
http://www.apvma.gov.au/publications/guidelines/docs/gl_69_analytical_methods.pdf [6 August 2012].

Bansal, S. & DeStefano, A. 2007. Key elements of bioanalytical method validation for small molecules. *The AAPS journal,* 9**:**109-114.

Barnes, S. 1998. Evolution of the health benefits of soy isoflavones. *Proceedings of the Society for Experimental Biology and Medicine,* 217**:**386-396.

Baynes, J. W. 2001. The role of AGEs in aging: causation or correlation. *Experimental gerontology,* 36**:**1527-1537.

Beinert, W. D. 1994. Function Control of HPLC Instruments According to Quality Standards. *LC GC International,* 7**:**568-568.

Bendich, A. 1993. Biological functions of dietary carotenoids. *Annals of the New York Academy of Sciences,* 691**:**61-67.

Bendich, A., Machlin, L., Scandurra, O., Burton, G. & Wayner, D. 1986. The antioxidant role of vitamin C. *Advances in Free Radical Biology & Medicine,* 2**:**419-444.

Berlett, B. S. & Stadtman, E. R. 1997. Protein oxidation in aging, disease, and oxidative stress. *Journal of Biological Chemistry,* 272**:**20313-20316.

Biehler, E. & Bohn, T. 2010. Methods for assessing aspects of carotenoid bioavailability. *Current Nutrition & Food Science,* 6**:**44-69.

Bielski, B. H. J., Richter, H. W. & Chan, P. C. 1975. Some Properties of the Ascorbate free radical. *Annals of the New York Academy of Sciences,* 258**:**231-237.

Bisby, R. H., Brooke, R. & Navaratnam, S. 2008. Effect of Antioxidant Oxidation Potential in the Oxygen Radical Absorption Capacity (ORAC) Assay. *Food Chemistry,* 108**:**1002-1007.

Block, G., Patterson, B. & Subar, A. 1992. Fruit, Vegetables, and Cancer Prevention: A Review of the Epidemiological Evidence. *Nutrition and cancer,* 18**:**1-29.

Blot, W. J., Li, J. Y., Taylor, P. R., Guo, W., Dawsey, S., Wang, G. Q., Yang, C. S., Zheng, S. F., Gail, M. & Li, G. Y. 1993. Nutrition intervention trials in Linxian, China: supplementation with specific vitamin/mineral combinations, cancer incidence, and disease-specific mortality in the general population. *Journal of the National Cancer Institute,* 85**:**1483-1491.

Bohr, V., Taffe, B. & Larminat, F. 1995. DNA repair, oxidative stress and aging. In: Cutler, R. G., Packer, L., Bertram, J. & Mori, A. (eds.) *Oxidative Stress and Aging.* Switzerland: Birkhauser Verlag Basel: 101-110.

Bourdel□Marchasson, I., Delmas□Beauvieux, M. C., Peuchant, E., Richard-Harston, S., Decamps, A., Reignier, B., Emeriau, J. P. & Rainfray, M. 2001. Antioxidant defences and oxidative stress markers in erythrocytes and plasma from normally nourished elderly Alzheimer patients. *Age and Ageing,* 30**:**235-241.

Boyer, J. & Liu, R. H. 2004. Apple phytochemicals and their health benefits. *Nutrition Journal,* 3**:**12.

Cadenas, S., Rojas, C. & Barja, G. 1998. Endotoxin increases oxidative injury to proteins in guinea pig liver: protection by dietary vitamin C. *Pharmacology & toxicology,* 82**:**11-18.

Calokerinos, A. & Hadjiioannou, T. 1983. Direct potentiometric titration of thiosulfate, thiourea, and ascorbic acid with lodate using an iodide ion-selective electrode. *Microchemical Journal* 28**:**464-469.

Cao, G., Alessio, H. M. & Cutler, R. G. 1993. Oxygen-radical absorbance capacity assay for antioxidants. *Free Radical Biology and Medicine,* 14**:**303-311.

Cao, G. & Prior, R. L. 1998. Comparison of different analytical methods for assessing total antioxidant capacity of human serum. *Clinical Chemistry,* 44**:**1309-1315.

Catala, A. 2009. Lipid peroxidation of membrane phospholipids generates hydroxy-alkenals and oxidized phospholipids active in physiological and/or pathological conditions. *Chemistry and physics of lipids,* 157**:**1-11.

Catani, M. V., Costanzo, A., Savini, I., Levrero, M., De Laurenzi, V., Wang, J. Y. J., Melino, G. & Avigliano, L. 2002. Ascorbate up-regulates MLH1 (Mut L homologue-1) and p73: implications for the cellular response to DNA damage. *Biochemical Journal,* 364**:**441.

Causey, A. G., Hill, H. M. & Phillips, L. J. 1990. Evaluation of criteria for the acceptance of bioanalytical data. *Journal of pharmaceutical and biomedical analysis,* 8**:**625-628.

Cermak, R. 2008. Effect of dietary flavonoids on pathways involved in drug metabolism. *Expert Opinion on Drug Metabolism & Toxicology,* 4**:**17-35.

Cerutti, P. & Trump, B. F. 1991. Inflammation and oxidative stress in carcinogenesis. *Cancer cells,* 3**:**1-7.

Chan, C. C. 2008. Analytical Method Validation: Principles and Practices. *Pharmaceutical Sciences Encyclopedia: Drug Discovery, Development, and Manufacturing***:**1-16.

Chen, Q., Espey, M. G., Krishna, M. C., Mitchell, J. B., Corpe, C. P., Buettner, G. R., Shacter, E. & Levine, M. 2005. Pharmacologic ascorbic acid concentrations selectively kill cancer cells: action as a

pro-drug to deliver hydrogen peroxide to tissues. *Proceedings of the National Academy of Sciences of the United States of America,* 102**:**13604-13609.

Cirak, B., Inci, S., Palaoglu, S. & Bertan, V. 2003. Lipid peroxidation in cerebral tumors. *Clinica chimica acta,* 327**:**103-107.

CITAC/EURACHEM. 2002. *Guide to quality in Analytical Chemistry*. http://www.european-accreditation.org/publication/citac-eurachem-ta [10 October 2012].

Crowther, J., Dowling, J., Hartwick, R. & Ciccone, B. 2008. Performance qualification of HPLC instrumentation in regulated laboratories. *LC GC North America***:**70-82.

Crozier, A., Burns, J., Aziz, A. A., Stewart, A. J., Rabiasz, H. S., Jenkins, G. I., Edwards, C. A. & Lean, M. E. J. 2000. Antioxidant flavonols from fruits, vegetables and beverages: measurements and bioavailability. *Biological research,* 33**:**79-88.

Dahan, K., Fennal, M. & Kumar, N. B. 2008. Lycopene in the prevention of prostate cancer. *Journal of the Society for Integrative Oncology,* 6**:**29.

Dávalos, A., Gómez-Cordovés, C. & Bartolomé, B. 2004. Extending applicability of the oxygen radical absorbance capacity (ORAC-fluorescein) assay. *Journal of Agricultural and Food Chemistry* 52**:**48-54.

De Souza, S. V. C. & Junqueira, R. G. 2005. A procedure to assess linearity by ordinary least squares method. *Analytica Chimica Acta,* 552**:**25-35.

Deutsch, J. C. 2000. Dehydroascorbic acid. *Journal of Chromatography A,* 881**:**299-307.

Dolan, J. W. 2001. *System suitability*. http://www.chromatographyonline.com/lcgc/data/articlestandard//lcgcasia/512005/261980/article.pdf [01 November 2012].

Donaldson, M. S. 2004. Nutrition and cancer: a review of the evidence for an anti-cancer diet. *Nutrition Journal,* 3**:**19.

Dong, M., Paul, R. & Gershanov, L. 2001. Getting the peaks perfect: System suitability for HPLC. *Todays Chemist At Work,* 10**:**38-42.

Esterbauer, H. 1996. Estimation of peroxidative damage. A critical review. *Pathologie-biologie,* 44**:**25-28.

Fairbairn, D. W., Olive, P. L. & O'Neill, K. L. 1995. The comet assay: a comprehensive review. *Mutation Research/Reviews in Genetic Toxicology,* 339**:**37-59.

Fearon, I. M. & Faux, S. P. 2009. Oxidative stress and cardiovascular disease: novel tools give (free) radical insight. *Journal of molecular and cellular cardiology,* 47**:**372-381.

Finaud, J., Lac, G. & Filaire, E. 2006. Oxidative stress: relationship with exercise and training. *Sports medicine,* 36**:**327-358.

Food and Drug Administration. 2001. *Guidance for Industry: Bioanalytical Method Validation*. http://www.fda.gov/downloads/Drugs/.../Guidances/ucm070107.pdf [10 August 2012].

Food and Drug Administration. 2011. *Guidance for Industry Process Validation: General Principles and Practices*. http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070336.pdf [15 November 2012].

Food and Drug Administration/ Center for Drug Evaluation and Research. 1994. *Reviewer Guidance: Validation of Chromatographic Methods*.
http://www.fda.gov/downloads/Drugs/.../Guidances/UCM134409.pdf [11 October 2012].

Formica, J. & Regelson, W. 1995. Review of the biology of quercetin and related bioflavonoids. *Food and chemical toxicology,* 33**:**1061-1080.

Fotsis, T., Pepper, M. S., Aktas, E., Breit, S., Rasku, S., Adlercreutz, H., Wähälä, K., Montesano, R. & Schweigerer, L. 1997. Flavonoids, dietary-derived inhibitors of cell proliferation and in vitro angiogenesis. *Cancer Research,* 57**:**2916-2921.

Galati, G. & O'Brien, P. J. 2004. Potential toxicity of flavonoids and other dietary phenolics: significance for their chemopreventive and anticancer properties. *Free radical biology and medicine,* 37**:**287-303.

García-Campaña, A. M., Bosque-Sendra, J. M., Rodríguez, L. C. & López, E. A. 2000. A framework for in house accuracy validation of analytical procedures. *Biomedical Chromatography,* 14**:**27-29.

Gaspar, J., Rodrigues, A., Laires, A., Silva, F., Costa, S., Monteiro, M., Monteiro, C. & Rueff, J. 1994. On the mechanisms of genotoxicity and metabolism of quercetin. *Mutagenesis,* 9**:**445-449.

Gaziano, J. M., Glynn, R. J., Christen, W. G., Kurth, T., Belanger, C., MacFadyen, J., Bubes, V., Manson, J. A. E., Sesso, H. D. & Buring, J. E. 2009. Vitamins E and C in the Prevention of Prostate and Total Cancer in Men. *JAMA: the journal of the American Medical Association,* 301**:**52-62.

Ghiselli, A., Serafini, M., Maiani, G., Azzini, E. & Ferro-Luzzi, A. 1995. A fluorescence-based method for measuring total plasma antioxidant capability. *Free radical biology and medicine,* 18**:**29-36.

Gilroy, J. J. & Dolan, J. W. 2004. Gradient performance checks. *LC GC Europe,* 17**:**566-572.
Glazer, A. N. 1990. Phycoerythrin fluorescence-based assay for reactive oxygen species. *Methods Enzymology,* 186**:**161-168.

Glazer, A. N. 1990. Phycoerythrin fluorescence-based assay for reactive oxygen species. *Methods Enzymology,* 186**:**161-168.

Gough, L. A. & Reynolds, T. M. 2000. Is Clinical Pathology Accreditation worth it? A survey of CPA-accredited laboratories. *British Journal of Clinical Governance,* 5**:**195-201.

Griendling, K. K. & FitzGerald, G. A. 2003. Oxidative Stress and Cardiovascular injury: Part II: Animal and Human studies. *Journal Of American Heart Association,* 108**:**2034-2038.

Grisanti, V. & Zachowski, E. J. 2002. Operational and performance qualification. *LC GC North America,* 20**:**356-363.

Guyton, K. & Kensler, T. 1993. Oxidative mechanisms in carcinogenesis. *British medical bulletin,* 49**:**523-544.
Guzik, T., Korbut, R. & Adamek-Guzik, T. 2003. Nitric oxide and superoxide in inflammation. *Journal of physiology and pharmacology,* 54**:**469-487.

Halliwell, B. 1994. Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? *Lancet,* 344**:**721-724.

Halliwell, B. & Aruoma, O. 1991. DNA Damage by Oxygen-Derived Species: Its Mechanism and Measurement in Mammalian Systems. *Federation of European Biochemical Societies* 281**:**9-19.

Halliwell, B. & Gutteridge, J. M. C. 1985. *Free radicals in biology and medicine,* New York: Oxford University Press.

Hanneken, A., Lin, F. F., Johnson, J. & Maher, P. 2006. Flavonoids protect human retinal pigment epithelial cells from oxidative-stress-induced death. *Investigative ophthalmology & visual science,* 47**:**3164-3177.

Hartmann, C., Smeyers-Verbeke, J., Massart, D. & McDowall, R. 1998. Validation of bioanalytical chromatographic methods. *J. Pharm. Biomed. Anal.,* 17**:**193-218.

Hertog, M. & Hollman, P. 1996. Potential health effects of the dietary flavonol quercetin. *European journal of clinical nutrition,* 50**:**63-71.

Hodnick, W. F., Kung, F. S., Roettger, W. J., Bohmont, C. W. & Pardini, R. S. 1986.

Inhibition of mitochondrial respiration and production of toxic oxygen radicals by flavonoids: A structure-activity study. *Biochemical pharmacology,* 35**:**2345-2357.

Hony, B. M. & Butler, J. 1984. The repair of oxidized amino acids by antioxidants. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology,* 791**:**212-218.

Huang, D., Ou, B., Hampsch-Woodill, M., Flanagan, J. A. & Deemer, E. K. 2002a. Development and validation of oxygen radical absorbance capacity assay for lipophilic antioxidants using randomly methylated β-cyclodextrin as the solubility enhancer. *Journal of Agricultural and Food Chemistry,* 50**:**1815-1821.

Huang, D., Ou, B., Hampsch-Woodill, M., Flanagan, J. A. & Prior, R. L. 2002b. High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. *Journal of Agricultural and Food Chemistry,* 50**:**4437-4444.

Huang, D., Ou, B. & Ronald, L. 2005. The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry,* 53**:**1841-1856.

Huber, L. 2007. *Validation of Analytical Methods*. http://www.chem.agilent.com/Library/primers/Public/5990-5140EN.pdf [10 October 2012].

Huber, L. 2009. *Analytical Instrument Qualification and System Validation*. http://www.chem.agilent.com/Library/primers/Public/5990-3288EN.pdf [11 November 2012].

Huber, L. n.d. *Validation of Analytical Methods and Procedures*. http://www.labcompliance.com/tutorial/methods/default.aspx?sm=d_d [10 October 2012].

International Conference on Harmonisation. 1996. *Guidance for Industry Q2B Validation of Analytical Procedures: Methodology*. http://www.fda.gov/downloads/Regulator%20yInformation/Guidances/UCM128049.pdf [15 September 2012].

International Conference on Harmonisation. 2000. *Good Manufacturing Practice Guide for Active Pharmaceutical Ingredients: Draft ICH Consensus Guideline*. http://dbase.aboutchem.com/ich/pdfdoc/q7estep2.pdf [25 August 2012].

International Conference on Harmonisation. 2005. *Validation of Analytical Procedures: Text and Methodology Q2(R1) Part I and II*. http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf [11 August 2012].

International Organization for Standardization/International Electrotechnical Commission. 2005. *ISO/IEC 17025: 2005, General requirements for the competence of testing and calibration laboratories* http://bbs.labsky.com/uploads/2005-8/2005-08-24_205709.pdf [11 October 2012].

Jenke, D. R. 1996. Chromatographic method validation: a review of current practices and procedures. I. General concepts and guidelines. *Journal of liquid chromatography & related technologies,* 19**:**719-736.

Jenkins, R. 1988. Free radical chemistry. Relationship to exercise. *Sports medicine (Auckland, NZ),* 5**:**156.

Ji, L. L. Antioxidants and oxidative stress in exercise. 1999. Royal Society of Medicine, 283-292.

Karadag, A., Ozcelik, B. & Saner, S. 2009. Review of Methods to Determine Antioxidant Capacities. *Food Analytical Methods,* 2**:**41-60.

Karlson, E. W., Shadick, N. A., Cook, N. R., Buring, J. E. & Lee, I. M. 2008. Vitamin E in the primary prevention of rheumatoid arthritis: the Women's Health Study. *Arthritis Care & Research,* 59**:**1589-1595.

Katiyar, S. K. 2003. Skin photoprotection by green tea: antioxidant and immunomodulatory effects. *Current Drug Targets-Immune, Endocrine & Metabolic Disorders,* 3**:**234-242.

Kenny, D. 2001. ISO and CEN documents on quality in medical laboratories. *Clinica chimica acta; international journal of clinical chemistry,* 309**:**121.

Kesavulu, M., Rao, B. K., Giri, R., Vijaya, J., Subramanyam, G. & Apparao, C. 2001. Lipid peroxidation and antioxidant enzyme status in Type 2 diabetics with coronary heart disease. *Diabetes research and clinical practice,* 53**:**33-39.

Kimura, H., Yamada, Y., Morita, Y., Ikeda, H. & Matsuo, T. 1992. Dietary ascorbic acid depresses plasma and low density lipoprotein lipid peroxidation in genetically scorbutic rats. *The Journal of nutrition,* 122**:**1904-1909.

Kohen, R. & Nyska, A. 2002. Invited review: Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. *Toxicologic pathology,* 30**:**620-650.

Konig, D., Wagner, K., Elmadfa, I. & Berg, A. 2001. Exercise and oxidative stress: significance of antioxidants with reference to inflammatory, muscular, and systemic stress. *Exercise immunology review,* 7**:**108.

Lee, I. M., Cook, N. R., Gaziano, J. M., Gordon, D., Ridker, P. M., Manson, J. A. E., Hennekens, C. H. & Buring, J. E. 2005. Vitamin E in the primary prevention of cardiovascular disease and cancer. *JAMA: the journal of the American Medical Association,* 294**:**56-65.

Levine, R. L., Wehr, N., Williams, J. A., Stadtman, E. R. & Shacter, E. 2000. Determination of carbonyl groups in oxidized proteins. *Methods in Molecular Biology* 99**:**15-24.

Li, Y. & Schellhorn, H. E. 2007. New developments and novel therapeutic perspectives for vitamin C. *The Journal of nutrition,* 137**:**2171-2184.

Lin, J. & Weng, M. S. 2006. Flavonoids as nutraceuticals. In: Grotewold, E. (eds.) *The science of flavonoids.* New York: Springer: 213-238.

Liu, R. H. 2004. Potential synergy of phytochemicals in cancer prevention: mechanism of action. *The Journal of nutrition,* 134**:**3479-3485.

Liu, T. Z., Chin, N., Kiser, M. D. & Bigler, W. N. 1982. Specific spectrophotometry of ascorbic acid in serum or plasma by use of ascorbate oxidase. *Clinical Chemistry,* 28**:**2225-2228.

Llesuy, S., Evelson, P., Campos, A. & Lissi, E. 2001. Methodologies for evaluation of total antioxidant activities in complex mixtures. A critical review. *Biological research,* 34**:**51-73.

Lussignoli, S., Fraccaroli, M., Andrioli, G., Brocco, G. & Bellavite, P. 1999. A microplate-based colorimetric assay of the total peroxyl radical trapping capability of human plasma. *Analytical Biochemistry,* 269**:**38-44.

Lutsenko, E. A., Cárcamo, J. M. & Golde, D. W. 2002. Vitamin C prevents DNA mutation induced by oxidative stress. *Journal of Biological Chemistry,* 277**:**16895-16899.

Machlin, L. J. 1995. Critical assessment of the epidemiological data concerning the impact of antioxidant nutrients on cancer and cardiovascular disease. *Critical Reviews in Food Science & Nutrition,* 35**:**41-49.

Madamanchi, N. R., Vendrov, A. & Runge, M. S. 2005. Oxidative stress and vascular disease. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 25**:**29-35.

Marnewick, J. L., Rautenbach, F., Venter, I., Neethling, H., Blackhurst, D. M., Wolmarans, P. & Macharia, M. 2011. Effects of rooibos (*Aspalathus linearis*) on oxidative stress and biochemical parameters in adults at risk for cardiovascular disease. *Journal of ethnopharmacology,* 133**:**46-52.

Massart, D. L., Vandeginste, B. G. M. & Buydens, L. 1997. *Handbook of chemometrics and qualimetrics,* Elsevier Science Ltd.

Merck Millipore. 2012. *CertiPUR® reference materials for ICP and AAS*. http://www.merckmillipore.co.za/chemicals/certipur-reference-materials-for-icp-and-aas/c_awisHfETb4UAAAE4KQBGnXUv [10 October 2012].

Metodiewaa, D., Jaiswal, A. K., Cenas, N., Dickancaité, E. & Segura-Aguilar, J. 1999. Quercetin may act as a cytotoxic prooxidant after its metabolic activation to semiquinone and quinoidal product. *Free radical biology and medicine,* 26**:**107-116.

Mgekn, I., Quinn, R. & Tabet, N. 2008. Vitamin E for Alzheimer's disease and mild cognitive impairment. *Cochrane Database Syst Rev*, 3. http://onlinelibrary.wiley.com/doi/10.1002/14651858.CD002854.pub2/abstract [Accessed 15 January 2013].

Middleton Jr, E. 1998. Effect of plant flavonoids on immune and inflammatory cell function. *Advances in experimental medicine and biology,* 439**:**175.

Miller, A. L. 1996. Antioxidant flavonoids: structure, function and clinical usage. *Alternative Medicine Review,* 1**:**126-127.

Miller III, E. R., Pastor-Barriuso, R., Dalal, D., Riemersma, R. A., Appel, L. J. & Guallar, E. 2005. Meta-analysis: high-dosage vitamin E supplementation may increase all-cause mortality. *Annals of Internal Medicine,* 142**:**37-46.

Mitic, S., Kostic, D., Naskovic-okic, D. & Mitic, M. N. 2011. Rapid and Reliable HPLC Method for the Determination of Vitamin C in Pharmaceutical Samples. *Tropical Journal of Pharmaceutical Research,* 10:105-111.

Morand, C., Dubray, C., Milenkovic, D., Lioger, D., Martin, J. F., Scalbert, A. & Mazur, A. 2011. Hesperidin contributes to the vascular protective effects of orange juice: a randomized crossover study in healthy volunteers. *The American journal of clinical nutrition,* 93**:**73-80.

Moure, A., Cruz, J. M., Franco, D., Domínguez, J. M., Sineiro, J., Domínguez, H., José Núñez, M. & Parajó, J. C. 2001. Natural antioxidants from residual sources. *Food Chemistry,* 72**:**145-171.

Mueller, C. F. H., Laude, K., McNally, J. S. & Harrison, D. G. 2005. Redox mechanisms in blood vessels. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 25**:**274-277.

Naidu, K. A., Karl, R. C. & Coppola, D. 2003. Antiproliferative and proapoptotic effect of ascorbyl stearate in human pancreatic cancer cells: association with decreased expression of insulin-like growth factor 1 receptor. *Digestive diseases and sciences,* 48**:**230-237.

Naidu, K. A., Liu Tang, J., Akhilender Naidu, K., Prockop, L. D., Nicosia, S. V. & Coppola, D. 2001. Antiproliferative and apoptotic effect of ascorbyl stearate in human glioblastoma multiforme cells: modulation of insulin-like growth factor-I receptor (IGF-IR) expression. *Journal of neuro-oncology,* 54**:**15-22.

Nguyen, L. A., He, H. & Pham-Huy, C. 2006. Chiral drugs. An overview. *International Journal of Biomedical Science,* 2**:**85-100.

Nigam, S. & Schewe, T. 2000. Phospholipase A2s and lipid peroxidation. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids,* 1488**:**167-181.

Noda, N. & Wakasugi, H. 2001. Cancer and oxidative stress. *Japan Medical Association Journal,* 44**:**535-539.

Novakova, L., Solich, P. & Solichova, D. 2008. HPLC methods for simultaneous determination of ascorbic and dehydroascorbic acids. *TrAC Trends in Analytical Chemistry,* 27**:**942-958.

Nowatzke, W. & Woolf, E. 2007. Best practices during bioanalytical method validation for the characterization of assay reagents and the evaluation of analyte stability in assay standards, quality controls, and study samples. *The AAPS journal,* 9**:**117-122.

Odriozola-Serrano, I., Hernández-Jover, T. & Martín-Belloso, O. 2007. Comparative evaluation of UV-HPLC methods and reducing agents to determine vitamin C in fruits. *Food Chemistry,* 105**:**1151-1158.

Ohno, H., Yahata, T., Sato, Y., Yamamura, K. & Taniguchi, N. 1988. Physical training and fasting erythrocyte activities of free radical scavenging enzyme systems in sedentary men. *European journal of applied physiology and occupational physiology,* 57**:**173-176.

Ou, B., Hampsch-Woodill, M. & Prior, R. L. 2001. Development and Validation of an Improved Oxygen Radical Absorbance Capacity Assay using Fluorescein as the Fluorescent Probe. *Journal of Agricultural and Food Chemistry,* 49**:**4619-4626.

Packer, L. 1991. Protective role of vitamin E in biological systems. *The American journal of clinical nutrition,* 53**:**1050-1055.

Padayatty, S. J., Katz, A., Wang, Y., Eck, P., Kwon, O., Lee, J. H., Chen, S., Corpe, C., Dutta, A. & Dutta, S. K. 2003. Vitamin C as an antioxidant: evaluation of its role in disease prevention. *Journal of the American College of Nutrition,* 22**:**18-35.

Padayatty, S. J., Riordan, H. D., Hewitt, S. M., Katz, A., Hoffer, L. J. & Levine, M. 2006. Intravenously administered vitamin C as cancer therapy: three cases. *Canadian Medical Association Journal,* 174**:**937-942.

Pham-Huy, L. A., He, H. & Pham-Huy, C. 2008. Free radicals, antioxidants in disease and health. *International Journal of Biomedical Sciences,* 4**:**89-96.

Pignatelli, P., Pulcinelli, F. M., Lenti, L., Gazzaniga, P. P. & Violi, F. 1998. Hydrogen peroxide is involved in collagen-induced platelet activation. *Blood,* 91**:**484-490.

Powers, S. K., Ji, L. L. & Leeuwenburgh, C. 1999. Exercise training-induced alterations in skeletal muscle antioxidant capacity: a brief review. *Medicine & Science in Sports & Exercise,* 31**:**987.

Prior, L., Hoang, H., Gu, L., Wu, X., Bacchiocca, M., Howard, L., Hampsch-Woodill, M., Huang, D., Ou, B. & Jacob, R. 2003. Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical

absorbance capacity (ORACFL)) of plasma and other biological and food samples. *Journal of Agricultural and Food Chemistry,* 51**:**3273-3279.

Prior, L., Wu, X. & Schaich, K. 2005. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry,* 53**:**4290-4302.

Prior, R. L. & Cao, G. 1999. In vivo total antioxidant capacity: comparison of different analytical methods1. *Free radical biology and medicine,* 27**:**1173-1181.

Rahman, I. & Adcock, I. M. 2006. Oxidative Stress and Redox Regulation of Lung Inflammation in Chronic Obstructive Pulmonary Disease. *European Respiratory Journal,* 28**:**219-242.

Ratnam, D. V., Ankola, D., Bhardwaj, V., Sahana, D. & Kumar, M. 2006. Role of antioxidants in prophylaxis and therapy: a pharmaceutical perspective. *Journal of controlled release,* 113**:**189-207.

Rebouche, C. J. 1991. Ascorbic acid and carnitine biosynthesis. *The American journal of clinical nutrition,* 54**:**1147-1152.

Reiter, R. J. 1995. Oxidative processes and antioxidative defense mechanisms in the aging brain. *The FASEB Journal,* 9**:**526-533.

Rezai-Zadeh, K., Ehrhart, J., Bai, Y., Sanberg, P. R., Bickford, P., Tan, J. & Shytle, R. D. 2008. Apigenin and luteolin modulate microglial activation via inhibition of STAT1-induced CD40 expression. *J Neuroinflammation,* 5**:**41.

Rice-Evans, C. & Burdon, R. 1993. Free radical-lipid interactions and their pathological consequences. *Progress in lipid research,* 32**:**71.

Rice-Evans, C., Diplock, A. T. & Symons, M. C. R. 1991. *Techniques in free radical research,* Elsevier Science Ltd.

Robaszkiewicz, A., Balcerczyk, A. & Bartosz, G. 2007. Antioxidative and prooxidative effects of quercetin on A549 cells. *Cell biology international,* 31**:**1245-1250.

Rona, C., Vailati, F. & Berardesca, E. 2004. The cosmetic treatment of wrinkles. *Journal of cosmetic dermatology,* 3**:**26-34.

Roper, P., Walker, R., Bedson, P., Lawn, R., Barwick, V. J., Burke, S 2001. *Applications of Reference Materials in Analytical Chemistry,* Cambridge, UK: Royal Society of Chemistry.

Sai, K., Umemura, T., Takagi, A., Hasegawa, R. & Kurokawa, Y. 1992. The protective role of glutathione, cysteine and vitamin C against oxidative DNA damage induced in rat kidney by potassium bromate. *Cancer Science,* 83**:**45-51.

Sánchez-Mata, M. C., Cámara-Hurtado, M., Díez-Marqués, C. & Torija-Isasa, M. E. 2000. Comparison of high-performance liquid chromatography and spectrofluorimetry for vitamin C analysis of green beans (*Phaseolus vulgaris*). *European Food Research and Technology,* 210**:**220-225.

Sandhar, H., Kumar, B., Prasher, S., Tiwari, P., Salhan, M. & Sharma, P. 2011. A review of phytochemistry and pharmacology of flavonoids. *Internationale Pharmaceutica Sciencia,* 1**:**25-41.

Schapira, A. H. V. 2008. Mitochondria in the aetiology and pathogenesis of Parkinson's disease. *The Lancet Neurology,* 7**:**97-109.

Sen, C. K. 1995. Oxidants and antioxidants in exercise. *Journal of Applied Physiology,* 79**:**675-686.

Serafini, M., Ghiselli, A. & Ferro-Luzzi, A. 1996. In vivo antioxidant effect of green and black tea in man. *European Journal of Clinical Nutrition,* 50**:**28.

Shabir, G. A. 2003. Validation of high-performance liquid chromatography methods for pharmaceutical analysis: Understanding the differences and similarities between validation requirements of the US Food and Drug Administration, the US Pharmacopeia and the International Conference on Harmonisation. *Journal of Chromatography A,* 987**:**57-66.

Shah, V. P. 2007. The history of bioanalytical method validation and regulation: evolution of a guidance document on bioanalytical methods validation. *The AAPS journal,* 9**:**43-47.

Sies, H. 1997. Oxidative stress: oxidants and antioxidants. *Experimental physiology,* 82**:**291-295.

Sies, H. & Stahl, W. 1995. Vitamins E and C, beta-carotene, and other carotenoids as antioxidants. *The American journal of clinical nutrition,* 62**:**1315-1321.

Sigma Aldrich. 2012. *Quality Management Systems*. http://www.sigmaaldrich.com/customer-service/quality-systems.html [10 October 2012].

Sitte, N. 2003. *Oxidative damage to proteins,* The Netherlands: Kluwer Academic Publishers.

Songara, R. & Prakashkumar, A. 2011. Overview of analytical method validation in pharmaceutical industries. *International Journal of PharmaInforma, Journal of Analytical Chemistry*, 1. http://www.slideshare.net/prarkash_dra/overview-of-analytical-method-validation [Accessed 7 October 2012].

South African National Accreditation System. 2008a. *Why Accreditation*. http://www.sanas.co.za/accreditation_why.php [12 December 2012].

South African National Accreditation System. 2008b. *History of the South African National Accreditation System*. http://www.sanas.co.za/history.php [12 December 2012].

Stadtman, E. R. 1992. Protein oxidation and aging. *Science,* 257**:**1220-1224.

Stadtman, E. R. & Berlett, B. S. 1998. Reactive oxygen-mediated protein oxidation in aging and disease. *Drug metabolism reviews,* 30**:**225-243.

Sweetman, S., Strain, J. & McKelvey-Martin, V. 1997. Effect of antioxidant vitamin supplementation on DNA damage and repair in human lymphoblastoid cells. *Nutrition and Cancer,* 27**:**122-130.

Talamona, A. 2005. *Laboratory Chromatography Guide,* Switzerland: Büchi Labortechnik AG.

Taverniers, I., De Loose, M. & Van Bockstaele, E. 2004. Trends in quality in the analytical laboratory. II. Analytical method validation and quality assurance. *TrAC, Trends in Analytical Chemistry,* 23**:**535-552.

Taylor, J. K. & Oppermann, H. V. 1986. *Handbook for the Quality Assurance of Meteorological measurements (Final Report),* Gaithersburg, Md: U.S National Bureau of Standards.

Temple, N. J. 2000. Antioxidants and disease: more questions than answers. *Nutrition Research,* 20**:**449-459.

Thompson, M., Ellison, S. L. R. & Wood, R. 2002. Harmonized guidelines for single-laboratory validation of methods of analysis. *Pure and Applied Chemistry* 74**:**835-855.

Tijburg, L., Mattern, T., Folts, J., Weisgerber, U. & Katan, M. 1997. Tea flavonoids and cardiovascular diseases: a review. *Critical Reviews in Food Science & Nutrition,* 37**:**771-785.

Uličná, O., Greksák, M., Vančová, O., Zlatoš, L., Galbavý, S., Božek, P. & Nakano, M. 2003. Hepatoprotective effect of rooibos tea (*Aspalathus linearis*) on CCl4-induced liver damage in rats. *Physiological Research,* 52**:**461-466.

United Nations Office on Drugs and Crime. 2009. *Guidance for the Validation of Analytical Methodology and Calibration of Equipment used for Testing of Illicit Drugs in Seized Materials and Biological Specimens*.
http://www.unodc.org/documents/scientific/validation_E.pdf [19 January 2013].

Valko, M., Izakovic, M., Mazur, M., Rhodes, C. J. & Telser, J. 2004. Role of oxygen radicals in DNA damage and cancer incidence. *Molecular and cellular biochemistry,* 266**:**37-56.

Valko, M., Rhodes, C. J., Moncol, J., Izakovic, M. & Mazur, M. 2006. Free Radicals, Metals and Antioxidants in Oxidative Stress-Induced Cancer. *Chemico-Biological Interactions,* 160**:**1-40.

Van de Velde, F., Pirovani, M. E., Cámara, M. S., Güemes, D. R. & Bernardi, C. M. H. 2011. Optimization and Validation of a UV–HPLC Method for Vitamin C Determination in Strawberries (*Fragaria ananassa Duch.*), Using Experimental Designs. *Food Analytical Methods***:**1-8.

Viswanathan, C., Bansal, S., Booth, B., DeStefano, A. J., Rose, M. J., Sailstad, J., Shah, V. P., Skelly, J. P., Swann, P. G. & Weiner, R. 2007. Quantitative bioanalytical methods validation and implementation: best practices for chromatographic and ligand binding assays. *Pharmaceutical research,* 24**:**1962-1973.

Vlachos, N., Michail, C. & Sotiropoulou, D. 2002. Is ISO/IEC 17025 accreditation a benefit or hindrance to testing laboratories? The Greek experience. *Journal of Food Composition and Analysis,* 15**:**749-757.

Vovk, T., Bogataj, M., Roškar, R., Kmetec, V. & Mrhar, A. 2005. Determination of main low molecular weight antioxidants in urinary bladder wall using HPLC with electrochemical detector. *International journal of pharmaceutics,* 291**:**161-169.

Westgard, J. O. 2008. *Basic Method Validation,* Madison: Westgard QC, Inc.

Willcox, J. K., Ash, S. L. & Catignani, G. L. 2004. Antioxidants and prevention of chronic disease. *Critical Reviews in Food Science and Nutrition,* 44**:**275-295.

Winter, W. 2006. Analytical Instrument Qualification. *BioProcess International*.

Wood, R. 1999. How to validate analytical methods. *TrAC Trends in Analytical Chemistry,* 18**:**624-632.

Wu, X., Beecher, G. R., Holden, J. M., Haytowitz, D. B., Gebhardt, S. E. & Ronald, L. 2004. Lipophilic and hydrophilic antioxidant capacities of common foods in the United States. *Journal of Agricultural and Food Chemistry* 52**:**4026-4037.

Yagi, K. 1982. Assay for serum lipid peroxide level and its clinical significance. *Lipid peroxides in biology and medicine,* 223**:**242.

Yagi, K. 1992. Lipid peroxides and exercise. *Medicine and Sport Science,* 37**:**40-40.

Yao, L. H., Jiang, Y., Shi, J., Tomas-Barberan, F., Datta, N., Singanusong, R. & Chen, S. 2004. Flavonoids in food and their health benefits. *Plant Foods for Human Nutrition (Formerly Qualitas Plantarum),* 59**:**113-122.

Yeom, C. H., Jung, G. C. & Song, K. J. 2007. Changes of terminal cancer patients' health-related quality of life after high dose vitamin C administration. *Journal of Korean medical science,* 22**:**7.

Yuan, Y.V., Kitts, D.D. 1997. Endogenous Antioxidants: Role of antioxidant enzymes in Biological Systems. *Natural Antioxidants: Chemistry, Health Effects, and Applications*. USA: AOCS Press.

Zafra-Stone, S., Yasmin, T., Bagchi, M., Chatterjee, A., Vinson, J. A. & Bagchi, D. 2007. Berry anthocyanins as novel antioxidants in human health and disease prevention. *Molecular nutrition & food research,* 51**:**675-683.

CHAPTER THREE: RESEARCH ARTICLE ONE

Validation of a manual oxygen radical absorbance capacity assay for the measurement of hydrophilic antioxidant capacity in food and beverage products

Olivia L. Parbhunath, Fanie Rautenbach, Glenda Davison and Jeanine L. Marnewick

Oxidative Stress Research Centre, Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology, P.O. Box 1906, 7538, South Africa.

Abstract

The oxygen radical absorbance capacity-fluorescein ($ORAC_{FL}$) assay, which remains the widely accepted method for total antioxidant capacity measurement in the food and beverage industry, has previously been adapted to a manual liquid handling system. In keeping with national and international regulatory guidelines, namely International Organization for Standardization/ International Electrotechnical Commission (ISO/IEC 17025), validation and standardization of the manually performed $ORAC_{FL}$ assay is essential.

This study aimed to optimize and validate the manually performed $ORAC_{FL}$ assay in accordance to ISO/IEC 17025 method performance parameters. As calculated for trolox, the method precision and accuracy, respectively was <6.0, expressed as coefficient of variation (COV) and 99.8%, expressed as percent recovery. The limits of detection and quantification were equivalent to 1.35 and 4.10 µM trolox standard solutions. The method performance parameters were applied to 12 samples (commercial fruit juices and teas, fresh fruit and vegetables and food extract products). The results demonstrate good linearity, accuracy, precision, specificity and robustness at varying levels of the trolox calibration curve of the $ORAC_{FL}$ assay when performed manually.

A noteworthy finding were the significantly ($p<0.05$) higher antioxidant yield recovered with ethanol/water and methanol/water extraction solvents compared to acetone/water/acetic acid extraction solvent for all samples tested. Another finding was the improved precision (COV=2.87) and reduction in outliers upon preheating (37 ºC) the AAPH buffer and micro-plate for 20 min prior to analysis and exclusion of some of the external plate wells. This work proves both the reliability and applicability of the manually performed $ORAC_{FL}$ assay to

various food and beverage samples and its compliance to ISO/IEC 17025 method validation requirements.

Keywords*: Validation, ORAC, antioxidant, method performance parameters, manual handling system*

To be submitted to the Journal of Agricultural and Food Chemistry. The referencing format of this chapter will reflect the format required for this journal.

3.1 Introduction

The putative health benefits associated with the consumption of antioxidants is rapidly assuming an appreciable niche in the food and beverage global market. This association is derived from overwhelming evidence verifying a reduced incidence of several pathological conditions related to antioxidant intake.[1-6]

Subsequently, the rise in food and beverage products displaying antioxidant claims has inundated the food industry, resulting in an apparent escalation in antioxidant measuring assays.[7] Moreover, various international standards emphasize the need for a sound accredited quality control system in laboratories to measure the quality and reliability of such food and beverage products.[8-12] In addition, with the inception of a newly revised labelling legislature, the South African government is making it compulsory for food and beverage manufacturers to verify antioxidant claims on products using South African National Accreditation System (SANAS) accredited assays.[13] Several assays have been developed and reviewed for measuring antioxidant capacity.[14] To date, the ORAC assay remains one of the most extensively utilized methods for the measurement of total antioxidant capacity in food, beverages and biological samples.[15]

The ORAC assay developed by Cao *et al,* based on the original work of Glazer *et al*, was first performed on an LS-5 fluorescence spectrophotometer using B-phycoerythrin as the oxidizable substrate.[16, 17] However, for a large amount of samples, the impracticality afforded by the labour intensity and long duration of the assay, prompted the adaptation of the ORAC assay to the COBAS Fara II centrifugal analyzer.[18] Later on, using the COBAS Fara II, fluorescein replaced B-phycoerythrin.[19] The COBAS Fara II was then replaced by a high through-put instrument set consisting of an automated multichannel pipetting system.[20] Due to the high cost and limited availability of an automated system, the need to adapt the $ORAC_{FL}$ assay to a manual handling system was apparent. In 2004, Davalos and co-workers adapted the ORAC assay to a manual handling system.[21]

Due to the uniqueness of the $ORAC_{FL}$ assay in simulating *in vivo* conditions such as pH 7.4, a temperature of 37 ^{o}C, and the presence of a peroxyl radical (AAPH), it not only extends its applicability to fruit and vegetables,[22, 23] fruit juices,[24] tea and tea extracts,[25-27] several types of herbs,[28] but also to biological samples.[29] Antioxidants have also spiked substantial interest in the cosmetics industry where some companies are using the $ORAC_{FL}$ assay to measure antioxidant capacity in their products.[30] Both “rooibos” (*Aspalathus linearis*) as well as “honeybush” (*Cyclopia species*) teas, indigenous to South Africa have rapidly become

sought after beverages globally, owing to their purported protective role against “free radicals”.[31, 32] As a result, the $ORAC_{FL}$ assay is increasingly being utilized for the analysis of indigenous teas.

The automated $ORAC_{FL}$ assay has been validated against a few method performance parameters, however validation of the manually performed $ORAC_{FL}$ assay has been minimally addressed. In 2001, the assay was validated against accuracy, precision, linearity and ruggedness using the COBAS Fara II.[19] Using an automated multichannel pipetting system, the assay was validated through focusing on method performance parameters which included accuracy, linearity, precision and ruggedness.[20] Davalos and co-workers subsequently validated the manually performed $ORAC_{FL}$ assay for specific method performance parameters [accuracy, precision, limit of detection (LOD) and limit of quantification (LOQ)] using trolox standards.[21] Later, Price and co-workers evaluated the manual performance of the $ORAC_{FL}$ assay using a panel of phylogenetically unrelated algae. However specific validation method performance parameters such as accuracy, ruggedness, precision, specificity were not addressed.[33] Prior and co-workers highlighted the importance of standardizing antioxidant capacity assays, and the inclusion of specific method performance characteristics some of which include accuracy, precision, range, ruggedness and specificity in validating these assays.[34] As there is no official standardized protocol for the ORAC assay, methodologies may differ depending on the use of manual or automated instrumentation.

The aim of this study was to validate the manually performed $ORAC_{FL}$ assay for measurement of hydrophilic antioxidants in food and beverage samples. This will be performed against recommended method performance parameters in accordance with ISO/IEC 17025 validation requirements.

3.2 Methods and materials

3.2.1 Chemicals and apparatus

The chemicals 2′2-azobis (2-amidino propane) dihydrochloride (AAPH), citric acid, fluorescein sodium salt (FL), glucose, sodium chloride (NaCl), potassium citrate, sucrose and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma-Aldrich (Johannesburg, South Africa). Acetone, ethanol, glacial acetic acid, methanol and di-sodium hydrogen orthophosphate dehydrate (Na_2HPO_4) were purchased from Merck (Johannesburg, South Africa). All H-$ORAC_{FL}$ analyses were performed on a Fluoroskan plate reader (Thermo Fischer Scientific, Waltham, Mass., U.S.A). Table 1 summarizes plate reader settings for sample analysis. Black flat bottom 96-well micro-plates were obtained from Greiner Bio-one (Germany).

Table 1. Typical instrument settings (Fluoroskan Ascent 2.5)

No. of cycles:	25
Measurement interval	5 min
Integration time:	20 ms
Filter wavelengths:	Excitation.filter: 485 nm Emission.filter: 538 nm
Temperature:	37^{o} C
Shaking duration:	10 s
Shaking diameter	1 mm
Shaking speed	1200 rpm

3.2.2. Sample types

Sample used in this study consisted of several commercial fruit juices (apple juice, dragon-fruit glacial vitamin water, mixed berry juice and tropical fruit juice), tea (organic honey-bush), fresh fruit and vegetables (apples, incan berries and tomatoes) and several food extracts (camu powder, chia seeds and cocoa paste) and were purchased from retail outlets within the Cape Town metropolitan area (South Africa). These samples were selected to assess the effect of different matrices on the method performance parameters of the assay and the optimal extraction conditions for different samples used. Depending on the method performance parameters investigated, different samples were used for different parameters tested. Beverage samples were aliquoted from fresh into 1.5 mL vials and stored at -80^{o} C. All food samples were extracted and stored until analyzed as described below.

3.2.3. Hydrophilic antioxidant extraction of food and food extracts

Approximately one gram of coco paste, incan berries, 500 mg of chia seeds and 100 mg of camu powder was accurately weighed and the mass noted and 25 mL of three separate solvents: 60% ethanol, 80% methanol and acetone/distilled water/acetic acid, (70:29.5:0.5, v/v/v) (AWA)[35] was added to samples and subsequently homogenized in a Polytron homogenizer (9000 rpm) for 5 min. After the homogenization step, the sample extraction mixtures were incubated at room temperature (RT) for 30 min, centrifuged (4000 rpm, 5 min) and the resulting supernatants removed, aliquoted and stored at -80 ºC until time of analysis. Prior to analysis, the samples were appropriately diluted and assayed in triplicate.

3.2.4. Optimization of hydrophilic antioxidant extraction

Hydrophilic antioxidants were extracted for all the above mentioned samples using 60% ethanol (n=7). After homogenization, the extraction mixtures were treated as follow for each sample: (a) two sample extraction mixtures were incubated at 60 ºC and 80 ºC, respectively for 30 min, centrifuged (4000 rpm, 5 min) and the resulting supernatants removed, aliquoted and stored at -80 ºC until time of analysis, (b) three sample extraction mixtures were centrifuged (4000 rpm, 5 min), the resulting supernatant transferred to a 50 mL screw cap tube, while the sample sediment was re-suspended with an additional 25 mL of 60% ethanol and incubated at 23 ºC, 60 ºC, 80 ºC, respectively for 30 min. Upon centrifugation (4000 rpm, 5 min), the resulting supernatant was combined with the supernatant from the first extraction, which was subsequently centrifuged (4000 rpm, 2 min), aliquoted and stored at -80 ºC until time of analysis, (c) the pH of the last two sample extraction mixtures were adjusted to pH 2.0 and 11.7, respectively and incubated at RT for 30 min and then centrifuged (4000 rpm, 5 min). The resulting supernatants were aliquoted and stored at -80 ºC until time of analysis. Prior to analysis the samples were appropriately diluted in phosphate buffer and assayed in triplicate.

3.2.5. Preparation of Quality Control (QC) samples

A 250 mL synthetic juice formulation comprising sucrose (19 g), citric acid (1 g) and sodium citrate (0.023g) was prepared. Subsequently, three different concentrations of trolox were added to provide final concentrations of 22 µM (QC1), 12.5 µM (QC2) and 6.25 µM (QC3).The QC samples were immediately aliquoted into 2 mL vials and stored at -40 ºC till the time of analysis.

3.2.6. Hydrophilic $ORAC_{FL}$ assay

The H-$ORAC_{FL}$ assay was performed on the Fluoroskan Ascent 2.5 plate reader (Thermo Fischer Scientific, Waltham, Mass., U.S.A) using the methodology applied by Wu and co-workers.[36] A standard stock solution of trolox (500 µM) was prepared in 75 mM phosphate buffer (pH 7.4) to provide calibration standards ranging from 5 to 25 µM. The fluorescein stock solution that was prepared in phosphate buffer, was diluted to a final concentration of 14 µM per well. The reaction commenced upon the incubation of 12 µL of diluted sample/sample extract in a black 96 well flat bottom plate with 138 µL of diluted fluorescein solution and 50 µL AAPH solution (150 mg dissolved in 6 mL of 75 mM phosphate buffer). A blank consisting of phosphate buffer, and five calibration standards were included in every run and all analyses were performed in triplicate. The decay in fluorescence was recorded every 5 min for 120 min and final H-$ORAC_{FL}$ values were calculated using the regression equation $y=ax^2+bx+c$ between trolox concentration and area under the curve (AUC).[37] The H-$ORAC_{FL}$ values were determined by comparing the sample AUC to that of the trolox standard and the results were expressed as µM trolox equivalents per gram or per mL (µM TE/g or ml) of sample/sample extract. One ORAC unit is assigned as being the net protection area provided by 1 µM trolox in final concentration.

3.2.7. Optimization of pre-validation components

3.2.7.1. Equipment and Analyzer qualification

All equipment utilized was serviced and calibrated by an accredited metrology laboratory (Cape Metrology Field, Cape Town, South Africa) prior to commencement of the study. Subsequently, the performance of all equipment and glassware were verified on a continuous basis to ensure functioning was optimal and in agreement with manufacturer specifications at all times of analysis. Verification procedures included cleaning, calibration and testing the performance of equipment with certified reference materials (CRMs). Verification forms (Appendix E) were created and all actions documented on a routine basis in terms of repeatability, intermediate precision, accuracy, possible deviations from acceptable criteria and any troubleshooting performed.

The installation, operation, and performance qualification of the Fluoroskan Ascent 2.5 fluorescent plate reader was performed at the laboratory site by AEC Amersham (Cape Town, South Africa).

3.2.7.2. Method Optimization

Differences in temperature have been reported in the exterior wells of the microplate, resulting in poor reproducibility of the assay.[38] This problem was evaluated by dispensing a blank and standards (10 µM trolox standard, 20 µM trolox standard), in all 96 wells of three micro-plates and assaying using the $ORAC_{FL}$ assay described above. Subsequently, the procedure was repeated as described by Prior and co-workers with a slight modification where a single trolox standard (10 µM) sample was pre-incubated with the fluorescein solution in the micro-plate for 20 min at 37 ^{o}C in the Fluoroskan plate reader, whilst 6 mL of phosphate buffer was warmed to 37 ^{o}C for 30 min in a water bath within close proximity of the plate reader.[35] After 20 min, the pre-warmed buffer was added to 150 mg accurately weighed AAPH and immediately pipetted into the wells of the pre-incubated sample micro-plate.

3.2.8. Validation parameters methodology

3.2.8.1. Robustness

The robustness of the H-$ORAC_{FL}$ was evaluated during the pre-validation stage. The preparation of analytical solutions (phosphate buffer, trolox stock solution and AAPH solution) were modified to produce small, deliberate changes and a 10 µM trolox standard was dispensed in the 96 well micro-plate and assayed as per the H-$ORAC_{FL}$ assay described above. Each tested robustness parameter was evaluated in triplicate. The phosphate buffer (75 mM, pH 7.4) was evaluated at varying pH levels (7.0, 7.2, 7.4, 7.6 and 7.8). Results were evaluated for significant differences. The stability of the trolox stock solution was evaluated by storing different trolox stock solution (500 µM) aliquots at RT, and at 4 ^{o}C for a period of 24 hr before being diluted (10 µM) and assayed. Results were compared to those obtained from analysis with fresh trolox stock solution and evaluated for significant differences. The thermal decomposition of AAPH was assessed by adding 50 mg AAPH to 2 mL phosphate buffer (75 mM, pH 7.4) and allowing it to stand for 30, 60, 90, 120 min at 37 ^{o}C. Subsequently the pre-incubated AAPH buffer solutions were assayed together with a freshly prepared AAPH buffer solution. Results were evaluated for significant differences.

3.2.8.2. Linearity and range

It is mandatory that calibration standards should encompass the entire analytical range and be evenly distributed across it.[39, 40] Generally a minimum of five to eight calibration points are required, however it is recommended to use fewer rather than more calibration levels and perform more replicates.[10, 41, 42]

Trolox calibration standards ranging 5 µM to 100 µM (5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µM) were diluted from 75 mM phosphate buffer (pH7.4). The trolox standards and a blank sample were all assayed in triplicate and the results obtained were used to evaluate and establish the linear range for the assay. To drive the reaction to completion within 2 hr, 600 mg of AAPH was added to 2 mL of phosphate buffer (75mM, pH7.4). The percentage relative standard deviation (%RSD) of the responses were plotted against the respective concentration levels, and the concentration range in which the %RSD is equal to or less than 2%, was used to establish the linear range.[43] Additionally, chia seeds, organic honeybush tea and apple juice samples were each appropriately diluted spanning four concentration levels within the expected linear working range. Their responses were evaluated for linearity with acceptable accuracy and precision by appropriate statistical methods such as least squares linear regression.

3.2.8.3. Selectivity/specificity

Glucose (166 µM) and NaCl (166 µM) solutions were prepared and added to 5 µM, 10 µM and 15 µM calibration standards. In addition, glucose and NaCl solutions were added to an appropriately diluted beverage (organic honeybush tea), hydrophilic extract (chia seeds), and QC samples (6.25 µM, 12.5 µM). All samples were diluted within the linear range of the trolox standard curve. All dilution factors were compensated for upon the addition of the glucose and NaCl solutions to the various samples. All samples were assayed both before and after the addition of the glucose and NaCl solutions and the results evaluated by appropriate statistical methods.

3.2.8.4. Accuracy and precision

The $ORAC_{FL}$ ability to measure the antioxidant capacity of a sample with acceptable accuracy and precision was determined by recovery studies using the standard addition and spiked placebo recovery methods.[44]

The standard addition recovery method was performed by the addition of varying concentrations (within the linear range) of trolox calibration standards (5 µM, 10 µM, 15 µM, 20 µM) to appropriately diluted beverage (organic honeybush tea) and food extract (chia seeds) samples (60% ethanol extracts at 60 ^{o}C) and a low concentration trolox sample (2.5 µM) which had already been analyzed prior to the trolox standard addition. The measured $ORAC_{FL}$ concentration was compared to the expected $ORAC_{FL}$ concentration of the trolox spiked samples and the percentage recovery was calculated.

The spiked recovery method is normally performed by spiking a formulation blank with the analyte of interest at varying concentrations of the expected working range. Hence, quality control samples of varying concentrations (6.25 µM, 12.5 µM, 22 µM) spanning the working range were run in triplicate over 3 days and net AUC of the samples were expressed as the percentage recovery. The results of the accuracy determinations were also evaluated for repeatability (intra-assay precision) and intermediate precision and the results expressed as the COV.[45]

3.2.8.5. Limit of detection and quantification

A series of blank samples (n=6) were assayed on three consecutive days in triplicate and the standard deviations determined. Limit of detection (LOD) and limit of quantification (LOQ) were determined from the standard deviation of response (y-intercept) of the blank samples and the slope (from the trolox calibration curve) of the regression equation using Equations 2.1 and 2.2.

$$LOD = [3.3 \times (\sigma)]/S \qquad \textbf{Equation 2.1.}$$

$$LOQ = [10.0 \times (\sigma)]/S \qquad \textbf{Equation 2.2}$$

where: σ: standard deviation of the response
S:the slope of the calibration curve

Subsequently, food and beverage samples (apple juice, chia seeds hydrophilic extract) and a QC sample (22.00 µM) were diluted to four concentration points at and around the established LOQ concentration level and evaluated for accuracy, precision and linearity.[11]

3.2.8.6. Sample stability

Trolox QC samples (6.25 µM and 12.5 µM) and several food and beverage samples (mixed berry juice, tropical fruit juice, dragon-fruit glacial vitamin water, tomatoe, apple and camu powder) were evaluated for long-term (24 hr stored at 4 ^{o}C) and short-term/bench top (6 hr at RT) stability, as well as freeze-thaw stability (3 cycles at -40 ^{o}C) to evaluate the extent of antioxidant deterioration, if any.[46] All samples were diluted within the linear standard concentration range and then assayed. Percentage recoveries were determined and compared to that of fresh samples spiked with fresh trolox standards (6.25 µM and 12.5 µM.

3.2.9. Statistical Analysis

The mean, standard deviation, and COV were determined for all data. The statistical Microsoft Excel® software package was used to analyze data. Analysis of Variance (ANOVA) was used to ascertain whether the means between sample/standard experimental groups differ significantly at a 95% confidence level ($p<0.05$ considered/ indicated significant differences). The Levene's Test was used to determine normality between sample/standard experimental groups. If data did not show a normal distribution, a logarithmic transformation was applied. Subsequently, if data did not demonstrate a normal distribution, the Kruskal-Wallis test was used. The Paired T-test was used to show differences between two sample/standard experimental groups.

3.3 Results

3.3.1. Robustness

Table 2 illustrates the evaluation of robustness of the $ORAC_{FL}$ assay with regard to pH, AAPH decomposition and trolox stability. The $ORAC_{FL}$ assay is known to perform optimally when the phosphate buffer is at pH 7.4.[47] In the current study, the $ORAC_{FL}$ results were constant when the buffer was at pH 7.2 to 7.6 [no significant ($p>0.05$) differences]. This indicates that the assay was robust over ± 0.2 variation in pH. This was further confirmed by a significant ($p<0.05$) increase and decrease of $ORAC_{FL}$ results at pH 7.0 and 7.8, respectively. Trolox stability data presented in Table 2 showed consistently similar net AUC values for freshly prepared trolox solution and one stored at 4 ^{o}C for 24 hrs. This confirmed that the trolox stock solution was stable for up to 24 hours under refrigerated conditions. Additionally, significantly ($p<0.05$) higher values were observed for the trolox solution stored at RT. Results of the AAPH decomposition study further confirmed that no thermal decomposition of AAPH occurred for up to 2 hrs, which was indicated by no significant ($p>0.05$) differences between net AUC values of 30 min testing intervals. The results of the robustness study demonstrated and confirmed that the $ORAC_{FL}$ assay remained robust, despite small changes in method parameters.

Table 2. Evaluation of robustness of the $ORAC_{FL}$ assay

	pH					AAPH decomposition					Trolox stability		
	7.0	**7.2**	**7.4**	**7.6**	**7.8**	**0 min**	**30 min**	**60 min**	**90 min**	**120 min**	**Fresh**	**RT**	**4°C**
Net AUC_1	46.9[a]	42.23[b]	43.9[b]	42.6[b]	39.13[c]	51.58[d]	52.16[d]	52.84[d]	51.34[d]	52.00[d]	45.71[e]	46.41[f]	44.17[e]
SD	1.39	0.25	0.95	1.14	0.78	1.61	1.83	2.43	2.79	1.93	1.22	1.09	0.95
%RSD	2.96	0.60	2.17	2.67	1.98	3.12	3.5	4.60	5.43	3.72	2.67	2.35	2.14

[1]The net AUC of a 10 µM trolox standard were calculated from readings that were made by the plate reader at five minute intervals. Same letter superscripts indicate no significant differences ($p>0.05$). Different letter superscripts indicate significant differences ($p<0.05$).

3.3.2. Optimization of hydrophilic antioxidant extraction

In selecting and optimizing an extraction solvent technique for the samples that were to be assayed, conditions such as solvent type and volume, extraction temperature, time and pH were considered and evaluated using four food samples (incan berries, chia seeds, camu powder, coco paste).[40] It was observed that significantly ($p<0.05$) higher hydrophilic antioxidant recoveries were obtained with ethanol/water extraction solvents at pH 7.4, compared to those obtained with AWA solvent for most sample conditions tested (Table 3). Additionally, high temperatures and increased total extraction solvent volume, relative to sample volume, generated good recoveries for coco paste and chia seeds. However at excessive temperatures (80 ^{o}C) antioxidant degradation may have occurred contributing to lower antioxidant yields as seen with incan berries and camu powder. Similarly, at extreme pH conditions (pH 2.0 and 11.7) antioxidant degradation was evident contributing to significantly ($p<0.05$) lower recoveries for some samples (cocoa paste and chia seeds) than those obtained at pH 7.4. Results obtained from the methanol extraction were significantly ($p<0.05$) lower for most samples (cocoa paste, incan berries and chia seeds) than those obtained with ethanol extraction, but significantly ($p<0.05$) higher for all samples than those obtained with AWA extraction.

Table 3. Optimization of hydrophilic antioxidant extraction

Samples	80% meth (µM TE/L)	AWA RT (µM TE/L)	60% ethanol (25 mL) RT (µM TE/L)$_1$	60% ethanol (50 mL) (RT) (µM TE/L)$_1$			60% ethanol (25 mL) (RT) (µM TE/L)	
			RT	60°C	80°C	RT	pH 2.0	pH 11.7
Coco paste	400.92 ± 13.29^{a}	344.08 ± 2.26^{b}	502.85 ± 20.49^{c}	707.57 ± 4.74^{d}	729.25 ± 15.22^{e}	589.36 ± 40.72^{f}	491.73 ± 12.94^{a}	431.89 ± 43.48^{a}
Incan berries	57.07 ± 0.55^{a}	50.93 ± 1.16^{b}	49.39 ± 0.26^{b}	86.16 ± 3.53^{c}	78.53 ± 0.84^{d}	153.03 ± 3.77^{e}	56.96 ± 0.84^{a}	53.10 ± 1.25^{f}
Chia seeds	97.92 ± 3.31^{a}	94.29 ± 2.99^{a}	128.74 ± 1.23^{b}	197.46 ± 6.16^{c}	242.30 ± 14.45^{d}	156.62 ± 1.35^{e}	101.12 ± 2.52^{a}	104.96 ± 2.43^{f}
Camu powder	769.52 ± 15.49^{a}	622.09 ± 20.06^{b}	497.25 ± 22.62^{c}	627.69 ± 19.28^{ab}	548.96 ± 10.90^{d}	450.80 ± 29.85^{e}	496.72 ± 7.48^{c}	413.78 ± 2.17^{f}

$ORAC_{FL}$ values in columns are means ± SD of three determinations (n = 3). $_1$Exthanol extraction solvents at pH 7.4. $ORAC_{FL}$ values in each row with the same letter are not significantly ($p>0.05$) different. Abbreviations: AWA, Acetone/Distilled Water/Acetic Acid; RT, Room temperature

3.3.3. Method optimization

Table 4 shows the optimization of the $ORAC_{FL}$ assay. Prior to optimization, blank and standards showed poor precision between wells of the micro-plate (Appendix B). Subsequent to the pre-heating step, the COV (3.58) showed a 51.30% reduction. Additionally, it was observed that lack of precision occurred mostly in wells of column 1, 2 and 12 of the micro-plate (Figure 1). Therefore, columns 1, 2 and 12 were excluded for further $ORAC_{FL}$ assay analysis. The exclusion of these wells provided expected results. Significant ($p<0.05$) lower COV (2.87) was obtained with 60.96% reduction as compared to initial values.

Table 4: Method optimization for the $ORAC_{FL}$ assay

	Before preheating$_1$		**After preheating$_2$**		**Exclusion of wells$_3$**	
	Average net AUC	**COV**	**Average net AUC**	**COV**	**Average net AUC**	**COV**
Blank (PB 75 mM)	21.27 ± 1.99	**9.37**	-	-	-	-
Trolox (10 µM) std run1	48.13 ± 2.98^a	**6.2**	42.83 ± 1.55^b	**3.62**	42.60 ± 1.40^b	**3.28**
Trolox (10 µM) std run2	47.06 ± 3.78^a	**8.02**	40.40 ± 1.40^b	**3.47**	40.20 ± 1.03^b	**2.55**
Trolox (10 µM) std run3	45.72 ± 3.58^a	**7.84**	45.24 ± 1.64^a	**3.63**	44.83 ± 1.24^a	**2.77**
Average (10 µM trolox runs)	46.97 ± 0.41^a	**7.35**	42.82 ± 1.53^b	**3.58**	42.54 ± 1.22^b	**2.87**

Mean values are ± SD of 96 determinations (n=96); $ORAC_{FL}$ values in each row with the same letter are not significantly ($p>0.05$) different. $_1$No pre-heating of prepared micro-plate and phosphate buffer prior to analysis; $_2$Pre-heating of prepared micro-plate and phosphate buffer for 20 min at 37^o C prior to analysis; $_3$Exclusion of wells in columns 1,2 and 12.

Figure 2 shows the significant ($p<0.05$) decrease in outliers observed upon optimization of the $ORAC_{FL}$ assay. It was observed that poor precision (COV=7.17) of results occurred within the 2 standard deviation (2SD) prior to method optimization. After optimization good precision (COV=2.20) of results were observed within the 2SD.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	3	1	1	1	1	1	1	1	1	1	2
B	1	2	1	1	1	1	1	1	1	1	1	2
C	1	2	1	1	1	1	1	1	1	1	1	2
D	2	2	1	1	1	1	1	1	1	1	1	2
E	1	2	2	1	1	1	1	1	1	1	1	2
F	1	2	1	1	1	1	1	1	1	1	1	1
G	1	2	2	1	1	1	1	1	1	1	1	1
H	1	1	1	1	1	1	1	1	1	1	1	1

	COV
± 1 SD	1.40
± 2 SD	2.81
± 3 SD	4.21

Figure 1: Microplate optimization of $ORAC_{FL}$ assay. Illustration of improved precision of a 10uM trolox standard occurring within the 96 well micro-plate subsequent to method optimization

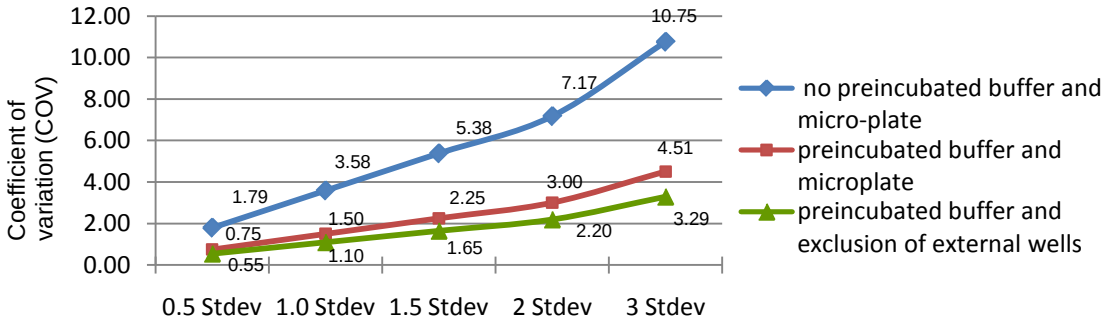
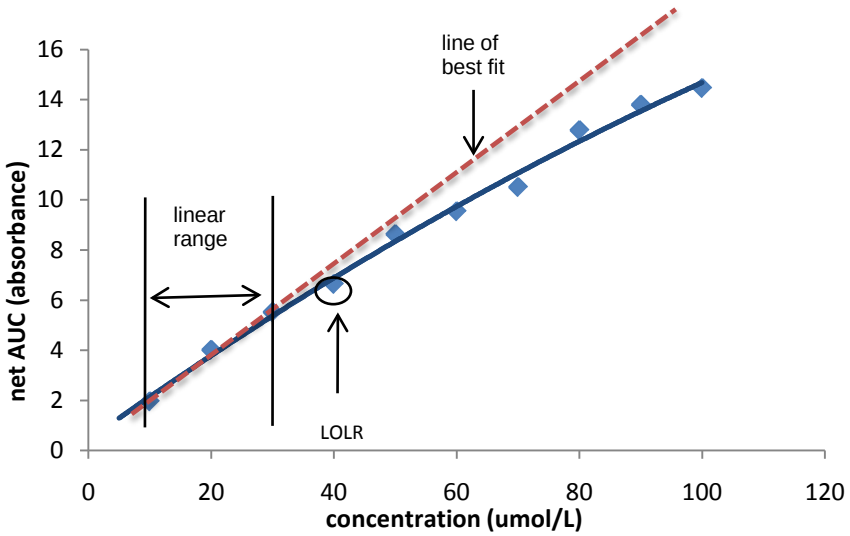


Figure 2. Method optimization of $ORAC_{FL}$ assay. Reduction in outliers observed upon inclusion of the pre-incubation step, and subsequently omitting wells in columns 1,2 and 12. A 10 µM trolox standard sample was assayed.

3.3.4. Linearity and Range

Figure 3a illustrates the linearity of the trolox standards. In this figure, the 40 µM trolox standard was the limit of linear response (LOLR). Poor linearity was observed at higher concentrations where response values showed a deviation from the line of best fit. Additionally, Figure 3b illustrates an increased imprecision at lower (<5 µM) and higher (>30 µM) concentrations and a good precision from 10 µM to 30 µM on the trolox standard curve. Hence, a linear calibration range comprising five trolox standards (5 to 25 µM) was chosen for routine analysis.

3a



3b

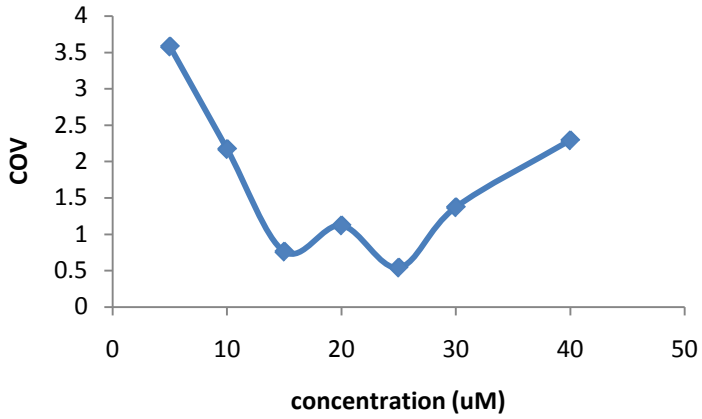


Figure 3. Linearity and precision of Trolox standards (a) Linearity of Trolox standard samples (10 to 100 µM). The limit of linear response (LOLR) occurred at 40 µM. **(b)** Precision (repeatability) of Trolox standards (5 to 40 µM) expressed as coefficient of variation. Imprecision increases at lower and higher concentrations.

Table 5 summarizes the accuracy, precision and linearity of the trolox standards. The linearity expressed as r^2 ranged from 0.987 to 0.999. The intermediate (day-to-day) precision expressed as COV, ranged from 2.83 to 6.06 with the highest imprecision, although still acceptable, observed at the 5 µM standard (6.06). Good repeatability was observed ranging from 1.14 to 4.58 for individual runs. The accuracy expressed as percent recovery ranged from 98 to 102% for all trolox standard samples.

Table 5. Accuracy, precision and linearity of Trolox standards.

Trolox standards (µM)	Intermediate precision [1]	Intra-assay precision [2]	Accuracy (µM TE/mL)[3]	REC (%)	r^2
5	6.06	4.58	4.97 ± 0.49	99.48	0.995
10	4.74	2.98	9.83 ± 0.28	98.29	
15	5.71	3.20	15.3 ± 0.67	101.97	
20	2.81	1.14	19.96 ± 0.52	99.81	
25	2.83	2.19	24.87 ± 0.49	99.46	

Data for all Trolox standards obtained from analysis over 8 days. [1]Precision between different days (n=8). [2]Precision within individual runs (n=3). [3]Concentration calculated using $y=mx+c$. Accuracy values expressed as means ± SD of eight determinations (n=8). Abbreviations: r^2, Mean correlation coefficient; REC, Recovery percentage.

Table 6 summarizes the optimization of the linearity of apple, chia seeds and organic honeybush samples. This optimization included a few modifications. Firstly, the measurement intervals of the plate reader were decreased to 1 min. Secondly, $ORAC_{FL}$ results were calculated using the linear equation $y=bx+c$, instead of the quadratic equation $y=ax^2+bx+c$. Lastly, samples were diluted to fit within the linear range of the standard curve. In this table, poor linearity was observed with samples prior to optimization. Subsequent to optimization, the linearity of samples improved significantly ($p<0.05$) (Appendix A). Additionally, the precision expressed as the COV showed a sizeable reduction (mean COVs <6%) for all samples compared to initial values (mean COVs >10%).

Table 6. Optimization of linearity of $ORAC_{FL}$ assay

	5 minute readings					1 minute readings				
Samples	**DF**	**$ORAC_{FL}$ (μM TE/L)$_1$**	**Mean**	**COV**	**r^2**	**DF**	**$ORAC_{FL}$ (μM TE/L)$_2$**	**Mean**	**COV**	**r^2**
Apple	25	5781.57 ± 4.45	5720.64	14.39	0.81	25	5370.91 ± 0.45	5531.94	3.64	0.94
	50	6013.01 ± 2.88				35	5438.67 ± 2.70			
	75	4573.88 ±3.41				50	5492.84 ± 3.62			
	10	6514.11 ± 2.70				60	5825.31 ± 4.33			
Chia seeds	7	288.41 ± 4.31	332.53	14.76	0.87	7	148.59 ± 5.84	142.49	4.92	0.97
	10	299.17 ± 2.36				10	140.80 ± 0.60			
	15	347.08 ± 6.53				15	133.28 ± 2.81			
	20	395.45 ± 1.06				20	147.29 ± 4.76			
Organic honeybush tea	5	2407.40 ± 6.58	2655.47	10.4	0.75	10	2536.95 ± 2.53	2758.73	5.66	0.98
	10	2536.95 ± 1.37				20	2764.00 ± 5.77			
	20	2631.16 ± 13.14				30	2848.46 ± 1.38			
	40	3046.36 ± 1.68				40	2885.51 ± 5.36			

Values in columns are means ± SD of three determinations (n = 3). $_1ORAC_{FL}$ values calculated using a quadratic equation ($Y=ax^2+bx+c$) and from measurements taken at 5 minute intervals by the plate reader. Linear calibration range not established. $_2ORAC_{FL}$ values calculated using a linear equation ($Y=mx+c$) and from measurements taken at 1 minute intervals by the plate reader. Linear calibration range established. Abbreviations: COV, Coefficient of variance; DF, Dilution factor; r^2, Coefficient of determination.

3.3.5. Selectivity/ specificity

The optimized H-$ORAC_{FL}$ assay was tested to show whether the method is specific/selective for antioxidants. This objective was achieved by spiking samples with a solution matrix that contain components that are most likely to be present in most food and beverage sample.[11] Table 7 summarizes the specificity of the $ORAC_{FL}$ assay. All samples showed good intra-assay precision (<5%) and accuracy (97 to 109%) with no significant ($p>0.05$) differences between samples before and after the addition of glucose and salt solutions. All trolox standards (5 µM, 10 µM, 15 µM) gave a linear response with correlation coefficients (r^2) of ≥0.993 upon the addition of glucose and NaCl solutions. The results show that the manual $ORAC_{FL}$ assay is selective/ specific for antioxidants in the presence of other sample matrix components producing accurate, precise and linear results for all sample types tested.

Table 7. Specificity of the $ORAC_{FL}$ assay using glucose and salt (NaCl) solutions

			Glucose solution spike (10 µM)			NaCl solution spike (10 µM)		
Sample concentration	**$ORAC_{FL}$ measured values (µM TE/L)**	**COV**	**$ORAC_{FL}$ measured values (µM TE/L)**	**COV**	**REC (%)**	**$ORAC_{FL}$ measured values (µM TE/L)**	**COV**	**REC (%)**
Chia seeds extract	97.92 ± 3.31[a]	3.38	95.03 ± 1.93[a]	2.03	97.04	95.32 ± 2.49 [a]	2.62	97.34
Organic honeybush tea	2727.95 ± 81.81[b]	3.00	2757.46 ± 16.89[b]	0.61	101.08	2806.36 ± 46.59[b]	1.66	102.87
QC 1 (6.25 µM)	6.49 ± 0.22[c]	3.40	6.81 ± 0.04[c]	0.68	104.96	6.58 ± 0.45[c]	6.88	101.43
QC 2 (12.5 µM)	12.83 ± 0.23[d]	1.81	12.50 ± 0.34[d]	2.70	97.40	12.29 ± 0.25[d]	2.04	95.76
Trolox standard (5 µM)	5.17 ± 1.97[e]	1.87	5.23 ± 4.89[e]	4.41	101.24	5.23 ± 1.18[e]	1.10	101.11
Trolox standard (10 µM)	9.65 ± 4.52[f]	2.44	9.53 ± 1.29[f]	0.69	98.76	9.54 ± 1.16[f]	0.62	98.89
Trolox standard (15 µM)	15.17 ± 2.61[g]	0.92	15.23 ± 5.30[g]	1.82	100.40	15.23 ± 0.07[g]	0.02	100.35

$ORAC_{FL}$ values in columns are means ± SD of three determinations (n=3). $ORAC_{FL}$ values in each row with the same letter are not significantly ($p>0.05$) different.
Abbreviations: COV, Coefficient of variance; REC, Percent recovery

3.3.6 Accuracy and precision

Table 8 summarizes the accuracy and precision of the manual $ORAC_{FL}$ assay using the standard addition recovery method. All samples and standards demonstrated an acceptable precision (<5%) which was expressed as the COV. The accuracy expressed as percentage recovery ranged from 93 to 110%. All samples and standards displayed a linear response (r^2 ≥ 0.994) for all trolox standard additions as illustrated in Figure 4.

Table 8. Standard addition recovery method for validating accuracy and precision

Samples (µM TE/L)	Trolox spike (µM TE/L)	Expected concentration with Trolox spike (µM TE/L)	Measured concentration with Trolox spike (µM TE/L)	REC (%)	COV (%)	r^2
Chia seeds (2.35 ± 1.12)	5.00	7.35	7.94 ± 0.28	108.03	3.55	0.994
	10.00	12.35	11.52 ± 0.10	93.28	0.82	
	15.00	17.35	16.17 ± 1.50	93.20	1.76	
Organic honeybush tea (3.07± 1.88)	5.00	8.07	8.62 ± 0.63	106.82	0.38	1.000
	10.00	13.07	12.47 ± 0.10	95.41	0.04	
	15.00	18.07	17.26 ± 1.50	95.52	0.50	
Trolox standard (2.53 ± 0.09)	5.00	7.50	8.20 ± 0.24	109.33	2.88	0.996
	10.00	12.50	12.71 ± 0.03	101.68	0.25	
	15.00	17.50	17.35 ± 0.45	100.62	2.62	
	20.00	22.50	22.28 ± 0.52	100	0.13	

$ORAC_{FL}$ values in columns are means ± SD of three determinations (n=3). Abbreviations: COV, Coefficient of variance; r^2, Coefficient of determination, REC, Recovery percentage.

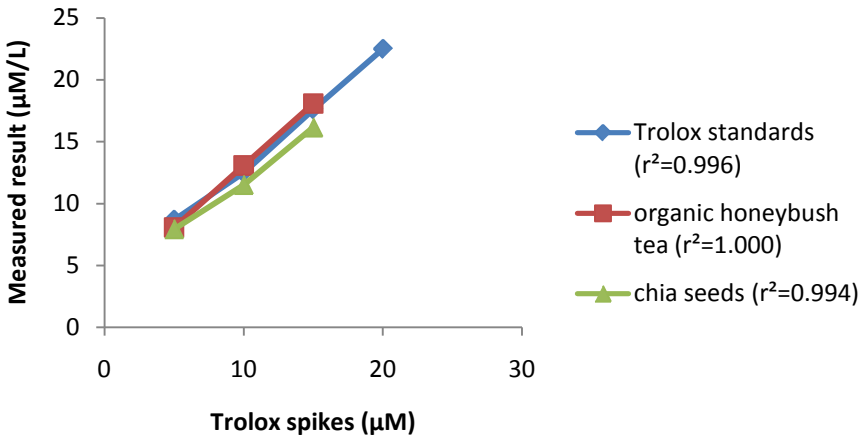


Figure 4. Linearity of samples following the standard addition method to validate accuracy

Table 9 summarizes the accuracy and precision of the manual $ORAC_{FL}$ assay using the spiked recovery method. Prepared QC samples spiked with three different Trolox concentrations produced recoveries ranging from 94.85 to 105.85% within individual runs and from 96.55 to 104.11% between all runs for the three days over which they were assayed. Moreover, good intra-assay and intermediate precision was observed with all COVs falling within 5%.

Table 9. Spiked recovery method for validating accuracy

		Day1			Day2			Day3			Average		
QC	**Expected concentration (µM)**	**Concentration (µM)**	**COV**	**REC (%)**	**Concentration (µM)**	**COV**	**REC (%)**	**Concentration (µM)**	**COV**	**REC (%)**	**Concentration (µM)**	**COV**	**REC (%)**
QC1	22	21.33 ± 0.39	1.83	96.98	21.52 ± 0.93	4.31	97.83	20.87 ± 0.46	2.22	94.85	21.24 ± 0.34	1.59	96.55
QC2	12.5	12.79 ± 0.43	3.39	102.31	12.43 ± 0.37	2.99	99.43	12.47 ± 0.20	1.62	99.80	12.56 ± 0.20	1.56	100.51
QC3	6.25	6.62 ± 0.10	1.45	105.85	6.33 ± 0.27	4.34	101.31	6.57 ± 0.11	1.68	105.16	6.51 ± 0.15	2.35	104.11

Values in columns are means ± SD of four determinations (n=4). Abbreviations: COV, Coefficient of variance; QC, Quality control; REC, Recovery percentage**.**

3.3.7. Limit of Detection and limit of quantification

Table 10 summarizes the LOD and LOQ of the manual $ORAC_{FL}$ assay. They were determined to be 1.35 and 4.10 µM, respectively. However, it has been recommended to demonstrate acceptable accuracy and precision at the LOQ level using samples, in order to establish the LOQ.[48] All samples (QC3, apple juice and chia seeds) demonstrated acceptable linearity ($r^2 \geq 0.970$), precision (COVs < 10%), and accuracy (percentage recoveries ranged from 94 to 110%) at and around the LOQ level as shown in Table 11.

Table 10. LOD and LOQ of the manual $ORAC_{FL}$ assay

	Net AUC of blank	COV	Slope	LOD_1(µM TE/L)	LOQ_2(µM TE/L)
Day 1	67.62 ± 1.51	2.24	15.93		
Day 2	60.78 ± 3.91	6.44	15.16		
Day 3	74.18 ± 4.06	5.47	16.22		
Average	67.53 ± 6.48	4.72 ± 2.19	15.77 ± 0.55	1.35	4.10

Values in columns are means ± SD of six determinations (n = 6) of the blank sample. $_1$LOD calculated using the formula LOD = [3.3 x (sigma)]/slope]. $_2$LOQ calculated using the formula LOQ = [10 x (sigma)]/slope].

Table 11. Accuracy, precision and linearity of samples analyzed at several points around the established LOQ level

Samples/ QC concentration	Dilution factor	Expected $ORAC_{FL}$ values (μM TE/L)	Measured $ORAC_{FL}$ values (μM TE/L)	COV (%)	Recovery (%)	r^2
Apple juice 336 μM	80	4.20	4.62 ± 4.20	5.45	110.03	0.981
	82	4.09	4.38 ± 4.11	5.62	107.24	
	85	3.94	4.12 ± 5.81	8.46	104.39	
	87	3.86	3.72 ± 0.46	0.75	96.43	
Chia seeds 325 μM	18	5.41	5.18 ± 8.40	9.57	97.41	0.970
	20	4.89	2.01 ± 1.46	1.74	102.60	
	22	4.44	4.43 ± 2.67	3.62	99.69	
	24	4.07	4.18 ± 3.20	4.60	102.65	
QC3 22 μM	3	7.33	6.91 ± 1.93	1.67	94.36	0.983
	4	5.49	5.68 ± 1.11	1.17	103.63	
	5	4.39	4.65 ± 1.46	1.89	105.95	
	6	3.66	3.98 ± 2.27	3.41	108.89	

Values in columns are means ± SD of three determinations (n = 3). Abbreviations: COV, Coefficient of variance; LOQ, Limit of quantification; QC, Quality control; r^2, Coefficient of determination

3.3.8. Sample stability

Table 12 summarizes the results of the stability of the QC samples and extract samples (mixed berry juice, tropical fruit juice, dragon-fruit glacial vitamin water, tomato, apple and camu powder). All QCs and samples were stable at 4 ^{o}C for 24 hr. This is confirmed by no significant ($p>0.05$) differences between fresh and long-term sample results. All samples were not stable at RT for 8 hrs. This was indicated by significantly ($p<0.05$) lower results obtained with short-term sample results, compared to fresh sample results. Additionally, all samples and QCs demonstrated poor stability after three freeze-thaw cycles. This observation was indicated by the significantly ($p<0.05$) lower results obtained for freeze-thaw sample and QC results compared to fresh sample results. These findings indicate that all QCs and samples demonstrated good long-term stability at a low temperature (4 ^{o}C). Additionally, QC samples were stable at RT over a short time period.

Table 12. Sample stability

	Fresh		Short-Term (8hr, RT)			Long-Term (24hr, 4°C)			Freeze-Thaw (3 cycles, -40°C)		
Samples/QC	**Measured values (µM TE/L)**	**COV**	**Measured values (µM TE/L)**	**COV**	**%REC**	**Measured values (µM TE/L)**	**COV**	**%REC**	**Measured values (µM TE/L)**	**COV**	**%REC**
Apple	45.15 ± 1.63 [a]	3.62	41.28 ± 1.23[b]	2.98	91.43	44.44 ± 0.40 [a]	0.90	98.42	41.24 ± 1.70[c]	4.13	91.36
Camu powder	693 ± 14[a]	2.11	609 ± 21 [b]	3.47	89.12	683 ± 14 [a]	2.08	101.38	586 ± 7[c]	1.20	84.64
Glacial vitamin water	3180 ± 86[a]	2.72	2689 ± 26[b]	0.99	83.96	3097 ± 35[a]	1.15	97.39	2224 ± 73[c]	3.31	69.94
Mixed berry	7372 ± 370[a]	5.03	5692 ± 406[b]	7.14	77.22	6808 ± 5.9[a]	0.09	92.35	4876 ± 549[c]	11.28	66.15
Tomato	22.01 ± 0.86[a]	3.90	18.89 ± 0.51[b]	2.69	88.00	21.47 ± 0.42[a]	1.97	97.55	17.19 ± 0.47[c]	2.74	78.10
Tropical	5129 ± 144 [a]	2.82	4531 ± 51[b]	1.13	88.35	4932 ± 176[a]	3.58	96.16	3555 ± 77[c]	2.18	69.32
QC 1 (6.25 µM)	6.34 ± 0.21[a]	3.38	6.43 ± 0.08[a]	1.21	101.34	6.48 ± 0.22[a]	3.40	102.34	5.12 ± 0.09[b]	1.94	80.77
QC 2 (12.5 µM)	12.76 ± 0.15[a]	1.21	12.32 ± 0.03[a]	0.23	96.54	12.62 ± 0.67[a]	5.29	98.91	10.97 ± 0.19[b]	1.80	85.93

Values in columns are means ± SD of three determinations (n = 3). $ORAC_{FL}$ values in each row with the same letter are not significantly ($p<0.05$) different. Abbreviations: COV, Coefficient of variance; REC, Recovery percentage; QC, Quality control.

3.4 Discussion:

This study focused on the optimization and validation of the manually performed H-ORAC$_{FL}$ assay in accordance with ISO 17025 method performance parameters. Conditions were optimized for extraction of hydrophilic antioxidants from various food matrices. Additionally, the effect of temperature and micro-plate usage on the manual performance of the ORAC$_{FL}$ assay was evaluated. Lastly, validation parameters tested and further optimized confirmed that the manually applied ORAC$_{FL}$ assay performed as expected, generating highly accurate and precise results. Moreover, all errors encountered were identified and minimized, contributing to the overall good performance of the assay.

While there are many methods that have been reported to extract antioxidants from various food matrices[50], industrial requirements within the food and beverage sectors, such as simplicity, efficiency and cost effectiveness of the extraction method, all contribute to the selection of solvent extraction as a preferred method.[51, 52] Several publications have reported the use of methanol/water (v/v) and ethanol/water (v/v) mixtures for extraction of antioxidants from plant and plant-based foods.[37] Other publications have reported the use of acetone/water mixture (v/v) and acetone/water/acetic acid (v/v/v) as extraction solvents for antioxidant recovery in various food products.[19, 20, 35, 53] However, no comparison between these extraction methods for recovering antioxidants from various samples is evident. In this study 60% ethanol/water mixtures is a desirable extraction solvent for good recovery of hydrophilic antioxidants in food samples and is in agreement with other publications using ethanol and methanol as preferred extraction solvents.[21, 54-57]

High temperatures generated good recoveries, however at excessive temperatures (80 ^{o}C) and extreme pH's (pH 2.0 and 11.7) antioxidant degradation may occur, contributing to lower antioxidant yields as seen with some of the study samples (Table 3).[39] Hence, the type of sample must be considered when using very high temperatures to extract antioxidants. Additionally, better yields were observed with some of the samples when the ethanol/water solvent was doubled. This finding differed from that reported by Prior *et al* who found that ethanol did not have much of an effect on antioxidant extraction from plasma, but then it should be noted that the matrix differed.[35]

Prior and co-workers reported disparities in net AUC (COVs ranged from 7.7 to 15.5) between wells of the 96 well micro-plate caused by temperature variations in parts of the plate.[35] Pre-heating the micro-plate and the phosphate buffer at 37 ^{o}C prior to initiation of the reaction, using a plate reader coupled to an automated handling system, substantially

improved consistency of net AUC between wells, decreasing the COV by approximately 50%.[35] Upon adapting this modification to a manual handling system, we observed a similar reduction in the COV of results. Similar findings have been reported by Lussignoli and co-workers as well as Milbury and co-workers and is believed to be caused by temperature variations that occur within the micro-plate once inside the plate reader.[38, 58] By excluding several external wells of the micro-plate, the repeatability improved significantly contributing to good precision of a manually applied assay.

The validation results demonstrated robustness of the manually applied $ORAC_{FL}$ assay when small variations in pH, AAPH and trolox preparation were applied. The $ORAC_{FL}$ assay has been reported to be very sensitive to changes in pH, however Ou and co-workers reported that small changes in pH did not have any effect on the $ORAC_{FL}$ assay.[19] Our observations fully support this latter finding in that only at extreme pHs (pH 7.0 and 7.8) the $ORAC_{FL}$ assay sensitive, however with small changes in pH (pH 7.2 to 7.6) the $ORAC_{FL}$ assay is robust. The thermal decomposition rate of AAPH is largely dependent on temperature as well as the pH of the solvent in which it is dissolved. At 37 ^{o}C and pH 7.4 there is consistent continuous radical generation for the first couple of hours.[59] Prior and co-workers reported reduced variability in $ORAC_{FL}$ results upon preheating the phosphate buffer at 37^{o}C for 10 min preceding the addition of AAPH.[35] However, there is no published data showing the effect phosphate buffer could have on $ORAC_{FL}$ results, upon standing for a period of time once dissolved in phosphate buffer prior to analysis. In the current study it was observed that AAPH decomposition of the prepared AAPH solution did not occur while the solution was maintained at 37 ^{o}C for up to 2 hr. This finding is further supported by Huang *et al* who reported that maintaining the temperature at 37 ^{o}C during the reaction is critical to constant radical generation and subsequently, reduces well to well variability of net AUC.[20]

The trolox standard range was found to be linear from 5 to 25 µM. Huang and co-workers have reported a linearity range for trolox between 6.25 and 50 µM, while Ou and co-workers reported a linearity range from 12.5 to 100 µM.[19, 20] However, in establishing a linear range for an assay, both accuracy and precision must be demonstrated to be acceptable at all points on the calibration curve.[11, 48] The results of the current study demonstrated good accuracy and precision for the trolox standards. The linearity (r^2=0.995) correlated well with other publications in which the $ORAC_{FL}$ assay was performed using an automated pipetting system (r^2=0.997)[19] and performed manually (r^2 ranged from 0.94 to 0.99)[33] and ($r^2 \geq$ 0.994).[21] The linearity of the samples improved significantly subsequent to the optimization. All samples showed good correlation ($r^2 \geq 0.94$) as compared to those obtained prior to

optimization (0.75 to 0.87). These results are comparable to Ou and co-workers and Davalos *et al* who observed good linearity (≥ 0.99) of some natural food products using an automated assay, and pure products using a manually applied assay, respectively.[19, 21]

The manually applied $ORAC_{FL}$ assay is specific/ selective for antioxidants. Ou *et al.* demonstrated the specificity of the $ORAC_{FL}$ assay using Fenton's reagent to destroy antioxidants present in a sample.[19] Due to the unavailability of Fenton's reagent, specificity was evaluated by adding a prepared salt and glucose solution to calibration standards and a few samples. The current finding confirmed the specificity and selectivity of the assay and is in agreement with those reported by Ou *et al.*[19]

The validation parameters, namely accuracy, precision, LOD and LOQ for the current study were acceptable. Davalos and co-workers reported accuracy and precision of less than 5% variation of the mean and less than 5% COV, respectively.[21] These values were reported for trolox standards for a manually applied H-$ORAC_{FL}$ assay. Similar findings were observed in the current study for accuracy and precision. Additionally, low values (0.5 and 1.0 µM) for LOD and LOQ were reported, however, no accuracy and precision were demonstrated for samples within the trolox standard range from Davalos *et al* employing a manually applied $ORAC_{FL}$ assay.[21] With the current method, COVs of less than 5% were observed for QC samples. These results can be compared to Ou and co-workers who reported COVs of up to 9.16 for QC samples.[19] Moreover the intra-assay precision achieved with our manual method for food and beverage samples (chia seeds and organic honeybush tea) was less than 5% and is comparable to the intra-assay precision observed by Huang and co-workers who reported COVs of greater than 10% for some food samples using a high through-put $ORAC_{FL}$ assay.[20] In the present study, the accuracy of samples, expressed as percent recoveries ranged from 94.85 to 105.85% within individual runs and from 96.55 to 104.11% between all runs. Using an automated handling system Ou and co-workers demonstrated within run and between run antioxidant recoveries (91 to 107% and 101 to 105%, respectively) for QC samples.[19]

The limit of detection (LOD) is the lowest concentration of antioxidant that can be detected but not necessarily quantified, whilst the limit of quantitation (LOQ) is the lowest concentration of antioxidant on the trolox standard calibration curve that can be quantified with acceptable accuracy and precision. Ou and co-workers and Huang and co-workers have reported LOD and LOQ values of 5 and 12.5 µM, respectively using an automated handling system.[19, 53] With the current study, lower LOD and LOQ values (1.35 and 4.10 µM) were achieved. Davalos and co-workers reported even lower LOD and LOQ values of 0.5

and 1.0µM, respectively however, accuracy and precision of samples were not demonstrated at and around the LOQ level.[21] The results of the current study showed acceptable accuracy, precision and linearity at and around the LOQ level.

These results indicate our optimization of the $ORAC_{FL}$ assay using a manual handling system is efficient in generating accurate and precise results.

3.5 Final Remarks

In this study we (1) optimized the hydrophilic antioxidant extraction for several food and beverage samples using a fully manual handling system (Appendix C); (2) optimized the method in terms of temperature and micro-plate usage for reproducible results and (3) successfully validated the manually performed $ORAC_{FL}$ for food and beverage products through accuracy, precision, range, linearity, LOD, LOQ, sample stability and selectivity in conjunction to the recommended international standards. The optimization and validation of the manually applied $ORAC_{FL}$ assay should make it highly desirable as a routine method for smaller research laboratories assaying food and beverage products.

3.6 References

1. Borek, C. Antioxidants and cancer. *Sci. Med.* **1997,** *4*, 51-62.

2. Borek, C.; Ong, A.; Mason, H.; Donahue, L.; Biaglow, J. E. Selenium and vitamin E inhibit radiogenic and chemically induced transformation in vitro via different mechanisms. *Proceedings of the National Academy of Sciences.* **1986,** *83*, 1490.

3. Engelhart, M. J.; Geerlings, M. I.; Ruitenberg, A.; van Swieten, J. C.; Hofman, A.; Witteman, J. C. M.; Breteler, M. M. B. Dietary intake of antioxidants and risk of Alzheimer disease. *JAMA, J. Am. Med. Assoc.* **2002,** *287*, 3223-3229.

4. Gaziano, J. M.; Manson, J. E.; Branch, L. G.; Colditz, G. A.; Willett, W. C.; Buring, J. E. A prospective study of consumption of carotenoids in fruits and vegetables and decreased cardiovascular mortality in the elderly. *Ann. Epidemiol.* **1995,** *5*, 255-260.

5. Joshipura, K. J.; Hu, F. B.; Manson, J. A. E.; Stampfer, M. J.; Rimm, E. B.; Speizer, F. E.; Colditz, G.; Ascherio, A.; Rosner, B.; Spiegelman, D. The effect of fruit and vegetable intake on risk for coronary heart disease. *Ann. Intern. Med.* **2001,** *134*, 1106-1114.

6. Steinmetz, K. A.; Potter, J. D. Vegetables, Fruit, and Cancer Prevention: A Review. *J. Am. Diet. Assoc.* **1996,** *96*, 1027-1039.

7. Kvidahl, M. Antioxidants for all. http://digitaledition.qwinc.com/publication/?i=46213&page=1 (September 2010).
8. CITAC/EURACHEM. Guide to quality in Analytical Chemistry. http://www.european-accreditation.org/publication/citac-eurachem-ta (10 October 2012).

9. Eurachem. The fitness for purpose of analytical methods. http://www.eurachem.org/images/stories/Guides/pdf/valid.pdf (10 October 2012).

10. International Conference on Harmonisation. Guidance for Industry Q2B Validation of Analytical Procedures: Methodology. http://www.fda.gov/downloads/Regulator%20yInformation/Guidances/UCM128049.pdf (15 September 2012).

11. International Conference on Harmonisation. Validation of Analytical Procedures: Text and Methodology Q2(R1) Part I and II. http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf (11 August 2012).

12. ISO/IEC. General requirements for the competence of testing and calibration laboratories. http://bbs.labsky.com/uploads/2005-8/2005-08-24_205709.pdf (12 July 2012).

13. Hayward, J. Understanding the new labelling legislation. http://www.foodreview.co.za/food-labelling-and-legislation/662-what-the-new-labelling-legislation-means-for-businesses-and-consumers (5 August 2012).

14. Cao, G.; Prior, R. L. Comparison of different analytical methods for assessing total antioxidant capacity of human serum. *Clin. Chem.* **1998,** *44*, 1309-1315.

15. Zulueta, A.; Esteve, M. J.; Frígola, A. ORAC and TEAC assays comparison to measure the antioxidant capacity of food products. *Food Chem.* **2009,** *114*, 310-316.

16. Cao, G.; Alessio, H. M.; Cutler, R. G. Oxygen-radical absorbance capacity assay for antioxidants. *Free Radical Biol. Med.* **1993,** *14*, 303-311.

17. Glazer, A. N. Phycoerythrin fluorescence-based assay for reactive oxygen species. *Methods Enzymol.* **1990,** *186*, 161-168.

18. Cao, G.; Verdon, C. P.; Wu, A.; Wang, H.; Prior, R. L. Automated assay of oxygen radical absorbance capacity with the COBAS FARA II. *Clin. Chem.* **1995,** *41*, 1738-1744.

19. Ou, B.; Hampsch-Woodill, M.; Prior, R. L. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *J. Agric. Food Chem.* **2001,** *49*, 4619-4626.

20. Huang, D.; Ou, B.; Hampsch-Woodill, M.; Flanagan, J. A.; Prior, R. L. High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. *J. Agric. Food Chem.* **2002b,** *50*, 4437-4444.

21. Dávalos, A.; Gómez-Cordovés, C.; Bartolomé, B. Extending applicability of the oxygen radical absorbance capacity (ORAC-fluorescein) assay. *J. Agric. Food Chem.* **2004,** *52*, 48-54.

22. Kevers, C.; Falkowski, M.; Tabart, J.; Defraigne, J. O.; Dommes, J.; Pincemail, J. Evolution of antioxidant capacity during storage of selected fruits and vegetables. *J. Agric. Food Chem.* **2007,** *55*, 8596-8603.

23. Wang, H.; Cao, G.; Ronald, L. Oxygen radical absorbing capacity of anthocyanins. *J. Agric. Food Chem.* **1997,** *45*, 304-309.

24. Wang, H.; Cao, G.; Ronald, L. Total antioxidant capacity of fruits. *J. Agric. Food Chem.* **1996,** *44*, 701-705.

25. Alarcon, E.; Campos, A.; Edwards, A.; Lissi, E.; Lopez-Alarcon, C. Antioxidant capacity of herbal infusions and tea extracts: A comparison of ORAC-fluorescein and ORAC-pyrogallol red methodologies. *Food Chem.* **2008,** *107*, 1114-1119.

26. Roy, M. K.; Koide, M.; Rao, T. P.; Okubo, T.; Ogasawara, Y.; Juneja, L. R. ORAC and DPPH assay comparison to assess antioxidant capacity of tea infusions: Relationship between total polyphenol and individual catechin content. *Int. J. Food Sci. Nutr.* **2010,** *61*, 109-124.

27. Wiseman, S. A.; Balentine, D. A.; Frei, B. Antioxidants in tea. *Crit. Rev. Food Sci. Nutr.* **1997,** *37*, 705-718.

28. Zheng, W.; Wang, S. Y. Antioxidant activity and phenolic compounds in selected herbs. *J. Agric. Food Chem.* **2001,** *49*, 5165-5170.

29. Serafini, M.; Ghiselli, A.; Ferro-Luzzi, A. In vivo antioxidant effect of green and black tea in man. *Eur. J. Clin. Nutr.* **1996,** *50*, 28-32.

30. Rappi, N. Demystifying ORAC (Antioxidant) Claims: The Gold Standard. http://www.naturopathydigest.com/archives/2006/mar/nappi.php (17 June 2012).

31. Marnewick, J. L.; Van der Westhuizen, F. H.; Joubert, E.; Swanevelder, S.; Swart, P.; Gelderblom, W. C. A. Chemoprotective properties of rooibos (*Aspalathus linearis*), honeybush (*Cyclopia intermedia*) herbal and green and black (*Camellia sinensis*) teas against cancer promotion induced by fumonisin B_1 in rat liver. *Food Chem. Toxicol.* **2009,** *47*, 220-229.

32. McKay, D. L.; Blumberg, J. B. A review of the bioactivity of South African herbal teas: rooibos (Aspalathus linearis) and honeybush (Cyclopia intermedia). *Phytother. Res.* **2006,** *21*, 1-16.

33. Price, J. A.; Sanny, C. G.; Shevlin, D. Application of manual assessment of oxygen radical absorbent capacity (ORAC) for use in high throughput assay of "total" antioxidant activity of drugs and natural products. *J. Pharmacol. Toxicol. Methods.* **2006,** *54*, 56-61.

34. Prior, L.; Wu, X.; Schaich, K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J. Agric. Food Chem. .* **2005,** *53*, 4290-4302.

35. Prior, L.; Hoang, H.; Gu, L.; Wu, X.; Bacchiocca, M.; Howard, L.; Hampsch-Woodill, M.; Huang, D.; Ou, B.; Jacob, R. Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity (ORACFL)) of plasma and other biological and food samples. *J. Agric. Food Chem. .* **2003,** *51*, 3273-3279.

36. Wu, X.; Beecher, G. R.; Holden, J. M.; Haytowitz, D. B.; Gebhardt, S. E.; Ronald, L. Lipophilic and hydrophilic antioxidant capacities of common foods in the United States. *J. Agric. Food Chem. .* **2004,** *52*, 4026-4037.

37. Peschel, W.; Sánchez-Rabaneda, F.; Diekmann, W.; Plescher, A.; Gartzía, I.; Jiménez, D.; Lamuela-Raventos, R.; Buxaderas, S.; Codina, C. An industrial approach in the search of natural antioxidants from vegetable and fruit wastes. *Food Chem.* **2006,** *97*, 137-150.

38. Lussignoli, S.; Fraccaroli, M.; Andrioli, G.; Brocco, G.; Bellavite, P. A microplate-based colorimetric assay of the total peroxyl radical trapping capability of human plasma. *Anal. Biochem.* **1999,** *269*, 38-44.

39. Escribano-Bailon, M. T.; Santos-Buelga, C. Polyphenol extraction from foods. *Methods Polyphenol Anal.* **2003**, 1-16.

40. Chirinos, R.; Rogez, H.; Campos, D.; Pedreschi, R.; Larondelle, Y. Optimization of extraction conditions of antioxidant phenolic compounds from mashua (*Tropaeolum tuberosum* Ruíz & Pavón) tubers. *Sep. Purif. Technol.* **2007,** *55*, 217-225.

41. Hartmann, C.; Smeyers-Verbeke, J.; Massart, D.; McDowall, R. Validation of bioanalytical chromatographic methods. *J. Pharm. Biomed. Anal.* **1998,** *17*, 193-218.

42. Thompson, M.; Ellison, S. L. R.; Wood, R. Harmonized guidelines for single-laboratory validation of methods of analysis. *Pure and Applied Chemistry* **2002,** *74*, 835-855.

43. Huber, L. Validation of Analytical Methods. http://www.chem.agilent.com/Library/primers/Public/5990-5140EN.pdf (10 October 2012).

44. Australian Pesticides and Veterinary Medicines Authority. Guidelines for the Validation of Analytical Methods for Active Constituent, Agricultural and Veterinary Chemical Products. http://www.apvma.gov.au/publications/guidelines/docs/gl_69_analytical_methods.pdf (6 August 2012).

45. Uličná, O.; Greksák, M.; Vančová, O.; Zlatoš, L.; Galbavý, S.; Božek, P.; Nakano, M. Hepatoprotective effect of rooibos tea (*Aspalathus linearis*) on CCl4-induced liver damage in rats. *Physiol. Res.* **2003,** *52*, 461-466.

46. Food and Drug Administration. Guidance for Industry: Bioanalytical Method Validation. http://www.fda.gov/downloads/Drugs/.../Guidances/ucm070107.pdf (10 August 2012).

47. Dávalos, A.; Bartolomé, B.; Gómez-Cordovés, C. Antioxidant properties of commercial grape juices and vinegars. *Food Chem.* **2005,** *93*, 325-330.

48. Chan, C. C. Analytical Method Validation: Principles and Practices. *Pharmaceutical Sciences Encyclopedia: Drug Discovery, Development, and Manufacturing.* **2008**, 1-16.

49. Sigma Aldrich. Quality Management Systems. http://www.sigmaaldrich.com/customer-service/quality-systems.html (10 October 2012).

50. Wang, L.; Weller, C. L. Recent advances in extraction of nutraceuticals from plants. *Trends Food Sci. Technol.* **2006,** *17*, 300-312.

51. Ballard, T. S.; Mallikarjunan, P.; Zhou, K.; O'Keefe, S. F. Optimizing the extraction of phenolic antioxidants from peanut skins using response surface methodology. *J. Agric. Food Chem.* **2009,** *57*, 3064-3072.

52. Pompeu, D.; Silva, E.; Rogez, H. Optimisation of the solvent extraction of phenolic antioxidants from fruits of *Euterpe oleracea* using Response Surface Methodology. *Bioresour. Technol.* **2009,** *100*, 6076-6082.

53. Huang, D.; Ou, B.; Hampsch-Woodill, M.; Flanagan, J. A.; Deemer, E. K. Development and validation of oxygen radical absorbance capacity assay for lipophilic antioxidants using randomly methylated β-cyclodextrin as the solubility enhancer. *J. Agric. Food Chem.* **2002a,** *50*, 1815-1821.

54. Antolovich, M.; Prenzler, P.; Robards, K.; Ryan, D. Sample preparation in the determination of phenolic compounds in fruits. *Analyst.* **2000,** *125*, 989-1009.

55. Lapornik, B.; Prošek, M.; Golc Wondra, A. Comparison of extracts prepared from plant by-products using different solvents and extraction time. *J. Food Eng.* **2005,** *71*, 214-222.

56. Lin, M. C.; Tsai, M. J.; Wen, K. C. Supercritical fluid extraction of flavonoids from *Scutellariae Radix*. *J. Chromatogr., A.* **1999,** *830*, 387-395.

57. Oszmianski, J.; Lee, C. Isolation and HPLC determination of phenolic compounds in red grapes. *Am. J. Enol. Vitic.* **1990,** *41*, 204-206.

58. Milbury, P. E. Microplate-based oxygen radical absorbance capacity (ORAC) assay of hydrophilic and lipophilic compartments in plasma. *Nutr. Sci.* **2006,** *9*, 48-54.

59. Niki, E. Free radicals initiators as source of water-or lipid-soluble peroxyl radicals. *Methods Enzymol.* **1990,** *186*, 100-108.

60. Food and Drug Administration/ Center for Drug Evaluation and Research. Reviewer Guidance: Validation of Chromatographic Methods. http://www.fda.gov/downloads/Drugs/.../Guidances/UCM134409.pdf (11 October 2012).

CHAPTER FOUR: RESEARCH ARTICLE TWO

Optimization and validation of a reverse-phase high performance liquid chromatography method with ultra-violet detection for the measurement of L-ascorbic acid in food and beverage products

Olivia L. Parbhunath, Fanie Rautenbach, Glenda Davison and Jeanine L. Marnewick

Oxidative Stress Research Centre, Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology, P.O. Box 1906, 7538, South Africa.

Abstract

In accordance with national and international regulatory standards, the validation of chromatography methods is becoming necessary. This study provides an optimized and fully validated reverse-phase high performance liquid chromatography (RP-HPLC) method with ultra-violet (UV) detection for the measurement of L-ascorbic acid (L-AA) in fruit, vegetable and food products.

Several commercial fruit juices and teas, fresh fruit and vegetables and food extract products were analyzed using a HPLCsystem with UV detection. Chromatographic separation of L-AA was achieved on a reverse phase C_{18} 150 mm x4.6 mm, 0.5 µm column with UV detection of 245 nm at room temperature (23 °C). Distilled water/acetonitrile/formic acid (99: 0.9: 0.1, v/v/v), pH 2.6 was used as the mobile phase, in isocratic mode (flow rate of 1ml/min). Samples were extracted in 4.5% metaphosphoric acid solution and filtered through a 0.45 µm membrane. The method was validated for accuracy, precision, linearity, range, limit of detection, limit of quantification, specificity, stability, robustness and system suitability in accordance with ISO 17025 validation requirements. Validation results demonstrated a linear response within a range of 5 to 125 µg/mL and a correlation coefficient of 0.999 was obtained. Mean recoveries ranged from 99 to 103% and 92 to 96% for L-AA standards and samples, respectively. The method was found to be precise (COVs < 5%) and specific with no interferences from coexisting peaks. The LOD and LOQ were 0.61 µg/mL and 1.84 µg/mL respectively.

The successful optimization and validation of the proposed method should make it easily applicable for routine laboratory analysis of L-AA measurement in various fruit and vegetable products.

Keywords: validation, L-ascorbic acid, HPLC, ISO 17025

To be submitted to the Journal of Chromatography A. The referencing format of this chapter will reflect the format required for this journal.

4.1 Introduction

Vitamin C or L-ascorbic acid (L-AA) plays a pivotal role in many biological and metabolic processes. Collagen, carnitine and hormone production; bone formation; protection of the immune system; reduction in cholesterol due to L-AA's involvement in cholesterol metabolism include some of L-AA's important physiological roles. Of paramount importance is L-AA's antioxidant role in counteracting reactive oxygen species (ROS) and reducing oxidative stress and possibly oxidative damage. The water-solubility of L-AA allows it to exert its antioxidant activities both within and outside the cell, subsequently protecting the cell from potential DNA, protein and lipid damage normally caused by ROS. Several studies have demonstrated a reduced incidence of cataracts, cardiovascular disease and cancer with the intake of L-ascorbic acid [1,2]. Therefore, it is evident that L-AA is vital to the normal functioning of human biological systems.

Aside from its biological benefits, L-AA also has application within the food and beverage industry. It has been widely used within the food industry as a preservative due to its powerful reducing action, thereby increasing the shelf-life of food and beverage products [3]. The incorporation of fatty esters of vitamin C in cosmetics is derived from the positive association between its intake/application and the reduced incidence of certain pathological diseases [4]. Dietary sources rich in vitamin C include green leafy vegetables, peppers, broccoli, brussel sprouts, citrus and tropical fruits [5].

The vast applications and health benefits associated with L-AA, have spiked a significant amount of interest within the food industry, resulting in an escalation in L-AA assay requests. Several analytical methods have been reported for the analysis of L-AA in food and beverage products, some of which include titration, electrochemical methods [6], spectrophotometry [7], potentiometric methods, enzymatic methods and chromatographic methods [8]. However, advantages such as optimal separation potential, ease of operation, rapid analysis time and high accuracy and sensitivity have contributed to the use of high performance liquid chromatography (HPLC) as a preferred method for vitamin C analysis [9,10]. Further, factors such as robustness, cost effectiveness, simplicity and a common frequency range for which many analytes absorb light, favours the use of ultra-violet (UV) detection as a preferred detection method [11].

Equally important to the selection of an analytical method is the quality, reliability, and regularity of results produced by such a method [12]. Hence, the process of method validation is clearly warranted as a means to verify that the HPLC method employed is

acceptable for the procedure/purpose it is intended for [13]. Additionally, national government legislation (Regulation 146/2010 as part of the Foodstuffs, Cosmetics and Disinfectants Act of South Africa, Act 54 of 1972) and several international regulatory organizations namely, the Food and Drug Administration (FDA), World Health Organization (WHO), U.S. Department of Agriculture (USDA), Centers for Disease Control and Prevention (CDC) and the Codex Alimentarius Commission (CAC) are compelling food and beverage manufacturers to use validated analytical methods to analyze products [14].
The literature reveals validation of UV-HPLC methods for the measurement of vitamin C has been performed largely on pharmaceutical products [15-17]. Few UV-HPLC methods have been validated for the measurement of vitamin C in food products [18,19].

The aim of this study is to validate a reversed-phase HPLC method in accordance with ISO/IEC 17025 requirements using UV detection for the quantification of L-AA in several food samples as well as commercial beverages.

4.2 Method and Materials

4.2.1 Chemicals and Reagents

The chemicals formic acid, sulphuric acid (H_2SO_4), hydrochloric acid (HCl), sodium hydroxide (NaOH) and methanol were purchased from Merck (Johannesburg, South Africa). Metaphosphoric acid (MPA) and L-AA was purchased from Sigma-Aldrich (Johannesburg, South Africa). Acetonitrile (gradient grade for liquid chromatography) was purchased from Saarchem (Johannesburg, South Africa). HPLC grade water was obtained from a Millipore Synergy water purification system (Cape Town, South Africa). A standard stock solution of L-AA (1 mg/ml) was prepared in 4.5% MPA in distilled water (v/v) prior to analysis each day and stored away from light at 4 ^{o}C when not in use.

4.2.2 Equipment

All analyses were carried out on an Agilent 1200 Series HPLC system purchased from Agilent Technologies (Johannesburg, South Africa). The chromatographic system was equipped with a reverse phase C_{18} column (150 mm x 4.6 mm, 0.5 µm in particle size) purchased from YMC Co., Ltd. (Cape Town, South Africa), a quaternary pump and a UV detector set at 245 nm. Two different isocratic mobile phases were tested: (a) 0.01% solution of sulphuric acid in distilled water (v/v) adjusted to pH 2.6 [20] and (b) distilled water/acetonitrile/formic acid (99: 0.9: 0.1, v/v/v) adjusted to pH 2.6. The flow rate was set at 1 ml/min and the injection volume was 20 µL. The analytical column temperature was maintained at RT (23 °C). Nylon 0.45 µm syringe filters were purchased from GVS Filter Technologies (Johannesburg, South Africa).

4.2.3 Preparation of Standards

Several standard solutions of varying concentrations (1 to 300 µg/ml) were prepared from diluting the L-AA stock solution (1 mg/mL) with the 4.5% MPA to determine a suitable calibration standard range for routine analysis.

4.2.4 Sample extraction and preparation

Onions, berries, apples, tomatoes, camu powder, breakfast cereal and several commercial beverages (dragonfruit flavoured vitamin water, orange flavoured vitamin water, pressed berry juice, pressed orange juice, tropical juice) were purchased from several retail outlets

within the Cape Town metropolitan area, South Africa. These samples were chosen to evaluate the effect of different matrices on the method performance parameters of the assay. Different samples were used for different parameters tested. The extraction of L-AA from food, fruit and vegetable samples was performed as described by Odriozola-Serrano and co-workers [18] with slight modifications . A representative portion of each food product (weight varied from 40 to 200 mg, depending on colour and texture) was added to 25 mL of 4.5% MPA solution and thoroughly homogenized for approximately 1 min. The homogenate was centrifuged at 4000 rpm for 2 min. The supernatant was filtered through a Nylon 0.45 µm syringe filter and the resulting extracts were aliquoted into 1.5 mL eppendorf tubes and stored at -80 ^{o}C until the time of analysis. Prior to analysis, the extracted samples were defrosted in a cold water bath, before being appropriately diluted with 4.5% MPA. Diluted sample extracts were stored away from light at 4 ^{o}C until the time of injection. Beverage samples were aliquoted into 1.5mL eppendorf tubes on the day they were purchased and stored at -80 ^{o}C until the time of analysis. Prior to analysis, they were appropriately diluted in 4.5% MPA and stored away from light at 4 ^{o}C until the time of injection.

4.2.5 Preparation of Quality Control (QC) samples:

A 250 mL synthetic juice formulation comprising sucrose (19 g), citric acid (1 g) and sodium citrate (0.023 g) was prepared, while 250 mg of L-AA was added to give a final concentration of 1 mg/mL. Three QC samples were subsequently prepared as shown in Table 1 and assayed in duplicate. Subsequently, several aliquots of each QC sample were prepared and stored at -40 ^{o}C until time of analysis. Prior to analysis, the frozen QC aliquots were defrosted in a cold (21 ^{o}C) water bath.

Table 1: L-ascorbic acid quality control preparation

QC samples	L-ascorbic acid synthetic juice formulation (1 mg/mL) (volume in µL)	4.5% Metaphosphoric acid solution (volume in µL)
QC 1	6.5	993.5
QC 2	55	945
QC 3	115	885

4.2.6 Pre-validation components

4.2.6.1 Equipment and analyzer qualification

The installation, operational and performance qualification of the Agilent 1200 Series HPLC system was performed at the laboratory site by Agilent Technologies (Cape Town, South Africa). All other equipment (pipettes, thermometers, analytical balances, pH meter, Millipore water purification system, water baths and centrifuges) and glassware were serviced and calibrated by an accredited metrology service (Cape Metrology Field, Cape Town, South Africa). Subsequently the performance of all equipment and glassware were verified on a continuous basis to ensure functioning was optimal and in agreement with manufacturer specifications at all times of analysis. Verification procedures included cleaning, calibration and testing the performance of equipment with certified reference materials (CRMs). Verification forms were created and all actions documented on a routine basis in terms of repeatability, intermediate precision, accuracy, possible deviations from acceptable criteria and any troubleshooting performed.

4.2.6.2 Reagent and standards stability

The stability of the extraction solvent (4.5% MPA stock solution) and L-AA standards (20 and 75 µg/mL) was tested. The L-AA standards were prepared in duplicate and one was stored at RT and the other at 4 ^{o}C prior to and between analyses. The standard samples were then assayed at 0, 4 and 8 hr. Results were evaluated for significant ($p<0.05$) differences for retention times and peak absorbance area (PAA).

The stability of the mobile phase [distilled water/acetonitrile/formic acid (99: 0.9: 0.1, v/v/v)] was established by analysis of a standard sample (10 µg/mL) at 24 hr intervals for 72 hr using the same mobile phase which was stored at room temperature, and comparing the results with that obtained from a freshly prepared standard (10 µg/mL) solution using a freshly prepared mobile phase. Results were evaluated for significant ($p<0.05$) differences for retention times and PAA.

4.2.6.3 QC: Monitoring of method performance

An aliquot of each QC sample was assayed in duplicate for a period of thirty days at 24 hr intervals. Twenty data points were then selected from thirty data points from which the mean, three standard deviations, coefficient of variation (COV) and acceptable tolerance

limits were determined [21]. Subsequently, the performance of the HPLC method was evaluated over twenty two days using the QC samples.

4.2.6.4 Mobile phase optimization

Both mobile phases [distilled water/acetonitrile/formic acid (99: 0.9: 0.1, v/v/v) and 0.01% solution of sulphuric acid] were evaluated for optimal separation of L-AA from other sample component peaks. A tomato sample extract was assayed with both mobile phases and the resulting chromatograms were evaluated.

4.2.7 Method performance parameters

4.2.7.1 Range and linearity

To determine the range for which L-AA can be quantified with acceptable accuracy, precision and linearity, a series of standards (1 to 300 µg/mL) prepared from a standard stock solution of L-AA (1 mg/mL) was assayed in duplicate over five days. The results were evaluated by two statistical approaches. The first approach involved plotting the relative peak absorbance area (mAU) against the logarithmic concentration (µg/mL) of L-AA standards. A horizontal line should encompass the entire linear range, with positive and negative digressions at low and high concentrations, respectively. Parallel lines were constructed at 95% and 105% of the horizontal relative response line and the intersection points illustrated where the method was non-linear [13]. In the latter approach, the peak absorbance areas were plotted against the L-AA concentrations (µg/mL) and the results were assessed using least squares linear regression [22]. Subsequently five samples (camu powder extract, dragonfruit flavoured vitamin water, orange flavoured vitamin water, pressed berry and tropical juices) were appropriately diluted at four different concentrations within the linear standard calibration range. Their responses were evaluated for acceptable linearity, accuracy and precision.

4.2.7.2 Precision and accuracy

The precision of the assay was evaluated by intermediate precision, intra-assay precision and repeatability of injection [22]. Intermediate precision was performed by assaying three L-AA standards (10, 50 and 125 µg/mL) in triplicate over three separate days. Intra-assay precision was performed by assaying QC samples (6.5, 55 and 115 µg/mL) in duplicate three times between other sample runs on the same day. Injection repeatability was

performed by injecting a QC sample (55 µg/mL) six times. The mean retention times and L-AA concentrations were calculated. The COVs were calculated and assessed for acceptable precision. The accuracy of the method was verified by carrying out recovery studies [23]. The spiked-placebo recovery method was performed by assaying three replicates of QC (6, 60 and 120 µg/mL) samples. The standard addition recovery procedure was performed at two concentration levels for each sample tested. The concentrations of L-AA added to the samples were: 55 and 115 µg/mL to camu powder extract, 7 and 60 µg/mL to tomato extract and 5 and 10 µg/mL to onion extracts. For each addition level, three determinations were performed and the recovery of L-AA was calculated.

4.2.7.3 Limit of detection (LOD) and quantification (LOQ):

The LOD and LOQ of the method were determined from the L-AA standard calibration lines that were used to establish linearity (5 to 125 µg/mL) and calibration lines containing concentration levels close to the approximate LOD [24]. The equations 3.1 and 3.2 were used to calculate LOD and LOQ respectively. Two samples (apple extract and a QC sample) were diluted to concentration levels at or around the LOQ concentration and assayed in triplicate. The responses were evaluated for accuracy, precision and linearity.

$LOD = [3.3 \times (\sigma)]/S$.....**Equation 4.1**

$LOQ = [10.0 \times (\sigma)]/S$.....**Equation 4.2**

where:
σ:standard deviation of the response
S:the slope of the calibration curve

4.2.7.4 Specificity

The specificity of the method was assessed in two ways. The chromatogram of the L-AA standard (50 µg/mL) was compared to those obtained for sample extracts (cereal and tomato extracts). They were evaluated for differences in retention times and the resolution of the L-AA peak from other peaks. In a second experiment, a tomato sample extract was exposed to stress conditions by incubating the sample at 80 ^{o}C for two hours to partially destroy L-AA and generate degradation products. The post stressed sample was injected and the resulting chromatogram was checked for the presence of interfering peak(s) from

degradation products close to the retention time of the L-AA peak. The photo-diode array detector was used to determine the peak purity.

4.2.7.5 Sample stability

The stability of samples was determined for short-term, long-term and freeze-thaw stability. Samples containing high and low concentrations of L-AA were evaluated. Short-term stability was established by storing samples at 4 ^{o}C for a period of 24 hr. Long-term stability was determined by storing samples at -80 ^{o}C and testing after one week, one month, and two month intervals. The sample extracts were allowed to thaw at RT prior to analysis. Freeze-thaw stability was assessed by thawing and freezing samples over three days. Three freeze-thaw cycles were performed. All samples were initially assayed fresh and the results were compared to results obtained from samples subjected to the stability conditions. Results were evaluated for significant differences.

4.2.7.6 Robustness

The ability of the HPLC assay to remain unaffected by small, but deliberate changes in chromatographic conditions was evaluated to assess the reliability of the method during routine sample analysis [25]. The method was subjected to a variety of conditions namely, changes in composition and pH of mobile phase and changes in column temperature. Results were compared to those obtained with the optimized HPLC method. Recoveries and precision between results of the optimized method and method with varied conditions were determined.

4.2.7.7 System suitability

System suitability parameters such as capacity factor, number of theoretical plates, resolution, peak asymmetry factor and selectivity were determined in accordance with the FDA: Reviewer Guidance [22]. A tomato extract sample (3 µg/mL L-AA) was injected five times and the results were evaluated for system suitability according the acceptance criteria set out in the FDA/CDER: Reviewer Guidance [22].

4.2.7.8 Statistical Analysis of Results:

The mean, standard deviation, and COV i.e. the relative standard deviation %SD were determined for all data. The statistical Microsoft Excel ® software package was used to

analyze data. Analysis of Variance (ANOVA) was used to ascertain whether the means between sample/standard experimental groups differ significantly ($p<0.05$, significant; $p>0.05$, not significant) at a 95% confidence level. The Levene's Test was used to determine normality between sample/standard experimental groups. If data did not show a normal distribution, a logarithmic transformation was applied. Subsequently, if data did not demonstrate a normal distribution, the Kruskal-Wallis test was used. The paired T-test was used to show differences between two sample/standard experimental groups.

4.3. Results

4.3.1 Reagent, standards and quality control stability

Table 2 summarizes the results of the MPA stability study. The MPA solvent is stable at RT and 4 ^{o}C for up to 8 hr. This is confirmed by no significant ($p>0.05$) differences observed between retention times and PAA for the two standards assayed. After 4 hr a slight decrease in PAA was observed for the L-AA standard (75 µg/mL) under both RT and 4 ^{o}C conditions as depicted in Figure 1, however, this decrease was not significant ($p>0.05$). Good repeatability (COV <5%) was achieved for PAA and retention time measurements. These results indicate that the extraction solvent (MPA) and the L-AA standards dissolved in the extraction solvent were stable for up to 8hr at both RT and 4 ^{o}C.

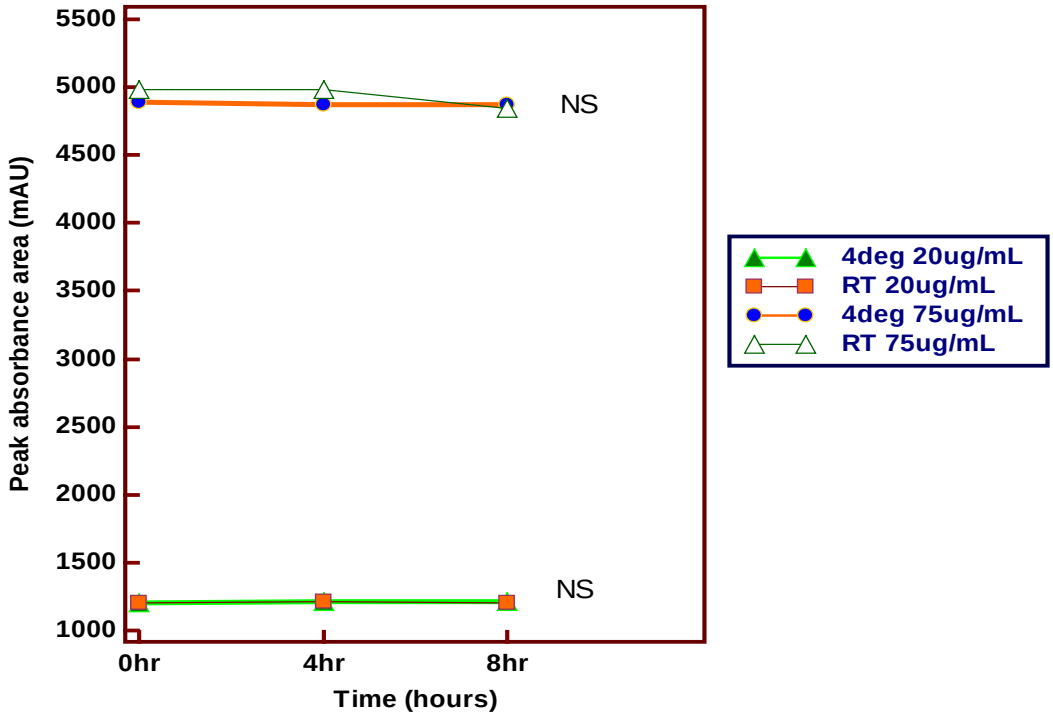


Figure 1: Stability of 4.5% MPA and L-AA standards under refrigerated (4^{o}C) and room temperature (RT) conditions. Abbreviation: NS, no significant ($p>0.05$) differences.

Table 2: Stability of metaphosphoric acid over an eight hour period under room temperature and refrigerated conditions

		0 hours (fresh)		4 hours				8 hours			
				RT		4ºC		RT		4 °C	
		Ret. time (min)	PAA (AU)	Ret. time (min)	PAA (AU)	Ret. time (min)	PAA (AU)	Ret. time (min)	PAA (AU)	Ret. time (min)	PAA (AU)
20 µg/mL Std	**Mean**	3.61 ± 0.03[a]	1207 ± 4[b]	3.58 ± 0.01[a]	1209 ± 21[b]	3.58 ± 0.01[a]	1210 ± 26[b]	3.58 ± 0.01[a]	1209 ± 25[b]	3.58 ± 0.01[a]	1210 ± 27[b]
	COV	0.87	0.38	0.15	1.77	0.15	2.20	0.16	2.12	0.15	2.30
75 µg/mL Std	**Mean**	3.05 ± 0.03[a]	4982± 54[b]	3.04 ± 0.01[a]	4978 ± 0.78[b]	3.03 ± 0.01[a]	4868 ± 163[b]	3.044 ± 0.01[a]	4846 ± 196[b]	3.035 ± 0.01[a]	4869 ± 153[b]
	COV	1.019	1.092	0.256	0.016	0.023	3.353	0.465	4.048	0.233	3.154

Values in columns are means ± SD of three determinations (n = 3). Retention times and peak area values in each row with the same letter are not significantly (p>0.05) different. Abbreviations: AU, absorbance unit; COV, coefficient of variation; RT, room temperature; Ret. Time, retention time; PAA, peak absorbance area. 0.01% sulphuric acid solution was used as the mobile phase.

4.3.2 Stability of the mobile phase

Table 3 summarizes the results of the stability study for the mobile phase. The results indicate that the mobile phase was stable for up to 48 hr at RT. This was confirmed by significantly ($p<0.05$) shorter retention times achieved with the 72 hr stored mobile phase standard compared to that obtained with the fresh, 24 and 48 hr stored mobile phase. Moreover, the good precision which was expressed as the COV was observed (<7%) for all runs performed. The PAA showed no significant ($p>0.05$) differences for all runs.

Table 3: Measurement of an L-AA standard (10 µg/mL) to test the stability of the mobile phase

	0 hours (fresh)		24 hours		48 hours		72 hours	
	Ret. time (min)	**PAA (AU)**	**Ret. time (min)**	**PAA (AU)**	**Ret time (min)**	**PAA (AU)**	**Ret. time (min)**	**PAA (AU)**
Mean	3.049 ± 0.021[a]	697 ± 35[c]	3.047 ± 0.004[a]	658 ± 42[c]	3.029 ± 0.004[a]	659 ± 45[c]	2.833 ± 0.046[b]	630 ± 2.0[c]
COV	0.672	5.033	0.143	6.398	0.133	6.844	1.641	0.319

Values in columns are means ± SD of three determinations (n = 3). Same letter superscripts indicate no significant ($p>0.05$) differences. Different letter superscripts indicate significant ($p<0.05$) differences. Abbreviations: AU, absorbance unit; COV, coefficient of variation PAA, peak absorbance area; Ret. time, retention time.

4.3.3 Stability of QC samples

Figure 2 illustrates the peformance of the QC samples over twenty two days. Figure 2a demonstrates one outlier on day 6 for QC 1 (6.5 µg/mL). From day 7 onwards, control data fell back into the ± 2SD. Figure 2b shows most data points falling within the ± 1SD, with a gradual negative trend occurring from day 17. Figure 2c shows data points are randomly distributed around the mean, with all points falling within ± 2SD. The precision of the QC samples reflects the degree of variation of all data points. The closeness of the data points to the true value determines the accuracy of the QC samples [26]. The intermediate precision expressed as the COV fell well within 5% and the accuracy expressed as percent recovery was satisfactory ranging from 99 to 102% (Table 4). The results indicate that L-AA QC samples were stable for approximately one month at -20 ^{o}C, demonstrating repeatable and accurate results at a 95% confidence level. A new batch of QC material was prepared at the beginning of each new month.

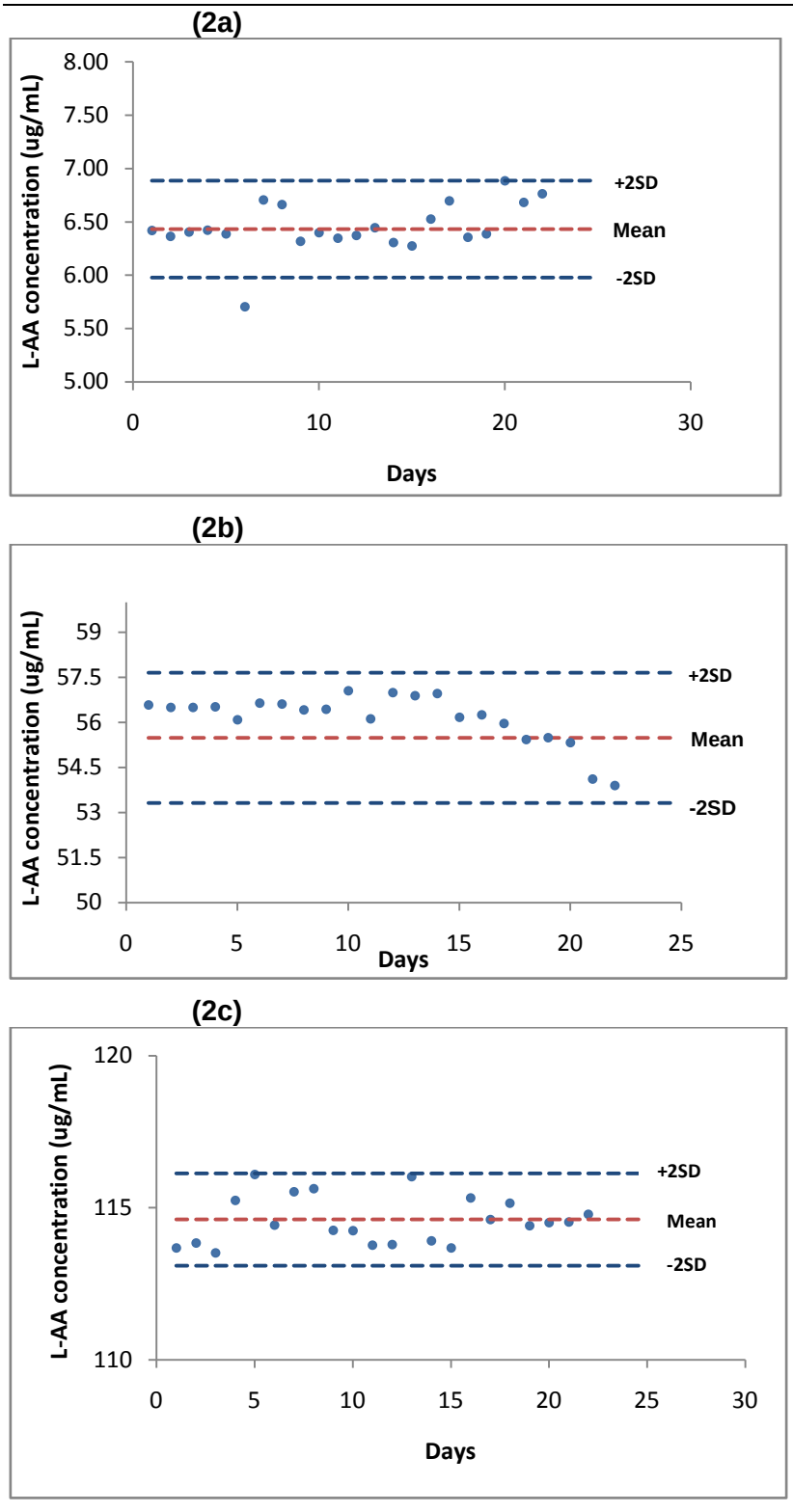


Figure 2: Evaluation of stability of QC samples: (a) QC1 (6.5 µg/mL); **(b)** QC2 (55 µg/mL); **(c)** QC3 (115 µg/mL) over twenty days.

Table 4. Accuracy, precision and stability of QC sample (60 µg/mL)

Expected concentration (µg/mL)	Acceptable tolerance limits (µg/mL)	Measured concentration (µg/mL)$_1$	COV	Average Recovery (%)
6.5	5.98 to 6.89	6.45 ± 0.23	3.69	99.21
55.00	53.32 to 57.65	56.14 ± 0.84	1.50	102.07
115	113.09 to 116.13	114.59 ± 0.79	0.69	99.64

$_1$Values are means ± SD of 22 determinations. Abbrevations: COV, coefficient of variation; QC, quality control.

4.3.4 Mobile phase optimization

Two mobile phases were tested to obtain optimal separation of L-AA from other existing sample components. The chromatograms of a tomato sample extract eluted with both mobile phases are shown in Figure 3a and 3b. Figure 3b illustrates significantly ($p<0.05$) shorter retention times of L-AA obtained for the tomato sample, showing poor resolution and many interfering peaks. Other sample component peaks are eluting at the same time as the L-AA peak, resulting in a poorly resolved L-AA peak. In contrast, Figure 3a demonstrated good separation and resolution of the L-AA peak using the mobile phase consisting of distilled water/acetonitrile/formic acid (99: 0.9: 0.1, v/v/v). Hence, the water/acetonitrile/formic acid mobile phase was subsequently used as the mobile phase for all further sample analysis.

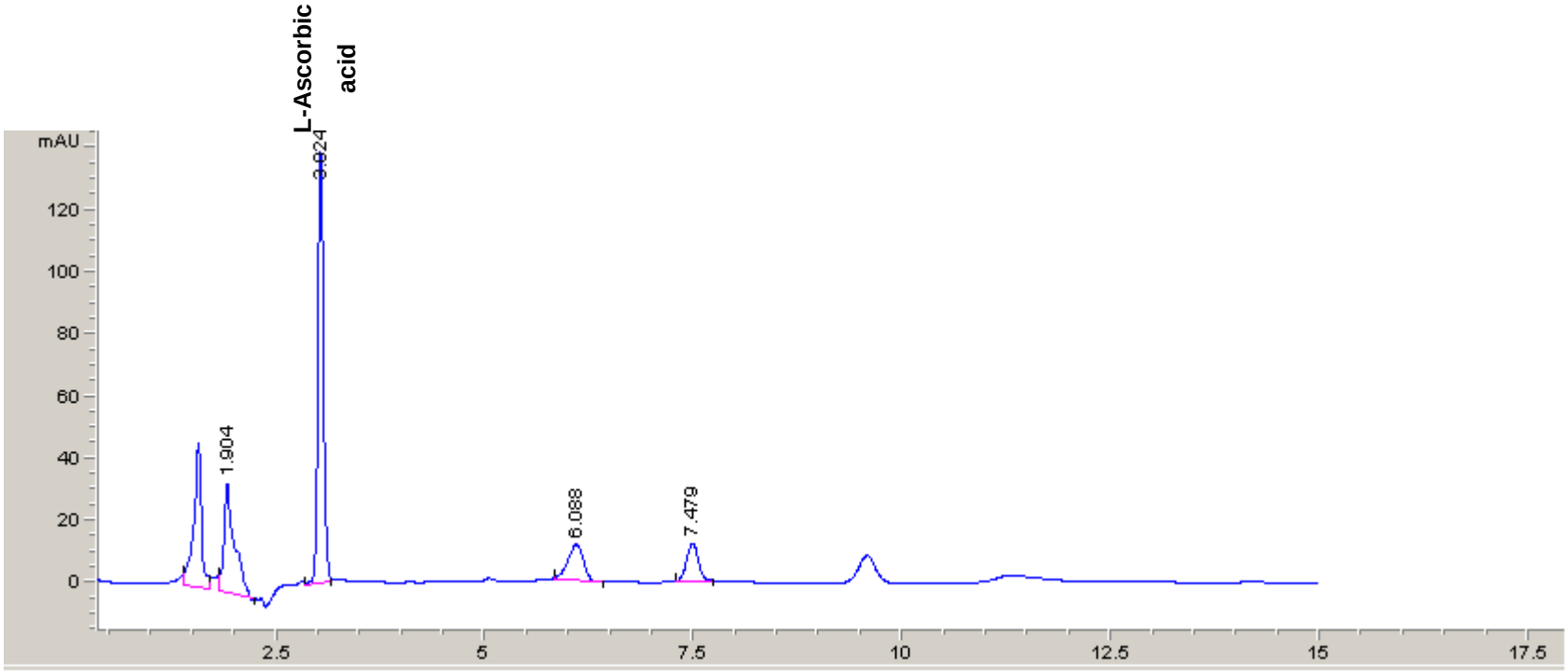


Figure 3a. Chromatogram of a tomato extract eluted with mobile phase consisting of distilled water/acetonitrile/formic acid (99: 0.9: 0.1, v/v/v)

.

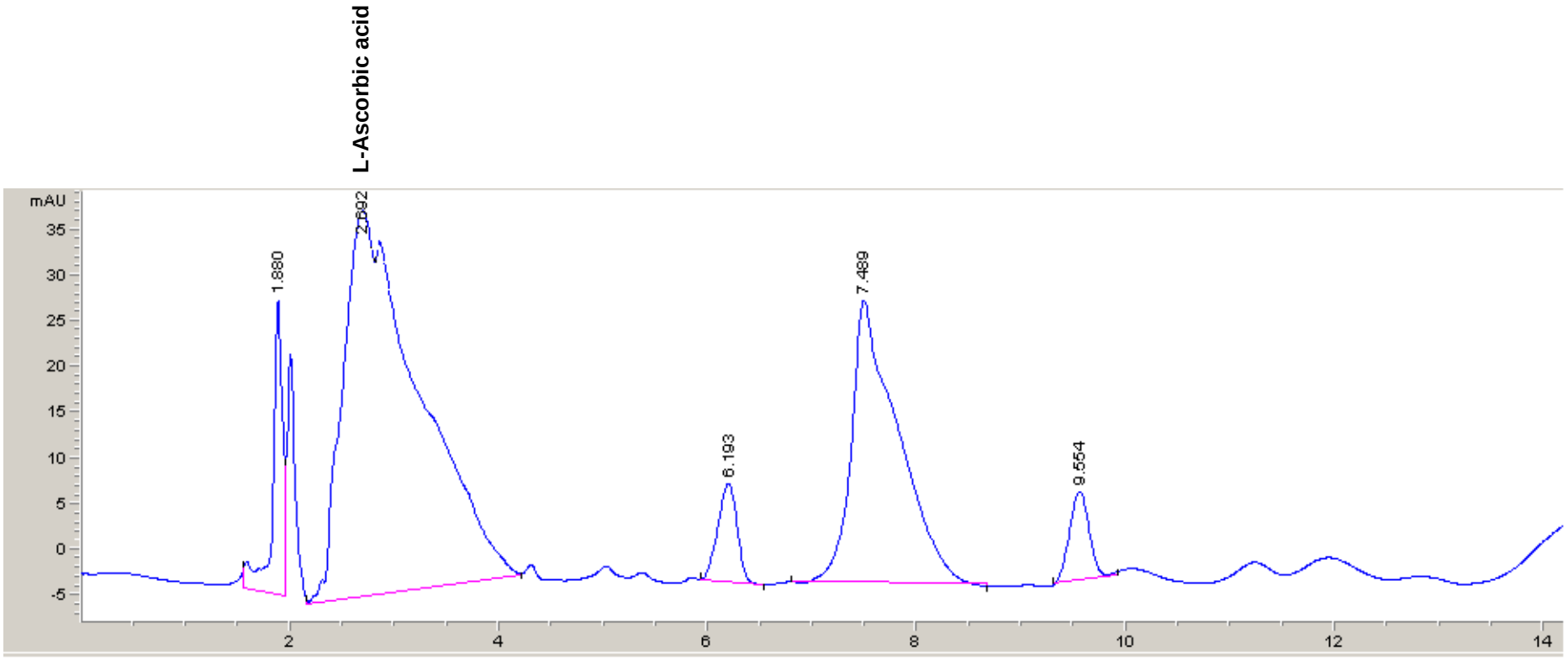


Figure 3b. Chromatogram of a tomatoe extract eluted with mobile phase consisting of 0.01% sulphuric acid solution.

4.3.5 Method performance parameters

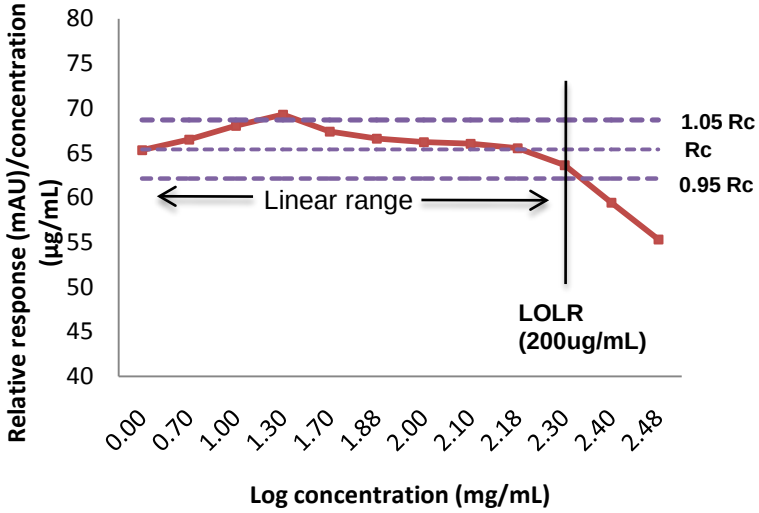
4.3.5.1 Linearity and range

Figure 4a illustrates the linear relationship between relative responses (mAU) and the logarithmic concentrations for the L-AA standards. The intersection point is at the 200 µg/mL, standard, after which the method becomes non-linear. In the second approach to determine linearity, Figure 4b illustrates the relationship between the peak absorbance area and concentration for the L-AA standards. The results indicate that good linearity was observed from 5 to 200 µg/mL for the L-AA standards, however, at high concentrations (>200 µg/mL) poor linearity was observed. In order to maintain good turn-around times for samples, a narrower calibration standard range of 5 to 125 µg/mL was utilized as the range for all further validation and sample analysis. Table 5 summarizes the accuracy, precision and linearity of the L-AA standards (5 to 125 µg/mL). The correlation coefficient (r^2=0.999) indicates that an excellent correlation exists between peak absorbance area and concentration of L-AA.

Furthermore, the intermediate and intra-assay precision expressed as the COV was acceptable with all runs falling within 10%. Similarly, good accuracy was observed with percent recovery ranging from 89 to102%.

Table 6 summarizes the accuracy, precision and linearity of several samples assayed at four concentration levels of the L-AA standard range. A good correlation coefficient (≥ 0.995) was observed for all samples. The precision expressed as the COV between all concentration levels tested was less than 10% and the accuracy expressed as percent recovery ranged from 92 to 120%. Hence, the proposed method showed acceptable precision and accuracy, and an excellent correlation between peak absorbance area and concentration for all samples and standards assayed.

4a



4b

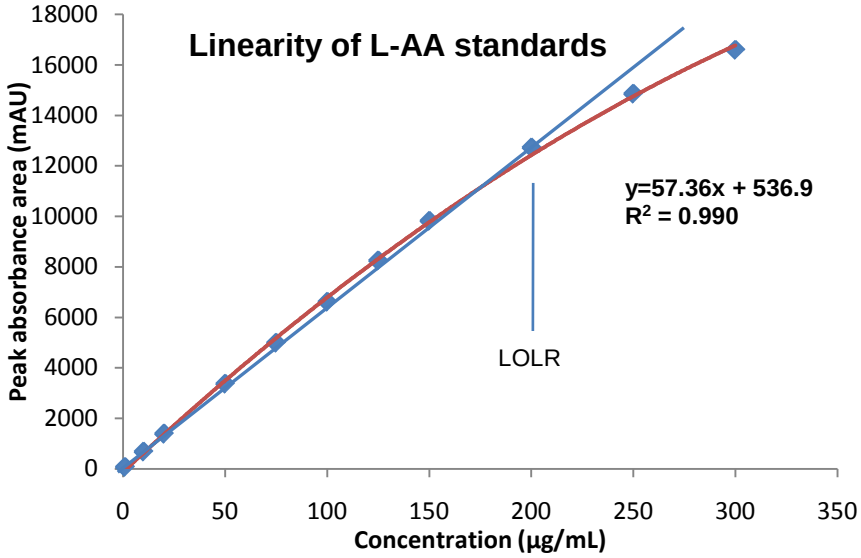


Figure 4. Graphical illustration of linearity plot for L-AA standards by HPLC analysis. (a) Relative response vs logarithmic concentration of L-AA standards. **(b)** Peak absorbance area vs concentration of L-AA standards. Abbreviations: Rc, Line of constant response, LOLR; Limit of linear response

Table 5. Linearity, accuracy and precision of L-AA standards (5 to 125 µg/mL)

L-AA standards (µg/mL)	PAA (AU) [1]	COV [2]	COV [3]	Accuracy(µg/mL)	Recovery (%)	r^2 [5]
5	332.66 ± 3.43	1.03	1.25	4.45	89	0.999
10	680.30 ± 50.38	7.4	0.91	9.73	97.3	
20	1385.93 ± 31.99	2.3	0.19	20.43	102	
50	3368.95 ± 32.24	0.96	0.32	50.55	101.1	
75	4994.81 ± 20.91	0.42	0.08	75.23	100.31	
100	6619.31 ± 44.02	0.67	0.07	99.89	99.89	
125	8254.59 ± 25.57	0.31	0.15	124.72	99.78	

[1]PA expressed as means ± SD of five determinations (n=5). [2]Precision between different days (n=5). [3]Precision within individual runs (n=3). [4]Concentration calculated using $y=mx+c$. [5] Mean correlation coefficient (r^2) n=5. Abbreviations: AU, absorbance unit; COV, coefficient of variation; PA, peak absorbance area; r^2, correlation coefficient.

Table 6. Linearity of samples (peak absorbance area vs dilution factor)

Samples (µg/mL)	Dilution factor	PAA (mAU)	Measured L-AA concentrations (µg/mL)$_1$	Recovery (%)	COV$_2$	r^2
Dragonfruit vitamin water	1/50	684.25 ± 1.41	489.50	102.13	2.46	0.997
(479.28)	1/25	1290.47 ± 0.44	474.50	99.00		
	1/9	3514.37 ± 3.27	461.34	96.26		
	1/4	7932.46 ± 0.02	479.32	100		
Camu powder extract	1/45	285.54 ± 3.15	168.30	95.22	2.12	0.999
(176.74)	1/8	1381.97 ± 71.00	163.04	92.25		
	1/4	2861.67 ± 9.69	171.40	96.97		
	1/2	5620.27 ± 21.69	169.44	95.86		
Orange vitamin water	1/4	7996.65 ± 7.03	480.00	100	1.87	0.999
(480.00)	1/10	3294.70 ± 0.11	494.20	102.96		
	1/25	1361.94 ± 0.13	502.00	104.58		
	1/50	684.25 ± 1.41	489.50	101.98		
Pressed berry juice	1/2	3784.89 ± 55.15	113.74	103.23	6.28	0.995
(110.14)	1/5	1774.24 ± 12.42	131.70	119.58		
	1/10	870.84 ± 3.67	126.30	114.67		
	1/20	435.99 ± 2.98	120.40	109.31		
Tropical juice	1/5	7548.66 ± 5.79	570.03	100.00	3.81	0.999
(570.03)	1/12	3230.86 ± 3.54	581.46	102.00		
	1/20	1993.46 ± 2.55	593.39	104.10		
	1/100	396.43 ± 3.71	542.43	95.16		

PAA values are means ± SD of two determinations (n = 2); $_1$ L-AA concentrations calculated using $y=mx+c$; $_2$Precision between four concentration levels.
Abbreviations: AU, absorbance unit; COV, Coefficient of variation; PAA, Peak absorbance area; r^2, Correlation coefficient

4.3.5.2 Precision and accuracy:

Table 7 summarizes the precision and accuracy of the current method using standards and QCs. The method showed satisfactory intermediate precision of the L-AA standards. All the COV values achieved for PAA and retention times were less than 1% and 3%, respectively. Additionally, good intra-assay precision (COV <1%) were observed for both retention times and L-AA concentrations of the QC samples. Similarly, the injection precision was acceptable demonstrating COVs of less than 1% for both retention times and peak absorbance areas. These results show excellent intermediate, intra-assay and injection precision for the current method.

Satisfactory results were obtained for accuracy of the current method with mean recovery percentages ranging from 99 to 103% for QC (Table 7). Similarly, acceptable mean recoveries were obtained for samples (camu powder, tomatoe and onion) assayed by the standard addition method (Table 8). Recoveries ranged from 92 to 96%. Furthermore, the ability of the current method to produce accurate results with good precision were confirmed by the low COVs (< 1%) achieved for results of samples reported in Table 8.

Table 7. Precision of the UV-HPLC method for the determination of L-AA

	L-AA QC/STD expected concentration (µg/mL)	Ret time (min)	COV	Measured concentration (µg/mL)	COV	REC_4 (%)
Intermediate precision$_1$	10 (L-AA STD)	3.038 ± 0.02	0.54	9.14 ± 2.46	2.46	
	50 (L-AA STD)	3.041 ± 0.01	0.30	48.61 ± 2.81	2.81	
	125 (L-AA STD)	3.042 ± 0.01	0.46	123.18 ± 1.96	1.96	
Intra-assay precision$_2$	6.5 (QC)	3.023 ± 0.004	0.12	6.59 ± 1.644	0.38	101.41 ± 0.40
	55 (QC)	3.023 ± 0.003	0.11	56.76 ± 6.780	0.19	103.19 ± 0.19
	115 (QC)	3.022 ± 0.003	0.09	114.05 ± 8.568	0.12	99.18 ± 0.14
Injection precision$_3$	55 (QC)	3.031 ± 0.02	0.52	54.11 ± 0.49	0.49	

$_1$Intermediate precision values are means ± SD of three determinations assayed over three separate days. $_2$Intra-assay precision values are means ± SD of three determinations assayed on the same day. $_3$Injection precision values are means ± SD of six determinations (n=6). $_4$Spiked recovery method for accuracy. Abbreviations: COV, coefficient of variation; L-AA STD, L-ascorbic acid standard; QC, quality control; REC, recovery; Ret, retention.

Table 8: Standard addition recovery method of the UV-HPLC assay to determine L-ascorbic acid in food products

Sample	Initial concentration (µg/mL)	Concentration after addition (µg/mL)		Recovery (%)		Mean Recovery (%)[3]	Mean COV
		Level I_1	Level II_2	Level I	Level II		
Camu powder extract	7.51	63.52 ± 0.28	118.09 ± 2.17	93.35	92.15	92.75 ± 0.85	0.92
Tomato extract	26.22	32.83 ± 3.68	82.21 ± 2.05	94.49	93.31	93.91 ± 0.84	0.89
Onion extract	1.24	6.02 ± 15.03	10.74 ± 25.25	95.59	94.98	95.29 ± 0.43	0.45

[1]55 µg/mL to camu powder extract; 7 µg/mL to tomato extract; 5 µg/mL to onion extracts. [2]115 µg/mL to camu powder extract ; 60 µg/mL tomato extract; 10 µg/mL to onion extracts. [3]Recovery mean ± standard deviation (n=3 in each level).

4.3.5.3 Limit of detection and quantification:

The current method showed good sensitivity with an LOD and LOQ of 0.61 µg/mL and 1.84 µg/mL, respectively (Table 9). The LOD and LOQ values were calculated from the regression equation obtained from the 0.1 to 5 µg/mL linear range, due to the lower standard error achieved for this linear range. The standard error for the intercepted point for the 0.1 to 5 µg/mL linearity range was significantly ($p<0.05$) lower (9.65) than that obtained for the 5 to 125 µg/mL linearity range (29.64).

Subsequently, samples diluted to the LOQ level demonstrated good linearity ($r^2 = 0.991$ and 0.972) for the QC and apple extract samples, respectively (Table 10). Accuracy which was expressed as percent recovery was satisfactory for QC (87 to 103%) and apple extract samples (91 to 99%) for all concentration levels tested (Table 10). Additionally, acceptable precision (<10%) within and between dilutions were observed. These findings demonstrate acceptable accuracy (within ± 20% of target value), precision (within 20% of the COV) and linearity at the limit of quantification for samples tested [26].

Table 9. LOD and LOQ for the UV-HPLC method to determine L-AA

Linearity range (µg/mL)	Regression equation$_1$	r^2 (%)	Standard error$_2$	LOD (µg/mL)	LOQ (µg/mL)
5 - 125	$y = 65.87x + 39.13$	0.999	29.64	1.48	4.50
0.1 - 5	$y = 73.67x + 5.30$	0.995	9.65	0.61	1.84

$_1y = mx+c$; $_2$Standard error of intercept point of calibration line. Abbreviations: LOD, Limit of Detection; LOQ, Limit of Quantification.

Table 10. Accuracy, precision and linearity of samples at limit of quantification concentration level

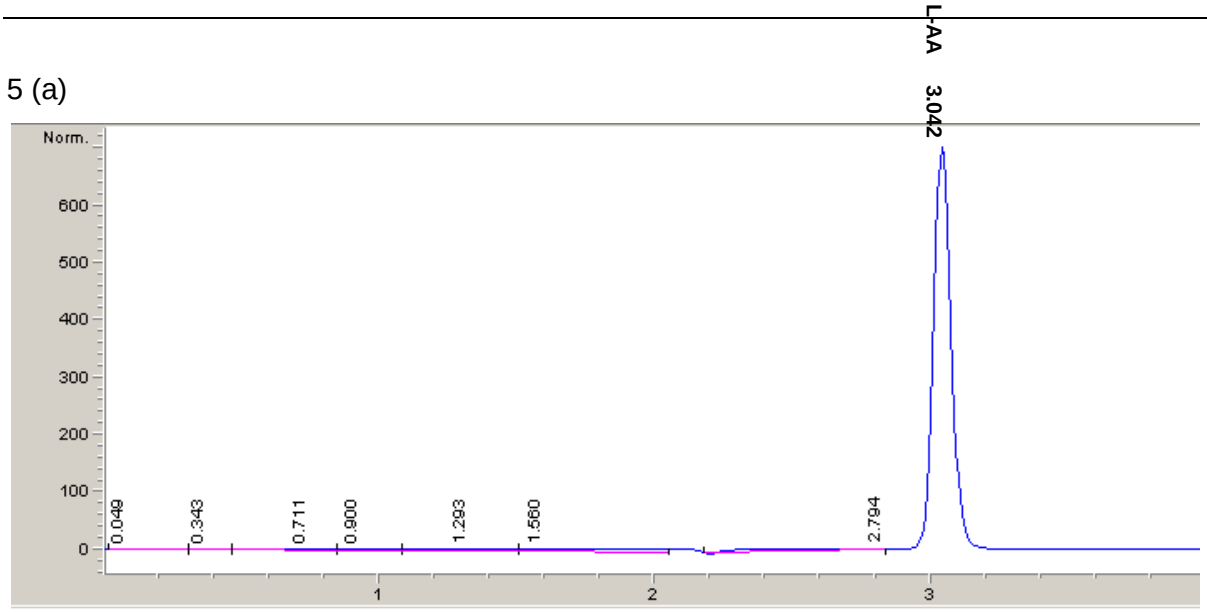
Samples (µg/mL)	Dilution factor (%)	PAA (AU)$_1$	COV$_2$	Concentration (µg/mL)	Recovery (%)	r^2	COV%$_3$
QC1 (6.5)	1/4	125.57 ± 1.88	0.99	1.31	87.48	0.991	3.10
	1/3	161.48 ± 1.47	0.61	1.86	92.86		
	1/2.5	201.73 ± 0.51	0.17	2.47	102.85		
	1/2	238.21 ± 0.83	0.23	3.02	100.75		
Apple extract (8.66 ± 44.64)	1/5	143.56 ± 3.93	2.74	1.59	91.54	0.972	7.41
	1/4.5	164.24 ± 0.97	0.59	1.90	98.70		
	1/4	175.83 ± 0.65	2.08	2.08	95.87		
	1/3.5	193.83 ± 3.12	2.35	2.34	94.92		

$_1$Mean ± standard deviation (n=3). $_2$Precision of replicates. $_3$Precision between dilution levels. Abbreviations: AU, absorbance unit; COV, coefficient of variation; PAA, peak absorbance area; r^2, correlation coefficient.

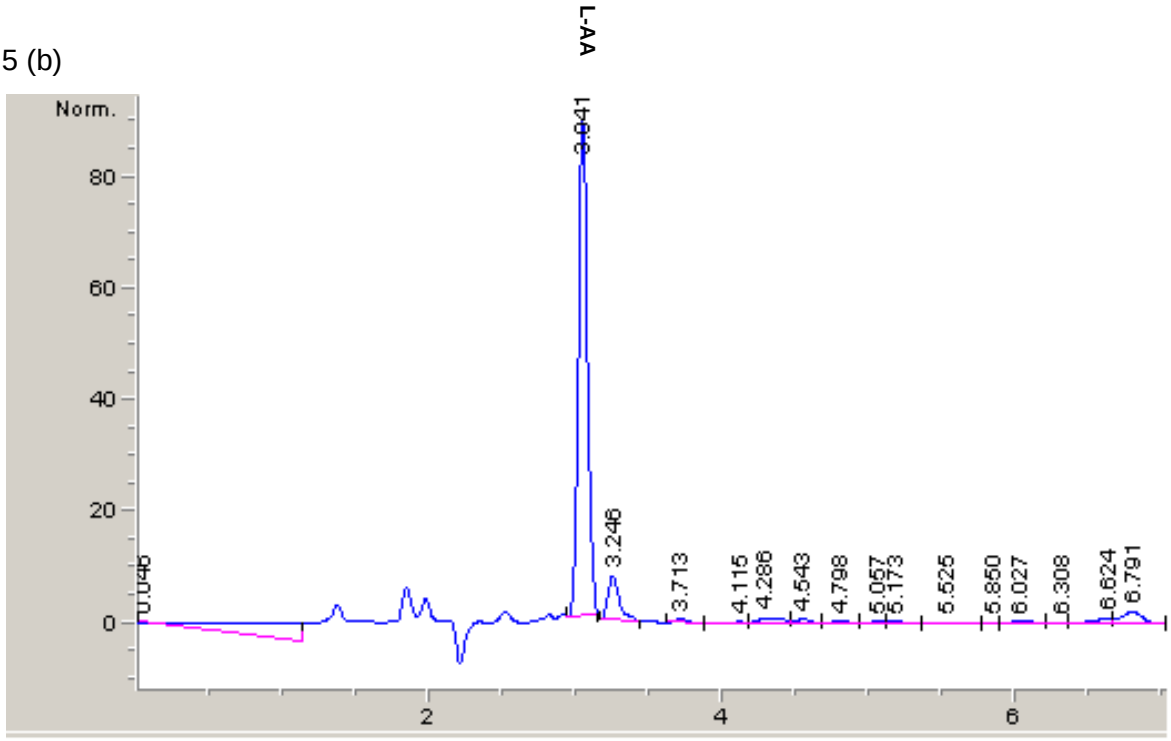
4.3.5.4 Specificity

Figure 5 shows representative chromatograms of samples (cereal and camu powder) and L-AA standard (50 µg/mL). The retention time of the L-AA standard (Figure 5a) was close to that obtained for the samples analysed (Figure 5b, c). The sample peaks were sharp and symmetrical and well resolved from other sample components with no co-eluting peaks. Peak purity was 98.25 and 99.64% for onion and cereal samples, respectively. Additionally, the UV-spectrum acquired for both sample extract peaks was the same as those obtained for L-AA standards. The chromatogram of the stress-induced tomato extract (Figure 5d) gave similar retention times to those obtained for the 50 µg/mL L-AA standard. Any degradation products and sample matrix components possibly present did not elute at the same time as the L-AA peaks. The UV-spectrum for the stress-induced sample was identical to that of the L-AA standards. Good peak purity was obtained for tomato (99.67%) sample. Under the method's test conditions, L-AA appeared to be well resolved from other sample components and thus proves the specificity of the method for the determination of L-AA.

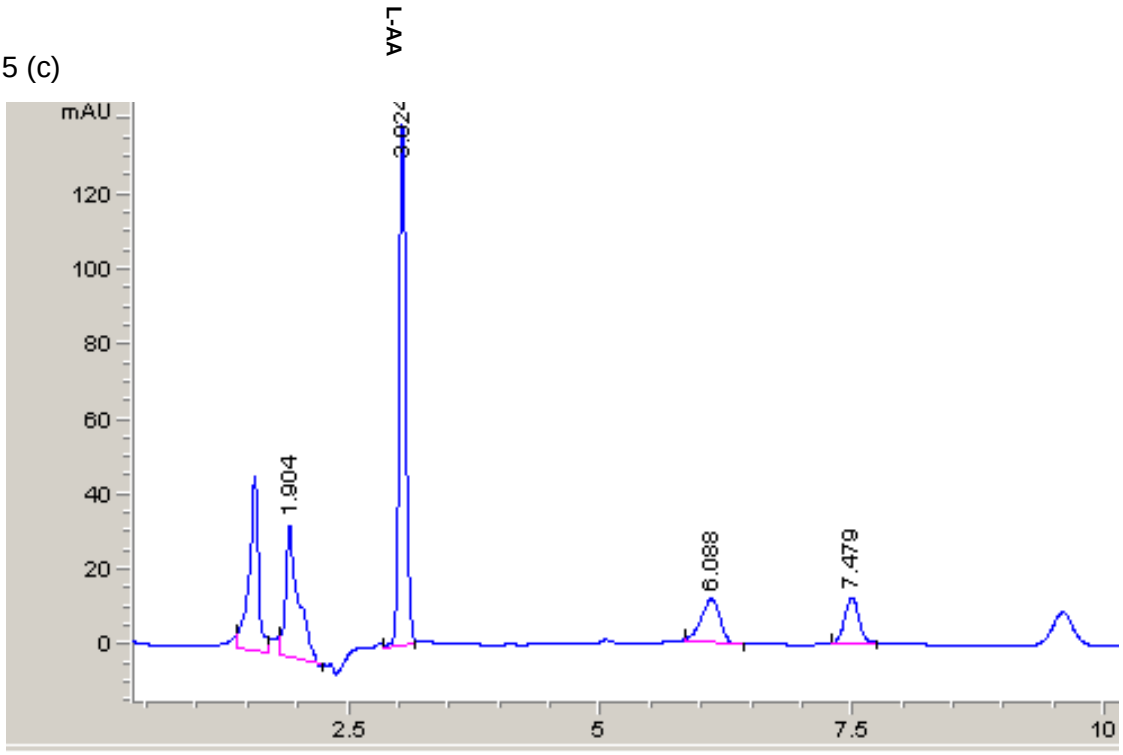
5 (a)


L-AA
3.042
Norm.
600
500
400
300
200
100
0
0.049
0.343
0.711
0.900
1.293
1.560
2.794
1
2
3

5 (b)


L-AA
3.041
Norm.
80
60
40
20
0
0.046
3.246
3.713
4.115
4.286
4.543
4.798
5.057
5.173
5.525
5.850
6.027
6.308
6.624
6.791
2
4
6

5 (c)



5 (d)

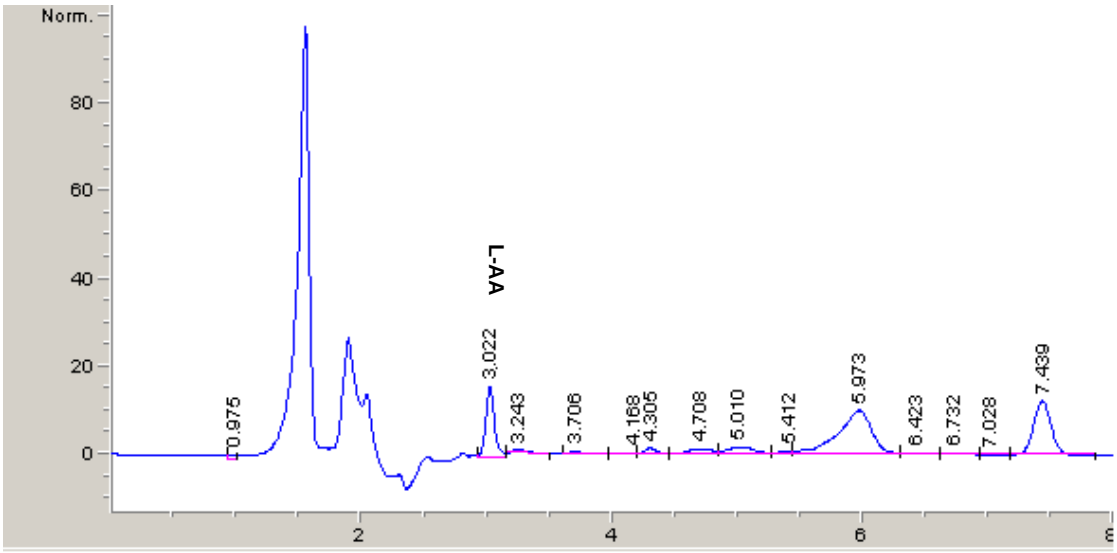


Figure 5. Representative chromatograms illustrating specificity of (a) 50µg/mL L-AA standard; (b) cereal extract (c) tomato extract; and (d) stressed tomato extract.

4.3.5.5 Sample stability

Table 11 summarizes the results of the stability study of sample extracts. All samples were found to be stable at 4 ºC for 24 hr (99 to 102% recovery). Similarly, no significant ($p>0.05$) differences were observed between results of fresh samples and samples stored at -80°C for a week, a month and two months. All beverage samples showed good preservation of L-AA at -80 ºC for up to two months. However, pressed berry juice was unstable after two months storage at -80 ºC indicating a significantly ($p<0.05$) lower recovery of L-AA concentration. This poor stability could possibly be attributed to the lack of commercially added preservatives.

Freeze-thaw stability of all samples was poor. Most samples showed significantly ($p<0.05$) lower recoveries when compared to fresh samples.

Table 11: Stability Studies of L-AA in sample extracts

Sample extracts	Fresh	Short-Term	Recovery (%)	Week 1	Recovery (%)	Month 1	Recovery (%)	Month 2	Recovery (%)	Freeze /Thaw	Recovery (%)
Pressed berry (µg/mL)	119.95 ± 20.37[a]	119.66 ± 72.55[a]	**99.75**	118.78 ± 184.97[a]	**99.02**	115.92 ± 105.09[a]	**96.64**	114.72 ± 20.58[b]	**95.63**	106.36 ± 20.00[c]	**88.67**
Tropical (µg/mL)	611.16 ± 42.57[a]	614.89 ± 22.33[a]	**100.61**	597.66 ± 181.49[a]	**97.79**	594.82 ± 28.96[a]	**97.33**	586.56 ± 633.43[a]	**95.97**	505.64 ± 1180.42[b]	**82.73**
Camu powder (µg/mL)	203.64 ± 9.87[a]	204.21 ± 112.47[a]	**100.28**	201.58 ± 66.02[a]	**98.99**	200.61 ± 12.40[a]	**98.51**	199.36 ± 19.59[a]	**97.89**	185.31 ± 719.93[b]	**91.00**
Onion (µg/mL)	2.20 ± 3.13[a]	2.25 ± 1.75[a]	**102.19**	2.24 ± 6.01[a]	**102.10**	2.10 ± 6.68[a]	**95.41**	2.07 ± 1.91[a]	**94.28**	1.12 ± 1.74[b]	**51.07**

Values in columns are means ± SD of three determinations. Paired samples T-test were used to determine statistical differences. L-AA values in each row with the same letter are not significantly ($p>0.05$) different, where different letters are indicative of significant ($p<0.05$) differences.

4.3.5.6 Robustness

The results of the robustness study summarized in Table 12 demonstrated that all varied conditions applied to the method, produced good recoveries of L-AA. Results were not significantly ($p>0.05$) different from those obtained from the optimized method for most samples tested. Camu powder extract was the only sample that produced significantly ($p<0.05$) higher results than that obtained with the standard optimized method when eluted with the adjusted mobile phase composition [distilled water/ acetonitrile/formic acid; (80.9: 19: 0.1, v/v/v)]. The precision which was expressed as the COV was acceptable (<5%) between results obtained with the optimized method and those achieved with the adjusted method. Therefore the ability of the optimized method to remain unaffected by small changes in parameters thereby producing accurate and precise results indicates the robustness of the method.

Table 12. Evaluation of the robustness of the HPLC method for L-AA determination.

Samples/ standards/QC	Changes	Retention time	Response	
	pH 2.8[1]	**COV**	**REC (%)**	**COV[4]**
Camu powder		0.075	102.13	2.42
Tropical juice		0.094	98.94	0.62
Mix berry juice		0.635	96.83	1.90
Pressed orange juice		0.099	102.48	1.42
	Distilled water/ acetonitrile/formic acid (80.9: 19: 0.1, v/v/v)[2]			
QC (60 µg/mL)		0.33	99.92	0.73
Camu powder		0.12	103.56[a]	2.03
Onion		4.64	99.47	3.89
	Column temperature: 20 °C[3]			
Camu powder		0.33	102.61	1.51
10 µg/mL L-AA standard		0.27	100.25	1.09
20 µg/mL L-AA standard		0.20	98.93	2.64
50 µg/mL L-AA standard		0.29	99.44	1.88
100 µg/mL L-AA standard		0.01	97.16	3.08
	Column temperature: 26 °C			
5 µg/mL L-AA standard		0.29	101.31	2.30
20 µg/mL L-AA standard		0.78	97.59	2.97
50 µg/mL L-AA standard		2.13	97.83	2.56
100 µg/mL L-AA standard		0.05	96.06	3.48
125 µg/mL L-AA standard		0.07	97.86	1.34
QC sample (60µg/mL)		0.18	99.83	0.72
Camu powder		0.26	101.71	1.00

[1]Optimized HPLC method at pH 2.6. [2]Mobile phase composition for optimized method: distilled water/acetonitrile/formic acid (99: 0.9: 0.1, v/v/v). [3]Column temperature of optimized method: 23 °C. [4]Precision between assay performed at optimal conditions and with variations. [a]Significantly ($p<0.05$) higher recovery obtained with modified mobile phase .

4.3.5.7 System suitability testing

Table 13 summarizes the results of the system suitability tests. The results show that all parameters evaluated fell within their respective limits. The precision, expressed as the COV of the retention time (0.018) was less than 1% and is in keeping with the FDA's acceptance limit [22]. The precision of the response (COV = 1.470) fell within 2% and is in compliance with the United States Pharmacopeia (USP) requirements [27]. Hence, the results of the system suitability tests indicate that the entire HPLC system is performing optimally and within the validated method performance limits.

Table 13: System suitability testing for the HPLC assay for the determination of L-AA in food and beverage products

		Retention time (min)[1]	PAA (AU)	Height (AU)	Capacity factor (K´)	Theoretical plates (N)	Resolution (Rs)	Peak asymmetry factor (A_s)	Selectivity factor (l_b)
Day	1	3.035	257.86	55.09	7.62	10561	7.91	0.89	1.45
	2	3.034	263.41	55.27	7.47	10749	7.53	0.86	1.45
	3	3.035	255.99	55.20	7.48	10850	7.89	0.92	1.45
	4	3.034	253.12	55.21	9.18	10747	7.91	0.88	1.46
	5	3.035	258.70	55.88	7.36	10752	7.7	0.9	1.46
Mean ± SD		3.035 ± 0.001	257.8 ± 3.8	55.3 ± 0.3	7.482 ± 0.092	10731.8 ± 104.9	7.788 ± 0.169	0.890 ± 0.022	1.454 ± 0.005
COV		0.018	1.470	0.568	1.234	0.978	2.172	2.512	0.377

[1]The retention time for the unresolved peak is 0.357 ± 1.33 SD. Abbreviations: AU, absorbance unit; PAA, peak absorbance area.

4.4 Discussion

The measurement of L-AA is well known in relation to many disease states. This is attributed to its antioxidant capacity in possibly preventing and controlling the progression of many disease conditions [28-30]. Hence, reliable and accurate monitoring of this vitamin contributes to an objective measurement, which may be necessary for assessment and management of such conditions. As with all analytical methods, a degree of intrinsic error may induce small or significant changes that could change measurement values. Subsequently, this implies that the method employed be tested and validated. Several international organizations namely, ISO/IEC, ICH and FDA provide comprehensive regulatory standards to implement and perform validation procedures [14]. This would provide documented evidence that the method performs within acceptable quality parameters, and the degree of error present does not affect method performance, resulting in accurate, precise and reliable results.

The current study evaluated the validation parameters, in accordance with ISO 17025, for optimal performance of an HPLC method with UV detection to measure L-AA. Additionally, mobile phase conditions were optimized to give the best separation of L-AA from other sample components.

Conditions such as pH and organic solvent component contribute to the degree of separation of anayte/s within a sample solution [31]. In the current study, two mobile phases were evaluated in an attempt to achieve the best separation and resolution between L-AA and other sample components. It was shown that the mobile phase containing an organic solvent component (distilled water/acetonitrile/formic acid) was more effective at achieving optimal separation and resolution. Similarly, Gorse *et al.* [32] and Biesaga *et al.* [33] have observed good separation and resolution of other analytes with various chromatographic methods using mobile phases consisting of organic modifiers .

Several authors have suggested the use of MPA for optimal extraction and preservation of L-AA [34-36]. Similarly, in the current study MPA was found to extract and stabilize L-AA with acceptable accuracy and precision. Additionally, it was observed that stability of L-AA in MPA, under room temperature conditions for up to eight hours contributes to its efficacy as an optimal extraction solvent for samples.

The inclusion of QC samples is imperative to detect deviation from prescribed tolerance limits. Any deviations outside acceptable tolerance limits implies that the HPLC method does not conform to pre-determined requirements [12]. The variations observed with the QC samples (outlier on day 6 for QC 1) were minimal. The single outlier result was possibly derived from analytical factors on that day namely, variation in analyst technique, variation in environmental conditions on different days and variability in performance of equipment used. These variations are inherent, however it is important to differentiate between variations of this kind and those that occur due to error. Such errors may be derived from contamination in the HPLC system, changes in reagents and consumables, poorly functioning equipment and poor analyst technique [37]. The results from the QC charts (Figures 3a, 3b and 3c) indicated ongoing, consistent good performance of the HPLC method in generating accurate and precise results.

The calibration range of the method was found to be linear from 5 to 200 µg/mL. The poor linearity observed at high concentrations (>200 µg/mL) may be as a result of saturation of the detector [38]. The calibration range consisted of five standards (5 to 125 µg/mL) and is in agreement with the ICH's recommendations for a quantitative analytical method. (ICH, 2005). The correlation coefficient (r^2=0.999) was good and is comparable to those obtained in other studies employing HPLC to measure L-AA in food products [18,24,34].

The low COVs (<3%) for various levels of precision for L-AA demonstrated that the method achieved good repeatability at various concentration levels. These results can be compared to those obtained by Kumar *et al.* [39] for validating various levels of precision (COVs less than 2%) of an HPLC method for L-ascorbic acid determination in health drinks. Similarly, Spinola *et al.* [24] obtained COVs less than 4% for an improved HPLC method for the measurement of L-AA in various fruit and vegetables. In another study, using food commodities to measure L-ascorbic acid content by HPLC, the average COV obtained was 8.7% and is comparable to those obtained in the present study [40].

The results from accuracy experiments reflect both the efficiency of the L-AA extraction from samples of the method in use and the effects of the sample matrices. Satisfactory recoveries ranging from 99 to 103% and 92 to 96% were achieved for L-AA from both QCs and food and vegetable samples, respectively. These results are similar to those obtained by Odriozola *et al.* [18] who demonstrated average recoveries of approximately 94 to 105% in fruit and vegetables, and is in agreement with the FDA/CDER's requirements of being within ±15% of the target value [26]. Additionally, the results obtained are comparable to average recoveries of 82.2 to 95.9% and 93.3% obtained by Valente *et al.* [41] and Sanchez *et al.*

[20] for L-ascorbic acid determination in fruit and vegetables , respectively. The results indicate that the extraction procedure employed was optimal, demonstrating almost complete recovery of L-AA by both recovery methods.

The LOD is the lowest concentration that is capable of producing a chromatographic response which usually cannot be determined with accuracy and precision. The LOQ is the lowest concentration that can produce a chromatographic response with acceptable precision and accuracy. In the current study, the LOD and LOQ corresponded to 0.61µg/ mL and 1.84 µg/mL, respectively. The LOQ value obtained in the current study implied that good sensitivity, accuracy and precision was achieved at this lower concentration level and is comparable to comparable to higher LOD and LOQ values (1.7 and 5.7 µg/mL) reported by Odriozola-Serrano *et al.* [18] who demonstrated the various UV-HPLC methodologies to analyze L-ascorbic acid containing fruits . In this previous study, a standard error of 36.98 was reported for the calibration standard line and can be compared to the current study in which a lower standard error of 9.65 was obtained. Similarly in another study, Sawant *et al.* [31] reported LOD and LOQ values of 1.42 and 4.32 µg/mL respectively for the analysis of L-ascorbic acid in *Phyllanthus Emblica*, which were calculated from the calibration standard as was demonstrated in the current study . Furthermore, the current method demonstrated that L-AA in QC and samples (apple) were measurable at this LOQ with satisfactory accuracy and precision.

The stability study demonstrated that L-AA is generally stable in beverage and some vegetable products for up to 2 months at -80ºC. These findings are in agreement with Scherer *et al.* [42] who reported stability of L-AA in fruit juices stored at 5 ºC for at least the first two days . Significant losses of vitamin C content during the freeze-thaw cycles in the current study were in agreement with some other studies that reported similar losses during the thaw-out process, despite a slight variation in storage and temperature conditions [43-45].

Additionally, a noteworthy finding in some studies revealed that microwave thawing prevented less vitamin C loss compared to thawing at room temperature [43,46,47]. In the current study, a similar loss in L-AA was observed when samples were thawed out three times at room temperature from -80 ºC. The onion sample demonstrated the most significant ($p<0.05$) loss in L-AA. This could be due to the possibility that no preservatives were added to the onion sample in comparison to commercially available fruit juices that may contain preservatives that protect L-AA [48]. Hernández *et al.* [43] recommended the addition of

antioxidants to slow down oxidation in certain fruit extracts. From the results of the stability study, it is evident that freezing vegetable and beverage products resulted in no significant ($p>0.05$) L-AA losses, however thawing out at room temperature resulted in significant ($p<0.05$) L-AA losses. Hence, it is recommended that frozen samples be thawed out in a microwave to reduce significant L-AA losses. The addition of an antioxidant should be considered during the extraction of L-AA in fruit and vegetables.

The current method demonstrated robustness and thus proves the reliability, and the ability of the method to remain unaffected with inherent day-to-day variations such as different analysts, equipment and environmental conditions. One important factor observed was that small changes of the organic component present in the mobile phase could result in significant changes in retention time. The results show that the retention times for samples eluted with the adjusted mobile phase were shorter than those obtained with the standard mobile phase, and was possibly due to the increased polarity of the mobile phase [49]. Hence, this should be taken into consideration when preparing the mobile phase.

4.5 Conclusion

The proposed optimized and validated method demonstrated an excellent technique for measurement of L-AA in food and beverage products (Appendix D). The extraction method proved an effective means for the isolation of L-AA from a variety of fruit and vegetable sample matrices. The results from the validation study confirmed a good performance of the method with regard to ISO 17025 validation requirements namely, accuracy, precision, linearity, specificity, robustness and stability. The successful optimization and validation of the proposed method should make it easily applicable for routine analysis of L-AA measurement in various fruit and vegetable products. Furthermore, the validation procedure applied in this study could be applied to samples other than food and beverage, such as pharmaceutical products and biological samples.

4.6 References

[1] J.E. Enstrom, in: L. Packer, J. Fuchs (Eds), Marcel Dekker Inc., New York, 1997, pp. 381.

[2] P. Weber, A. Bendich, W. Schalch, Int. J. Vitam. Nutr. Res. 66 (1996) 19.

[3] C.L. Rock, R.A. Jacob, P.E. Bowen, J. Am. Diet. Assoc. 96 (1996) 693.

[4] Y. Yan, U.T. Bornscheuer, R.D. Schmid, Biotechnol. Lett. 21 (1999) 1051.

[5] M.W. Davey, M. Montagu, D. Inzé, M. Sanmartin, A. Kanellis, N. Smirnoff, I.J.J. Benzie, J.J. Strain, D. Favell, J. Fletcher, J. Sci. Food Agric. 80 (2000) 825.

[6] A. Calokerinos, T. Hadjiioannou, Microchem. J. 28 (1983) 464.

[7] T.Z. Liu, N. Chin, M.D. Kiser, W.N. Bigler, Clin. Chem. 28 (1982) 2225.

[8] S. Arya, M. Mahajan, P. Jain, Anal. Chim. Acta. 417 (2000) 1.

[9] A. De Quirós, M. Fernández-Arias, J. López-Hernández, Food Chem. 116 (2009) 509.

[10] M.C. Sánchez-Mata, M. Cámara-Hurtado, C. Díez-Marqués, M.E. Torija-Isasa, Eur. Food Res. Technol. 210 (2000) 220.

[11] N. Toomula, A. Kumar, S.D. Kumar, V.S. Bheemidi, J. Anal. Bioanal. Tech. 2 (2011) 127.

[12] P. Masson, J. Chromatogr., A. 1158 (2007) 168.

[13] L. Huber, BioPharm. 12 (1999) 64.

[14] I. Taverniers, M. De Loose, E. Van Bockstaele, TrAC, Trends Anal. Chem. 23 (2004) 535.

[15] R. Dudhwal, P. Jagadish, K. Bhat, A. Mane, Pharma Chem. 3 (2011) 375.

[16] A.M. Maia, A.R. Baby, W.J. Yasaka, E. Suenaga, T.M. Kaneko, M.V.R. Velasco, Talanta 71 (2007) 639.

[17] S. Mitic, D. Kostic, D. Naskovic-okic, M.N. Mitic, Trop. J. Pharm. Res. 10 (2011) 105.

[18] I. Odriozola-Serrano, T. Hernández-Jover, O. Martín-Belloso, Food Chem. 105 (2007) 1151.

[19] F. Van de Velde, M.E. Pirovani, M.S. Cámara, D.R. Güemes, C.M.H. Bernardi, Food Analytical Methods 5 (2012) 1097.

[20] M.C. Sánchez-Mata, M. Camara-Hurtado, C. Diez-Marques, M.E. Torija-Isasa, Eur. Food Res. Technol. 210 (2000) 220.

[21] AOAC. (2002). http://www.aoac.org/Official_Methods/slv_guidelines.pdf [7 October 2012].

[22] FDA/CDER. (1994). http://www.fda.gov/downloads/Drugs/.../Guidances/UCM134409.pdf [11 October 2012].

[23] APVMA. (2004). http://www.apvma.gov.au/publications/guidelines/docs/gl_69_analytical_methods.pdf [6 August 2012].

[24] V. Spínola, B. Mendes, J.S. Câmara, P.C. Castilho, Anal. Bioanal. Chem. 403 (2012) 1049.

[25] G.A. Shabir, J. Chromatogr., A 987 (2003) 57.

[26] FDA. (2001). http://www.fda.gov/downloads/Drugs/.../Guidances/ucm070107.pdf [10 August 2012].

[27] J.W. Dolan. (2004). http://www.chromatographyonline.com/lcgc/data/articlestandard//lcgcasia/512005/261980/article.pdf [01 November 2012].

[28] J.C. Chambers, A. McGregor, J. Jean-Marie, O.A. Obeid, J.S. Kooner, Circulation 99 (1999) 1156.

[29] H.C. Gorton, K. Jarvis, J Manipulative Physiol. 22 (1999) 530.

[30] J.Y. Lin, M.A. Selim, C.R. Shea, J.M. Grichnik, M.M. Omar, N.A. Monteiro-Riviere, S.R. Pinnell, J. Am. Acad. Dermatol. 48 (2003) 866.

[31] L.P. Sawant, S. Prabhakar, N. Pandita. (2010). http://www.omicsonline.org/2155-9872/2155-9872-1-111.pdf [15 February 2013].

[32] J. Gorse, A. Balchunas, D. Swaile, M. Sepaniak, J. High Resolut. Chromatogr. 11 (2005) 554.

[33] M. Biesaga, U. Ochnik, K. Pyrzynska, J. Sep. Sci. 30 (2007) 2929.

[34] G. Burini, J. Chromatogr., A. 1154 (2007) 97.

[35] M. Gennaro, P. Bertolo, J. Liq. Chromatogr. 13 (1990) 1419.

[36] W.D. Graham, D. Annette, J. Chromatogr., A. 594 (1992) 187.

[37] J.O. Westgard, Basic Method Validation, Westgard QC, Inc., Madison, 3rd, 2008, pp. 114-115.

[38] B.A. Bidlingmeyer, Practical HPLC methodology and applications, Wiley & Sons, New York, 1992, pp. 416.

[39] K.K. Ravi, K.P. P, M. Rao, J. Chem. Pharm. Res. 3 (2011) 363.

[40] A.G. Frenich, M.E.H. Torres, A.B. Vega, J.L.M. Vidal, P.P. Bolanos, J. Agric. Food Chem. 53 (2005) 7371.

[41] A. Valente, T.G. Albuquerque, A. Sanches-Silva, H.S. Costa, Food Res. Int. 44 (2011) 2237.

[42] R. Scherer, A.C.P. Rybka, C.A. Ballus, A.D. Meinhart, H.T. Godoy, Food Chem. (2012) 150.

[43] Y. Hernández, M.G. Lobo, M. González, Food Chem. 96 (2006) 654.

[44] B. Nursal, S. Yücecan, Mol. Nutr. Food Res. 44 (2000) 451.

[45] M. Pilar Cano, C. Fuster, M. Antonia Marín, Z Lebensm Unters Forsch. 196 (1993) 142.

[46] M. Holzwarth, S. Korhummel, R. Carle, D.R. Kammerer, Food Res. Int. (2012) 241.

[47] J. Oszmiański, A. Wojdyło, J. Kolniak, Food Sci. Technol. 42 (2009) 581.

[48] P. Haddad, J. Chem. Educ. 54 (1977) 192.

[49] Z. Hao, B. Xiao, N. Weng, J. Sep. Sci. 31 (2008) 1449.

CHAPTER FIVE: GENERAL DISCUSSION

As with all analytical methods, there exists a certain degree of intrinsic error. Additionally, procedures employed in an analytical method contribute random and systematic errors which subsequently affect the precision and accuracy. These are possibly derived from variations in technique, environmental conditions, poor performance of equipment and instrumentation. Of importance is not only a measure of these errors, but how it influences the method's ability to perform within acceptable limits. Hence, in validating an analytical method, the total amount of error and its effect on the method performance is determined (Westgard, 2008). Several international standards provide regulatory guidelines for validation of analytical procedures (Taverniers *et al.*, 2004).

The H-ORAC$_{FL}$ assay has gained tremendous attention within the food industry and recently, research laboratories for its ability to detect and measure total antioxidant capacity in food products and biological samples. Moreover, there is a large amount of studies that have been performed to investigate the effects of both endogenous and exogenous antioxidants on various systems of the body under various conditions (Dreher and Maibach, 2001; Elsayed and Bendich, 2001; Hughes, 1999; Masella *et al.*, 2005; Steenvoorden and Beijersbergen van Henegouwen, 1997). More attention has been placed on the performance characteristics of the automated ORAC assay in comparison to the manually performed assay (Huang *et al.*, 2002b; Ou *et al.*, 2001; Prior *et al.*, 2005). The automated ORAC assay alleviates errors associated with pipetting and temperature control, resulting in improved accuracy and precision (Huang *et al.*, 2002b; Ou *et al.*, 2001; Prior *et al.*, 2005). The manual performance of the ORAC$_{FL}$, which is still practised largely in research and smaller laboratories are prone to such errors. In the absence of a validation study, the quantification of these errors and its effect on methods performance may be overlooked.

The first study investigated the method performance parameters in accordance with ISO 17025 validation requirements for the manually performed H-ORAC$_{FL}$ assay. Price *et al* reported acceptable linearity and precision for both trolox standard curves and algal extracts for a manually applied ORAC$_{FL}$ assay (Price *et al.*, 2006). Similarly, in another manually applied ORAC$_{FL}$ assay, good linearity, accuracy and precision were observed for Trolox standards (Dávalos *et al.*, 2004). However, in both these studies, validation performance parameters have been applied mostly to trolox standards, whilst accuracy and precision of samples have been minimally addressed. In this study, the method produced acceptable

linearity for both trolox standards and various samples demonstrating good precision at different concentration levels within the optimized linear range.

In the present study, pre-heating the phosphate buffer and prepared micro-plate, and excluding several exterior wells within the micro-plate significantly reduced outliers in the exterior wells of the micro-plate and significantly lowered COVs in the final $ORAC_{FL}$ results. These findings are in agreement with those reported by Prior *et al* who observed improved COVs after the pre-heating step using an automated $ORAC_{FL}$ assay (Prior *et al.*, 2003).

Several studies have demonstrated the use of acetone/water and acetone/water/acetic acid mixtures as solvents for extraction of antioxidants in food and beverage samples (Huang *et al.*, 2002a; Huang *et al.*, 2002b; Ou *et al.*, 2001; Prior *et al.*, 2003). In the current study, the antioxidant extraction method was optimized in terms of temperature, solvent type and volume. In contrast to these previous studies, the current findings revealed significantly lower antioxidant yields with the acetone/water/acetic acid solvent for most samples tested, making it the least desirable extraction solvent. Methanol and ethanol/water mixtures recovered significantly ($p<0.05$) higher antioxidant yields.

The selectivity/specificity of the $ORAC_{FL}$ assay was demonstrated and results confirm those reported by Ou *et al* (Ou *et al.*, 2001). The results also suggest that the presence of other existing sample components do not interfere with the accuracy of the result and justifies the applicability of the assay to various food and beverage matrices.

Results from the validation study demonstrated good accuracy, precision and robustness of the assay for both standards and samples, correlating well with studies using both the manual and automated $ORAC_{FL}$ assays (Dávalos *et al.*, 2004; Huang *et al.*, 2002a; Huang *et al.*, 2002b; Price *et al.*, 2006). Moreover, the mean recoveries for all accuracy experiments fell within 15% of the target value and the COVs did not exceed 15% for all precision experiments. These results demonstrated compliance with the FDA/CDER's validation requirements (Food and Drug Administration, 2001).

The second study investigated the method performance parameters in accordance with ISO 17025, of the automated RP-HPLC for L-AA determination in food and beverage products. Due to the automated nature of the assay, minimal error was encountered with little impact on the method's performance. Low COV values (<10%) and recoveries falling within 15% for most samples are evidence of the excellent accuracy and precision demonstrated by the current method and correlates well with previous studies (Odriozola-Serrano *et al.*, 2007;

Sánchez-Mata *et al.*, 2000; Spínola *et al.*, 2012; Valente *et al.*, 2011). Additionally, the accuracy, precision and stability achieved with the QC samples further justified the robustness of the method.

Adding an organic modifier (acetonitrile) to the mobile phase improves resolution and contributes to sharp, symmetrical peaks (Helaleh *et al.*, 2005). The robustness experiments demonstrated that small changes in acetonitrile composition in the mobile phase could vary retention times significantly, without necessarily affecting peak absorbance areas. Hence, this could create problems of other peaks co-eluting at the same time of L-AA. The optimized mobile phase increased retention time to approximately 3.0 min, providing well resolved symmetrical sharp peaks of L-AA from various food and beverage matrices.

The results for all samples and L-AA standards demonstrated excellent linearity over the concentration range (5 to 125 µg/mL) studied. The mean r^2 obtained for all samples and standards was ≥ 0.995 for this concentration range. The presence of other components in the samples did not interfere with the L-AA peak. The L-AA showed good resolution from other matrix components and potential stress degradation products. Hence, the method is selective and specific for L-AA. Low LOD (0.61 µg/ mL) and LOQ (1.84 µg/mL) values indicated that the method is highly sensitive with acceptable accuracy and precision.

The automated RP-HPLC can be compared to the manual performance of the H-$ORAC_{FL}$ assay. Better precision and linearity was observed for the RP-HPLC assay. Additionally, the RP-HPLC demonstrated superior sensitivity with a lower standard error at concentrations occurring around the limit of quantification.

CHAPTER SIX: CONCLUSION AND RECOMMENDATIONS:

This study investigated the method performance characteristics, in accordance with ISO 17025, for the validation of two bio-analytical methods, H-$ORAC_{FL}$ and RP-HPLC methods for TAC and L-AA measurement, respectively. The results showed that both assays were successfully validated demonstrating good performance with regard to accuracy, precision, linearity, range, limit of detection, limit of quantification, specificity, stability, robustness and system suitability. Additionally, the results confirmed and provided documented evidence that the assays were optimized to minimize total error and its effect on method performance.

Results from both these studies provided greater insight and information regarding the validation issues surrounding both manually performed and automated assays for the determination of antioxidants in food and beverage products. Results generated from the $ORAC_{FL}$ validation study could contribute significantly to the minimal literature available for the performance characteristics of the manually performed $ORAC_{FL}$ assay.

The antioxidant field is growing rapidly and has spawned a cumulative demand for AOC assays. However, the existing quality approach has not yet been routinely adapted to the area of AOC assays. At present no comprehensive standardized guidelines exist for routine validation of AOC assays. In this thesis, guidelines provided by the ISO 17025 standard for analytical method validation have been applied to a manually performed H-ORAC assay for hydrophilic antioxidant measurement. The results of the validation study exhibit proof that holds great potential and can be applied to other AOC assays namely, ferric ion reducing antioxidant power (FRAP), trolox equivalent antioxidant capacity (TEAC), and 2,2-diphenylpicrylhydrazyl (DPPH) assays to obtain reliable, high quality results. Furthermore, extraction conditions were optimized for hydrophilic antioxidants in samples. Lipophilic antioxidants have not been addressed in the current study. Further studies may be useful to optimize the extraction of lipophilic antioxidants from various food, beverage and cosmetic commodities. These further studies may evaluate the validation parameters for the manually performed $ORAC_{FL}$ assay for the determination and measurement of lipophilic antioxidants.

The information gained from the second study serves as valuable tool for other future studies to accurately and reliably measure L-AA and other vitamins by HPLC in food and beverage products. This validation approach may also be applied to the measurement of L-AA in biological samples, cosmetic formulations and pharmaceuticals.

The successful optimization and validation of both methods suggests their successful application as routine methods in food and research laboratories.

References

Dávalos, A., Gómez-Cordovés, C. & Bartolomé, B. 2004. Extending applicability of the oxygen radical absorbance capacity (ORAC-fluorescein) assay. *Journal of Agricultural and Food Chemistry* 52**:**48-54.

Dreher, F. & Maibach, H. 2001. Protective effects of topical antioxidants in humans. In: Thiele, J. & Elsner, P. (eds.) *Oxidants and Antioxidants in Cutaneous Biology.* Switzerland: S Karger AG: 157-164.

Elsayed, N. M. & Bendich, A. 2001. Dietary antioxidants: potential effects on oxidative products in cigarette smoke. *Nutrition Research,* 21**:**551-567.

Food and Drug Administration. 2001. *Guidance for Industry: Bioanalytical Method Validation*. http://www.fda.gov/downloads/Drugs/.../Guidances/ucm070107.pdf [10 August 2012].

Helaleh, M., Al-Omair, A., Tanaka, K. & Mori, M. 2005. Ion chromatography of common anions by use of a reversed-phase column dynamically coated with fluorine-containing surfactant. *Acta Chromatographica,* 15**:**247.

Huang, D., Ou, B., Hampsch-Woodill, M., Flanagan, J. A. & Deemer, E. K. 2002a. Development and validation of oxygen radical absorbance capacity assay for lipophilic antioxidants using randomly methylated β-cyclodextrin as the solubility enhancer. *Journal of Agricultural and Food Chemistry,* 50**:**1815-1821.

Huang, D., Ou, B., Hampsch-Woodill, M., Flanagan, J. A. & Prior, R. L. 2002b. High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. *Journal of Agricultural and Food Chemistry,* 50**:**4437-4444.

Hughes, D. A. 1999. Effects of dietary antioxidants on the immune function of middle-aged adults. *Proceedings of the nutrition society,* 58**:**79-84.

Masella, R., Di Benedetto, R., Varì, R., Filesi, C. & Giovannini, C. 2005. Novel mechanisms of natural antioxidant compounds in biological systems: involvement of glutathione and glutathione-related enzymes. *The Journal of nutritional biochemistry,* 16**:**577-586.

Odriozola-Serrano, I., Hernández-Jover, T. & Martín-Belloso, O. 2007. Comparative evaluation of UV-HPLC methods and reducing agents to determine vitamin C in fruits. *Food Chemistry,* 105**:**1151-1158.

Ou, B., Hampsch-Woodill, M. & Prior, R. L. 2001. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *Journal of Agricultural and Food Chemistry,* 49**:**4619-4626.

Price, J. A., Sanny, C. G. & Shevlin, D. 2006. Application of manual assessment of oxygen radical absorbent capacity (ORAC) for use in high throughput assay of "total" antioxidant activity of drugs and natural products. *Journal of Pharmacological and Toxicological Methods,* 54**:**56-61.

Prior, L., Hoang, H., Gu, L., Wu, X., Bacchiocca, M., Howard, L., Hampsch-Woodill, M., Huang, D., Ou, B. & Jacob, R. 2003. Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity (ORACFL)) of plasma and other biological and food samples. *Journal of Agricultural and Food Chemistry,* 51**:**3273-3279.

Prior, L., Wu, X. & Schaich, K. 2005. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry,* 53**:**4290-4302.

Sánchez-Mata, M. C., Cámara-Hurtado, M., Díez-Marqués, C. & Torija-Isasa, M. E. 2000. Comparison of high-performance liquid chromatography and spectrofluorimetry for vitamin C analysis of green beans (*Phaseolus vulgaris*). *European Food Research and Technology,* 210**:**220-225.

Spínola, V., Mendes, B., Câmara, J. S. & Castilho, P. C. 2012. An improved and fast UHPLC-PDA methodology for determination of L-ascorbic and dehydroascorbic acids in fruits and vegetables. Evaluation of degradation rate during storage. *Analytical and Bioanalytical Chemistry,* 403**:**1049-1058.

Steenvoorden, D. & Beijersbergen van Henegouwen, G. M. J. 1997. The use of endogenous antioxidants to improve photoprotection. *Journal of Photochemistry and Photobiology B: Biology,* 41**:**1-10.

Taverniers, I., De Loose, M. & Van Bockstaele, E. 2004. Trends in quality in the analytical laboratory. II. Analytical method validation and quality assurance. *TrAC, Trends in Analytical Chemistry,* 23**:**535-552.

Valente, A., Albuquerque, T. G., Sanches-Silva, A. & Costa, H. S. 2011. L-ascorbic acid content in exotic fruits: A contribution to produce quality data for food composition databases. *Food Research International* 44**:**2237-2242.

Westgard, J. O. 2008. *Basic Method Validation,* Madison: Westgard QC, Inc.

APPENDICES

Appendix A: Linearity of samples before and after the optimization of the trolox standard range for the $ORAC_{FL}$ assay

Figure 1 depicts a graphical presentation of the linearity of the samples prior and subsequent to optimization of the trolox standard range. Four dilutions of each sample were prepared and assayed. After optimization final $ORAC_{FL}$ values were similar between dilutions for each sample resulting in good linearity. Prior to optimization, final $ORAC_{FL}$ values were significantly ($p<0.05$) different between dilutions for each sample contributing to the poor linearity obtained.

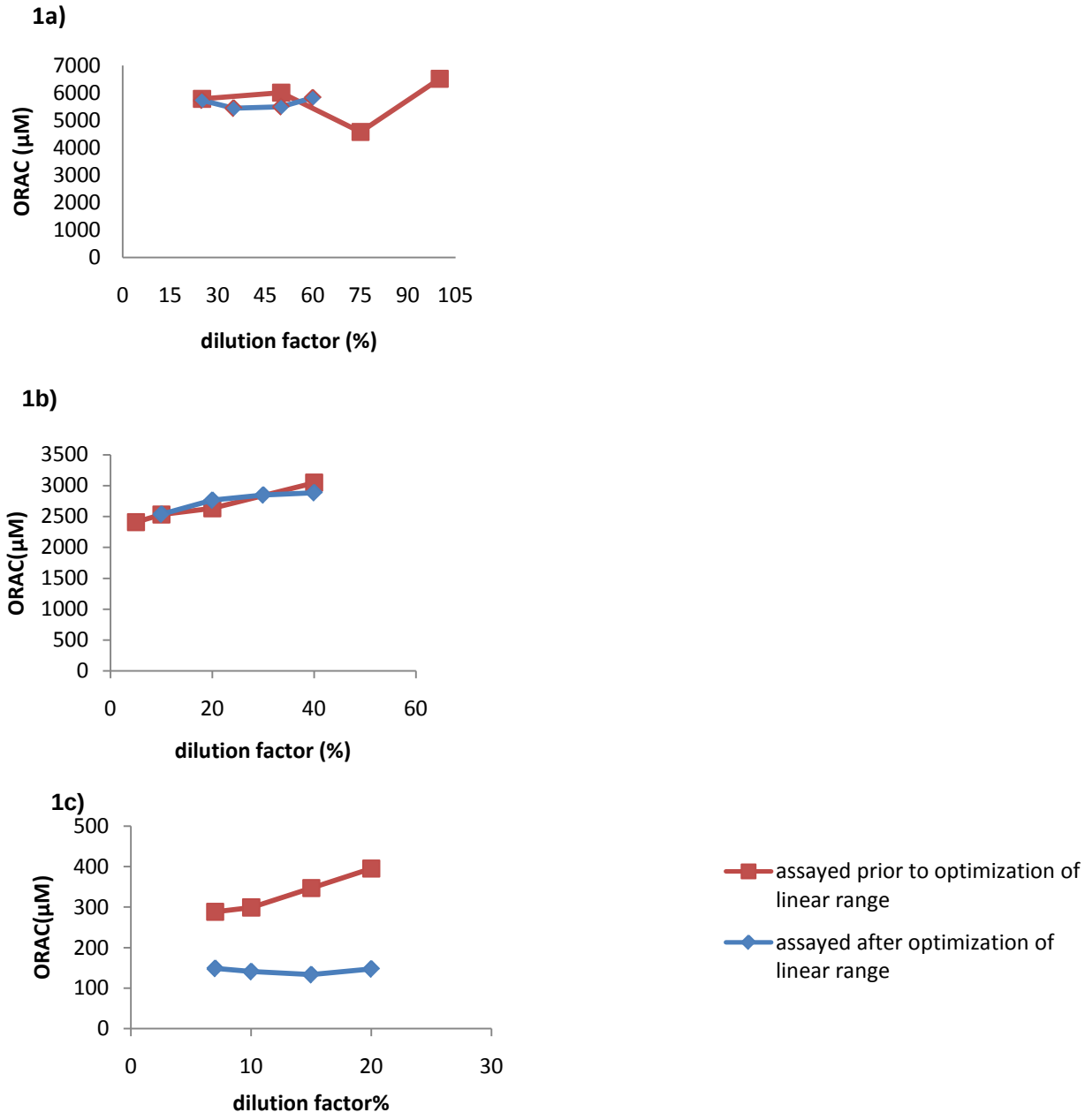


Figure 1 . Linearity of samples a) apple juice, **b)** organic honey-bush tea, an**d c)** chia seeds before and after optimization of trolox standards range.

Appendix B: Method optimization of the $ORAC_{FL}$ in terms of temperature control

The final $ORAC_{FL}$ values have taken into account the effect of the dilution factors. Prior to optimizing the method (no pre-incubation of the phosphate buffer and prepared micro-plate at 37ºC), poor repeatability of results were observed within the micro-plate as illustrated in Figure 1. A large proportion of outliers were evident within the internal wells of the micro-plate (columns 5 and 6). Subsequent to method optimization, these outliers occurring towards the centre of the micro-plate were eliminated.

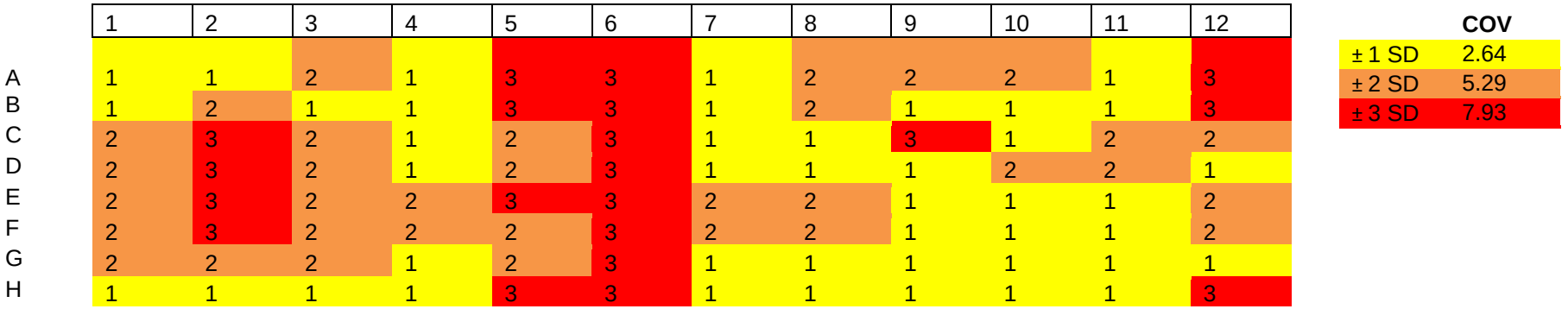


Figure 1. Illustration of poor precision of a 10 µM trolox standard occurring within the 96 well micro-plate prior to method optimization

Appendix C: Standard operating procedure (SOP): OXYGEN RADICAL ABSORBANCE CAPACITY ASSAY (ORAC) for measurement of hydrophilic antioxidants – Revision 1

All personnel concerned with this PROCEDURE must sign the PROCEDURE to indicate that he/she is familiar with its contents.

DOCUMENT CHANGE RECORD:

Date	Revision	Section	Description of Change
15 November 2012	1	**ALL**	***STANDARDIZATION OF SOP***
(Next revision: June 2013)			

Table of contents:

1. Purpose

This document defines the methodology for the $ORAC_{FL}$ assay used for the determination of hydrophilic antioxidants in food and beverage commodities.

2. Scope and application

This procedure is applicable to all laboratory analysts and students at the OSRC.
Free radicals and reactive oxygen species (ROS) are highly reactive molecules that are generated by normal cellular processes, environmental stresses, and UV irradiation. ROS react with cellular components, damaging DNA, carbohydrates, proteins, and lipids generally causing cellular and tissue injury. Excess production of reactive oxygen species can also lead to inflammation, premature aging disorders, and several disease states, including cancer, diabetes, and atherosclerosis. Organisms have developed complex antioxidant systems to protect themselves from oxidative stress.
The ORAC method is a simple, sensitive, and reliable way to measure the peroxyl radical absorbing capacity (with AAPH) of antioxidants in serum and other biological fluids. Hydroxyl radical absorbing capacity of serum has been performed successfully using the ORAC method with H_2O_2-Cu^{2+}. The method can be used with a fluorometry microplate reader using a 96-well plate to perform simultaneous kinetic analysis of many samples and to reduce the amount of sample required. Although the $ORAC_{FL}$ method was originally developed for plasma samples, it has been successfully applied in other fields of study.

3. Principle

The assay measures the loss of fluorescein fluorescence over time due to peroxyl-radical formation by the breakdown of AAPH (2,2'-azobis-2- methyl-propanimidamide, dihydrochloride). Trolox [6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid], a water soluble vitamin E analog, serves as the antioxidant inhibiting fluorescein decay in a concentration dependent manner. The ORAC assay is a kinetic assay measuring fluorescein decay and antioxidant protection over time. The antioxidant activity in biological fluids, cells, tissues, food, beverage and natural extracts can be normalized to equivalent Trolox units to quantify the composite antioxidant activity present.

A peroxyl radical (ROO**.**) is formed from the breakdown of AAPH (2,2'-azobis-2-methyl-propanimidamide, dihydrochloride) at 37 °C. The peroxyl radical can oxidize fluorescein

(3',6'-dihydroxy-spiro[isobenzofuran-1[3H], 9'[9H]-xanthen]-3-one) to generate a product without fluorescence. Antioxidants suppress this reaction by a hydrogen atom transfer mechanism, inhibiting the oxidative degradation of the fluorescein signal. The fluorescence signal is measured over 120 min by excitation at 485 nm, emission at 538 nm. The concentration of antioxidant in the test sample is proportional to the fluorescence intensity through the course of the assay and is assessed by comparing the net area under the curve to that of a known antioxidant, trolox. The ORAC value is calculated by dividing the area under the sample curve by the area under the trolox curve with both areas being corrected by subtracting the area under the blank curve. One ORAC unit is assigned as being the net protection area provided by 1 μM trolox in final concentration. When the area under the curve for the sample is compared to the area under the curve for trolox, the result is given in Trolox equivalents.

4. Samples

4.1 Food, beverages, dietary supplements, powder extracts.

4.2 The assay should be carried out within five days of receipt of samples into the laboratory and stored at the appropriate temperature for the duration up until they are processed.

5. Equipment

Centrifuge 5810R
Standard Balance (4 decimal places)
15 mL Conical tubes with screw cap
Eppendorf pipettes and tips
Multichannel pipette and solution reservoir
Eppendorf tubes (1.5 and 2.0 mL)
Fluorescence plate reader (Fluoroskan Ascent)
Black 96-well plate
pH meter
Measuring cylinders (1 L, 50 mL, 100 mL, 250 mL)
Tube rotator
Gilson pipetting aid with 10 mL disposable serological pipettes

6. Chemicals and Reagents

6.1 Extraction Chemicals for hydrophilic antioxidants

60% Ethanol solution: In a 1 L media bottle add the following:

600 mL ethanol (Merck, Johannesburg, South Africa)

400 mL distilled water

This solution is stable @ room temperature for up to one month.

.

6.2 Reagents:

6.2.1 Phosphate Buffer: Make up to 75 mM at pH 7.4

Weigh 10.36g of sodium di-hydrogen monophosphate ($NaH_2PO_4.H_2O$) and add to a 1L media bottle. Add 1L of distilled water and mix until dissolved. Check pH and adjust to pH7.4. Buffer is stable at 4^{o}C for a month.

6.2.2 Fluorescein stock solution:

Dissolve 0.0225 g $C_{20}H_{10}Na_2O_5$ in 50 mL of phosphate buffer. Mix and protect from light (wrap bottle with foil). Fluorescein stock solution is stable at 4 °C in dark container for 1 year.

6.3.3 Peroxyl radical:

AAPH (2,2'-Azobis (2-methylpropionamidine) dihydrochloride : 25 mg/mL. (Store at -20^{o}C)

Weigh 150 mg (0.150g) of AAPH into a 15 mL screw cap tube. Prepare fresh every day. Do not add any solution until last step of assay.

6.3.4 Trolox: 500 µM Stock solution:

Weigh 0.00625 g 6-Hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid in 50 mL screw cap tube, add 50 mL phosphate buffer with Gilson pipetting aid, mix until dissolved. Dispense trolox stock solution into 2 mL eppendorf tubes and store at -20^{o}C. Trolox stock solution is stable at -20^{o}C for approximately 6 months.

Quality Control Check: Dilute a single aliquot 2x. This solution should give an absorbance of 0.670 ±0.015 at 289nm.

7. Sample extraction

7.1 Food

Weigh approximately 1.0 g (note the exact weight) of the sample in a 50 mL screw-cap tube. IMPORTANT: The sample weight of 1.0 g is not fixed and may need to be changed depending on the nature of the sample. This weight should be decreased if it is suspected that the sample contains high amount of antioxidants or increased if it is suspected to contain few antioxidants.

Homogenize samples in 50 mL ethanol solution (60%). Depending on the sample type, extraction mixtures may be treated as follows:

(a) Incubate at 60 °C for 30 min, centrifuge (4000 rpm, 5 min) and remove the resulting supernatants, aliquot and stored at -80 °C until time of analysis.

(b) Incubate at RT for 30 min, centrifuge (4000 rpm, 5 min) and remove the resulting supernatants, aliquot and stored at -80 °C until time of analysis.

7.2 Wine, juices and herbal teas

Can be used directly after a suitably dilution. If there are obvious precipitates centrifuge the sample for 3 min at 4000 rpm and use supernatant in analysis.

7.3 Crude powder extract

Weigh approximately 100 mg (0.10 g) (note exact weight) of the sample in a 50 mL screw-cap tube. IMPORTANT: The sample weight of 100 mg is not fixed and may need to be changed depending on the nature of the sample. This weight should be decreased if it is suspected that the sample contains high amount of antioxidants or increased if it is suspected to contain few antioxidants. Add 50 mL ethanol (60%) with the Gilson pipetting aid. Mix until dissolved. Sonicate if necessary. Centrifuge at 4000 rpm for 5 min. Supernatant can be used directly after a suitable dilution.

8. Sample Analysis

Switch the computer and the fluoroskan plate reader on. IMPORTANT: The fluoroskan should be switched on at least 30 min before starting the assay to allow the machine to reach a temperature of 37°C. A detailed procedure for using the program software to commence analysis is available in the full version of this SOP at the OSRC laboratory.

9. Sample and Reagent Preparation

9.1 Trolox standard series

Take 6 Eppendorf tubes and mark them A-F. Take one 2 mL aliquot Trolox stock solution and place in a 37°C water bath for approximately 10min and allow to defrost completely. Add the required amount of standard stock solution and diluent [phosphate buffer; (75 mM; pH7.4] to each tube as described in the table below:

Table 1: Trolox standard series preparation

Tube	Standard concentration μM	Trolox stock solution μL	Phosphate Buffer μL
A(Blank)	0	0	750
B(STD1)	83	125	625
C(STD2)	167	250	500
D(STD3)	250	375	375
E(STD4)	333	500	250
F(STD5)	417	625	125

9.1.2 Fluorescein working solution

Prepare fluorescein working solution from the stock solution by transferring 2 mL of phosphate buffer to an eppendorf tube (2 mL) and adding 12 µL stock fluorescein solution. Transfer 300 µL of this solution to 14.7 mL phosphate buffer in a 15 mL conical tube and mix.

9.1.3 Samples

All samples depending on their type must be made up in phosphate buffer to an appropriate dilution. The following serves as a guide for diluting samples:

Table 2: Sample dilution guide

Sample	Dilution
Fruit juices	100x
Teas	50x
Wines	200x
Vitamin waters	20x
Fruit and vegetables, powder extracts	50 to 100x

Since very slight amount of turbidity interfere with the determination, samples showing visible turbidity should be clarified by centrifugation. Alternately, samples may be filtered using a 0.45 µm syringe filter.

9.1.4 Quality Control (QC)

Accurately weigh 19g sucrose, 1g citric acid and 0.023g sodium citrate and add to a 250 mL volumetric flask. Fill to the mark with phosphate buffer and mix thoroughly. Accurately weigh out 0.02288 mg of trolox and add to the above formulation (concentration = 366.66 µM). Adjust pH to 8.0 to allow trolox to fully dissolve. When 12 µL is added to the prepared micro-well plate the concentration is 22 µM. Add 568 µL of this solution to 432 µL of phosphate buffer (concentration = 208.3 µM). When 12 µL of this solution is added to the prepared micro-well plate the concentration is 12.5 µM. Finally add 500 µL of this second solution and add to 500 µL of phosphate buffer (concentration = 104.15 µM). When 12 µL of this solution is added to the prepared micro-well plate the final concentration is 6.25 µM.

9.2 Micro-well Plate Preparation

9.2.1 Use a new micro-well plate for every assay performed. Transfer working fluorescein solution to a solution reservoir and dispense 138 µL to all 96 wells in the micro-well plate using a multichannel pipette.

9.2.2 Dispense 12 µL of blank/standard/sample in triplicate to the designated wells. (As shown in the plate layout diagram below)
N.B At this stage the final volume in assay wells are 150 µL.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Empty_00 Assay	Empty_00 Assay	Blank_Ass Assay	Blank_Ass Assay	Blank_Ass Assay	Cal_0001 Assay 5 umol/L	Cal_0001 Assay 5 umol/L	Cal_0001 Assay 5 umol/L	Cal_0002 Assay 10 umol/L	Cal_0002 Assay 10 umol/L	Cal_0002 Assay 10 umol/L	Empty_0017 Assay
B	Empty_00 Assay	Empty_00 Assay	Cal_0003 Assay 15 umol/L	Cal_0003 Assay 15 umol/L	Cal_0003 Assay 15 umol/L	Cal_0004 Assay 20 umol/L	Cal_0004 Assay 20 umol/L	Cal_0004 Assay 20 umol/L	Cal_0005 Assay 25 umol/L	Cal_0005 Assay 25 umol/L	Cal_0005 Assay 25 umol/L	Empty_0018 Assay
C	Empty_00 Assay	Empty_00 Assay	Ctrl_0001 Assay	Ctrl_0001 Assay	Ctrl_0001 Assay	sample _0 Assay 1:1	sample _0 Assay 1:1	sample _0 Assay 1:1	sample _0 Assay 1:1	sample _0 Assay 1:1	sample _0 Assay 1:1	Empty_0019 Assay
D	Empty_00 Assay	Empty_00 Assay	sample _0 Assay 1:1	sample _0 Assay 1:1	sample _0 Assay 1:1	sample _0 Assay 1:1	sample _0 Assay 1:1	sample _0 Assay 1:1	sample _0 Assay 1:1	sample _0 Assay 1:1	sample _0 Assay 1:1	Empty_0020 Assay
E	Empty_00 Assay	Empty_00 Assay	sample _0 Assay 1:1	sample _0 Assay 1:1	sample _0 Assay 1:1	Ctrl_0002 Assay	Ctrl_0002 Assay	Ctrl_0002 Assay	sample _0 Assay 1:1	sample _0 Assay 1:1	sample _0 Assay 1:1	Empty_0021 Assay
F	Empty_00 Assay	Empty_00 Assay	sample _0 Assay 1:1	sample _0 Assay 1:1	sample _0 Assay 1:1	sample _0 Assay 1:1	sample _0 Assay 1:1	sample _0 Assay 1:1	sample _0 Assay 1:1	sample _0 Assay 1:1	sample _0 Assay 1:1	Empty_0022 Assay
G	Empty_00 Assay	Empty_00 Assay	sample _0 Assay 1:1	sample _0 Assay 1:1	sample _0 Assay 1:1	sample _0 Assay 1:1	sample _0 Assay 1:1	sample _0 Assay 1:1	sample _0 Assay 1:1	sample _0 Assay 1:1	sample _0 Assay 1:1	Empty_0023 Assay
H	Empty_00 Assay	Empty_00 Assay	sample _0 Assay 1:1	sample _0 Assay 1:1	sample _0 Assay 1:1	sample _0 Assay 1:1	sample _0 Assay 1:1	sample _0 Assay 1:1	Ctrl_0003 Assay	Ctrl_0003 Assay	Ctrl_0003 Assay	Empty_0024 Assay

Figure 1. ORAC$_{FL}$ Plate Layout

9.2.3 Allow plate to warm up to 37^{o}C by inserting the microwell plate into the fluorometer. Place the plate with the A1 well facing top and left. Close the door. Set the timer to 20 mins and press start.

9.2.4 Measure 7 mL of phosphate buffer in a conical tube and allow to warm up to 37^{o}C by incubating in a waterbath set at 37^{o}C for approximately 20mins.

9.2.5 Add 6 mL of pre-warmed phosphate buffer to the 150 mgAAPH weighed out earlier (step 7.3.3).

9.2.6 Remove the microwell plate from the fluorometer and immediately add 50µL of AAPH solution to all 96 wells of the microwell plate. This step should take no more than 60seconds (to prevent the temperature from dropping excessively). At this stage the final volume in the relevant wells are 200 µL.

9.2.7 Insert the microwell plate back into the fluorometer and press start to begin the analysis.

9.2.8 Once the analysis is complete, discard the contents of the micro-well plate and rinse with plenty of distilled water.

9.2.9 Save the date by clicking on “sheet“ and “save sheet as” to the flash drive (E) with a XLS extension.

10. Data analysis and calculations

The calculation steps have been omitted from this thesis. A detailed procedure for data analysis and calculations are available in the full version of this SOP at the OSRC laboratory.

10.1 The ORAC values are calculated using a regression equation (y=mx+c) between Trolox concentration (Y) (μM) and the net area under the fluorescence decay curve (X). Data are expressed a micromoles of Trolox equivalents (TE) per liter or per milligram of sample (units, μmole Trolox/mL for blood or μmole Trolox/g wet weight for food). The area under the curve (AUC) is calculates as:

$$AUC = (0.5 + f_2/f_1 + f_3/f_1 + f_4f_1 + \ldots + f_i/f_1) \times CT$$

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

10.2 If any of the ORAC values falls outside of the range of the standard curve, repeat assay after sample was diluted. Pipette 100 μl of sample supernatant into a new eppendorf. Add 900 μl of phosphate buffer to effect a 10-fold dilution.

11. Post analysis sample storage

11.1 Wines, oils and powder extracts: Store at RT in a dark cupboard for 30 days before being discarded.

11.2 Food, beverages and seeds: Store at 4^{o}C for a week before being discarded.

12. Precautions

12.1 Reagents and standards must be prepared fresh on the day of analysis.

12.2 Before pipetting each reagent, equilibrate the pipette tip and do not expose the pipette tip to the reagent(s) already in the well.

12.3 Standard laboratory protective clothing must be worn.

12.4 Dispose of all disposable materials (tubes, pipette tips etc.) into a Biohazard bag.

12.5 Wipe all work surfaces clean with 70% ethanol before and after completion of the procedure.

13. Data records

13.1 All records are to be stored in the OSRC laboratory.

14. References

14.1 Prior, R.L.; Huang, H.; Gu, L.; Wu, X.; Bacchiocca, M.; Howard, L.; Hampsch-Woodill, M.; Huang, D.; Ou, B.; Jacob, R. 2003. Assays for hydrophilic and lipophilic antioxidant capacity ($ORAC_{FL}$) of plasma and other biological and food samples. *Journal of Agricultural and Food Chemistry,* 51:3273-3279.

14.2 Cao, G.; and Prior R.L. 1998. Measurement of oxygen radical absorbance capacity in biological samples. *Methods in Enzymology*, 299:50-62.

Appendix D: Standard operating procedure: High performance Liquid Chromatography with UV detection (UV-HPLC) for L-ascorbic acid measurement-Rev1

All personnel concerned with this PROCEDURE must sign the PROCEDURE to indicate that he/she is familiar with its contents.

DOCUMENT CHANGE RECORD:

Date	Revision	Section	Description of Change
25 November 2012	1	**ALL**	***STANDARDIZATION OF SOP***
(Next revision: June 2013)			

Table of Contents

1. Purpose

This document defines the methodology for the UV-HPLC assay used for the measurement of L-ascorbic acid in food and beverage commodities.

2. Scope and Application

L-sscorbic acid (Vitamin C), an antioxidant and free radical scavenger, is found ubiquitously in fruit and vegetables such as citrus fruits (oranges, lemons, limes, tangerines etc.), melons, tomatoes, peppers, broccoli, green leafy vegetables such as spinach, potatoes and turnips. Its quantitative determination is especially important in the production of wine, beer, milk, soft drinks and fruit juices, where it can be a quality indicator. Given the essential role played in the human diet, L-ascorbic acid and salt derivatives are commonly used as food additives, with the additional advantage of their antioxidant and flavour enhancing properties. In the wine industry, L-ascorbic acid can be used to prevent oxidation of wine.

3. Summary of Method

The assay is a chromatographic measurement using HPLC with a UV detector. L-ascorbic acid is identified by comparing the retention time of the sample peak with that of the ascorbic standard at 254nm. Quantification is carried out using external standardization.

4. Health and Safety Warnings

4.1 Standard laboratory protective clothing and eye covering is required.
4.2 Dispose of all disposable materials (tubes, pipette tips etc.) into a Biohazard bag.
4.3 Wipe all work surfaces clean with 70% ethanol before and after completion of the procedure.

5. Interferences

5.1 Since very slight amount of turbidity interfere with the determination, samples showing visible turbidity should be clarified by centrifugation. Alternately, samples may be filtered.

5.2 If the sample contains heavy metals (e.g. copper or iron) it is essential to prepare the sample solution immediately before pipetting into the cuvettes as L-ascorbic acid is unstable in solutions containing metal ions.

6. Cautions

6.1 Meta-phosphoric acid solution and standards must be prepared fresh on the day of analysis unless otherwise stated.

6.2 The mobile phase may be prepared and stored both at room temperature or 4ºC for up to 48 hr.

6.3 Keep all samples/standards at 4ºC when not handling them during analysis.

6.4 Before pipetting each reagent, equilibrate the pipette tip and do not expose the pipette tip to the reagent(s) already in the container.

6.5 The accuracy of the results will be greatly affected by the accuracy of the volumetric measurements. Make sure that any volumetric flasks or pipettes used for obtaining the appropriate dilutions are calibrated correctly.

7. Personnel Qualifications

7.1 Technicians should be trained at least one week in the method before initiating the procedure alone.

8. Equipment and Chemicals Required

8.1 Equipment

Centrifuge 5810R
Balance (4 decimal places)
15 mL Conical tubes with screw cap
Eppendorf pipettes and tips
Eppendorf tubes (1.5 mL)

pH meter

Media bottle (1L)

HPLC with UV detector and C18 column

HPLC glass vials

Sample homogenizer

Nylon 0.45 µM syringe filters

8.2 Chemicals

Extraction solvent: 4.5% meta-phosphoric acid solution: accurately weigh 4.5 g meta-phosphoric acid and add to a 100 mL volumetric flask. Fill to the mark with distilled water and mix thoroughly.

Mobile phase: Distilled water/acetonitrile/formic acid (99:0.9:0.1,v/v/v) adjusted to pH 2.6. Use acetonitile gradient grade for liquid chromatography.

Standard stock: Accurately weigh 250 mg (0.250 g) of L-Ascorbic (Sigma Cat Nr.: A5960) acid into a 250 mL volumetric flask. Fill to the mark with 4.5% meta-phosphoric acid and mix thoroughly (1 mg/mL). Prepare fresh. Use this solution as the stock.

L-AA standards: Dilute the above stock solution with 4.5% meta-phosphoric acid solution to prepare 5 to 125 µg/mL L-AA standards as follows:

Table 1: L-AA standard series preparation

Concentration (µg/mL)	L-AA (µl)	5% MPA soln (µl)
5	5	995
10	10	990
20	20	980
50	50	950
75	75	925
100	100	900
125	125	875

Quality Control (QC): (Synthetic juice formulation) Accurately weigh 19 g sucrose, 1 g citric acid and 0.023g sodium citrate and add to a 250 mL volumetric flask. Fill to the mark with 4.5% meta-phosphoric acid solution and mix thoroughly. Add 250 mg of L-AA and mix thoroughly (final concentration: 1 mg/mL). Dilute further with 4.5%

meta-phosphoric acid to prepare three concentration levels of QCs as follows:

Table 2: L-AA QC preparation

Qc samples (µg/l)	L-ascorbic acid synthetic juice formulation (1 mg/ml) (volume in µL)	4.5% metaphosphoric acid solution (volume in µL)
QC 1	**6.5**	**993.5**
QC 2	**55**	**945**
QC 3	**115**	**885**

9. Sample Collection, Handling and Preservation

9.1 All beverage samples must be appropriately diluted with 4.5% meta-phosphoric acid solution if required, and filtered through 0.45 µm filters and stored away from light at 4°C.

9.2 Solid samples: Homogenise or crush solid samples in 4.5% meta-phosphoric acid solution, filter through 0.45 µm filters and stored away from light at 4°C.

9.3 Baobab powder: 100 mg in 10 mL water. The following serves as a guide for diluting samples.

Table 1: Sample dilution guide

Sample	Dilution
Fruit juices	100x
Teas	50x
Wines	200x
Vitamin waters	20x
Fruit and vegetables, powder extracts	50 to 100x

10. Sample Analysis

10.1 Switch on the HPLC (Computer, pumps, auto sampler, UV detector, fluorescence detector, degasser and control box). Must be switched on 20 min prior to analysis commences.

10.2 Place the mobile phases in the degasser holder. Let the mobile phases degas for at least an hour.

10.3 Start the “A” pump by pressing the green “run” button on the P2000 box. Let the pump run for at least half an hour to achieve a stable baseline.

10.4 Click on “solvent bottle filling”. Check and change volumes accordingly. Remember to switch off bottles that are not in use.

10.5 Check temperature and set at 23ºC.

10.6 Check that the diode array detector (DAD) is set on the correct wavelength (245nm)

10.7 Click on sample tray and then on “sequence table”. Fill on relevant sample details.

10.8 Once all parameters are highlighted in green, and the baseline has stabilized, press “start”

10.9 Transfer the samples, standards and quality control each to HPLC glass vials. Place them in the preferred order into the auto-sampler starting at position A1.

10.10 Each standard, control and sample is injected in duplicate in a chromatograph with UV detection, equipped with a C18 column (250x4.6 mm, with 5-μm particles) and pre-column with the same stationary phase. Mobile phase consists of an distilled water/ acetonitrile/formic acid (99:0.9:0.1, v/v/v) solution, with a flow rate of 1 mL/min. Injection volume is 20 μL. Detection is performed at 254 nm wavelength. Run time is 15 min.

10.11 After the analysis is complete, discard the standards/samples/control vials in a biohazard bag.

10.12 Opening a data file: On the Chromquest program, select “File”, then select “Data” and click on “Open”. Double click on the appropriate file. To view the chromatogram, select the appropriate detector on the detector button (between the print button and the cut button on the Chromquest program.

Printing a data file: On the Chromquest program, select “Reports”, then select “View” and click on “Area%”. To print the report, select “Reports”, then select “Print” and click on “Area%”. This procedure has to be peformed for each standard, control and sample.

11. Data Analysis and Calculations

11.1 Start the Microsoft excel program.

11.2 The concentration of the sample is calculated using the straight line equation y=mx+c. The dilution factors and weights are subsequently multiplied to this concentration to give the final answer in the desired units.

11.3 Quantification is based on the peak area method, with results integrated and displayed by an integrator.

12. Data Records and Management

All laboratory records must be maintained in the proper file designated for the method.

13. References

Romeu-Nadal, M., Morera-Pons, S., Castellote, A.I., Lopez-Sabater, M.C. 2006. Rapid high-performance liquid chromatographic method for Vitamin C determination in human milk versus an enzymatic method. *Journal of Chromatography B*, **830**(1):41-46.

Appendix E: Verification forms for verification procedures performed.

Figure 1 depicts an example of a temperature chart utilized to monitor and plot freezer temperatures. Document 1 depicts a verification form for the reporting of calibration of measuring cylinders. Document 2 depicts a verification form for the reporting and recording of instrument and analyzer troubleshooting and repair. Document 3 depicts a verification form for maintaining and recording performance of analytical balances.

-20°C REFRIDGERATION TEMPERATURE CHART

Fridge ID: Thermometer ID: Month/Year:

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
-10.0ºC																															
-12.0ºC																															
-14.0ºC																															
-16.0ºC																															
-18.0ºC																															
-20.0ºC																															
-22ºC																															
-24.0ºC																															
-26.0ºC																															
-28.0ºC																															
-30.0ºC																															
Lab Analyst																															
Supervisor																															

INSTRUCTIONS:

a) Plot the **MAXIMUM** and **MINIMUM** temperature **DAILY** and initial chart.

b) If the temperature falls outside acceptable limits (-14 to-26°C), the corrective action taken must be logged and signed.

c) Ensure that this chart is marked with the identification number of the Incubator.

Figure 1. -20 °C refrigerator temperature chart

Calibration Report: Measuring Cylinders:

Calibration Date: **Description:**

Graduations: Single line at indicated capacity **Serial No:**

Class: **Volume Range:**

Environmental Factors:

Room Temperature:_____

Calibration Details:

Indicated volume(mls)	Mass (g)	Density factor	Actual Mass(g)	%deviation

Indicated volume(mls)	Mass (g)	Density factor	Actual Mass(g)	%deviation

Summary Statistics:

Volume (mls)	Mean Mass (g)	%CV	%inaccuracy:

Acceptable Limits:

Measuring cylinders<100mls	**Imprecision(%CV):**	**≤5%**
	Inaccuracy(%error):	**≤5%**
Measuring cylinders>100mls	**Imprecision(%CV):**	**≤5%**
	Inaccuracy(%error):	**≤3%**

Analyst Signature: Date:

This document must be filed for accreditation purposes.

Document 1. Verification form for calibration of measuring cylinders

INSTRUMENT TROUBLESHOOTING AND REPAIR REGISTER

Complete the following form for all instrument repair and problems, with corrective actions taken

Laboratory: Oxidative Stress Research Lab **Model:**

Instrument Name: **Year of Purchase:**

Serial No:

Date:	Indicate problem experienced:	Document troubleshooting performed:	Problem corrected: Yes/No	Worksheet/ Invoice No.:	Date and Sign:

This document must be filed for accreditation purposes

Document 2. Instrument troubleshooting and repair register

MAINTENANCE AND QUALITY CHECKS FOR ANALYTICAL BALANCE

Balance Name: Sartorius **Serial Number: _____**

Daily: Accuracy check **Control weights:** **Month:…… Year: ……..**

Date	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
Mass (1g)																															
Mass (50g)																															
Initials																															

Acceptable range for mass weighed: ± 0.1%

Weekly: Cleaning

Date	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
Exterior																															
Interior																															
Initials																															

1. Clean exterior with a damp cloth

2. Clean the interior with a brush or dry tissues. Carefully remove weighing pan to clean beneath it as well.

Service:

1. **The balance must be serviced every 6 months by a service technician (e.g. from Cape Metrology Field).**
2. **If considered necessary, he will calibrate the balance.**
3. **The calibration certificate must be filed in the balance maintenance file.**

Please file this document for accreditation purposes

Document 3. Maintenance and quality checks for analytical balance