DEVELOPMENT OF A RAPID AND ROBUST METHOD TO MEASURE ANTIOXIDANT ACTIVITY IN A LIPID SYSTEM

MD AHSAN GHANI

B. Sc. (Chem.), M. Sc. (Chem.)

The thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

School of Agricultural and Wine Sciences
Charles Sturt University
Australia

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CERTIFICATE OF AUTHORSHIP

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DEDICATION

To my parents, wife and son,

Without you all this long journey would not have been possible, love you all so much
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## GLOSSARY AND ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>A. implexa</td>
<td><em>Acacia implexa</em></td>
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<tr>
<td>AAPH</td>
<td>2, 2’-azobis (2-amidinopropane) dihydrochloride</td>
</tr>
<tr>
<td>ABTS</td>
<td>2,2’-azinobis (3-ethyl-benzothiazolline-6-sulfonic acid)</td>
</tr>
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<td>BHA</td>
<td>Butylated hydroxyanisole</td>
</tr>
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<td>BHT</td>
<td>Butylated hydroxytoluene</td>
</tr>
<tr>
<td>BCB</td>
<td>β-carotene bleaching</td>
</tr>
<tr>
<td>CD</td>
<td>Conjugated diene</td>
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<td>DCM</td>
<td>Dichloromethane</td>
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<td>DPPH</td>
<td>2,2-diphenyl-1-picrylhydrazyl</td>
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<td>DAD</td>
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<td>E. rossii</td>
<td><em>Eucalyptus rossii</em></td>
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<td>E. cupressiformis</td>
<td><em>Exocarpos cupressiformis</em></td>
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<td>FRAP</td>
<td>Ferric reducing antioxidant power</td>
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<td>FTC</td>
<td>Ferric thiocyanate</td>
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<td>FOX</td>
<td>Ferrous oxidation xylenol orange</td>
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<td>FC</td>
<td>Folin-Ciocalteu</td>
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<td>GAE</td>
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<tr>
<td>4-HNE</td>
<td>4-hydroxy-2- nonenal</td>
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<td>IDF</td>
<td>International Dairy Federation</td>
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<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
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<td>LSD</td>
<td>Least significant difference</td>
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<td>LA</td>
<td>Linoleic acid</td>
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<td>NMR</td>
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<td>Redox</td>
<td>Reduction-oxidation</td>
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<td>SPSS</td>
<td>Statistical Package for Social Scientists</td>
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<td>TCA</td>
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<td>TBARS</td>
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<td>Ultra High Pressure Liquid chromatography-quadrupole-time of flight mass spectrometry</td>
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ABSTRACT

Lipid oxidation is problematic in both the food industry (oxidative rancidity) and human health (diseases such as atherosclerosis). Some lipids such as polyunsaturated fatty acids (PUFAs, e.g. linoleic acid (LA)), are very susceptible to oxidation in the presence of heat, light or trace amount of metal ions. This oxidation produces harmful primary oxidation products such as peroxides, which breakdown to numerous toxic secondary oxidation products such as aldehydes, conjugated dienes, and furans. Of the products, aldehydic malondialdehyde (MDA) is most studied as it is associated with off-flavours and aromas in meat products, as well as being a marker of oxidative damage in physiological systems. MDA, and some other lipid oxidation products react with thiobarbituric acid (TBA) to give pink/red coloured adducts, which are measured in the thiobarbituric acid reactive substances (TBARS) assay. On the other hand, peroxides may be measured by the ferric thiocyanate (FTC) assay. These two assays are of the several methods such as conjugated diene (CD), ferrous oxidation xyl enrol orange (FOX) and hexanal to measure lipid oxidation products.

Antioxidants are compounds that can inhibit the formation of oxidation products when present at a much lower concentration compared to an oxidisable substrate. Both FTC and TBARS assays can be used to measure the effectiveness of an antioxidant (i.e., antioxidant activity) in protecting a lipid system from oxidative damage. The above assays consist of two steps: lipid oxidation and colour development, and the former was found to be the source of variability in the widely
used TBARS assay. The variability in the TBARS assay includes imprecision of results in intra and inter-laboratory trials. Both FTC and TBARS assays have the same oxidation step, which needs a long time for the lipid substrate to be oxidised. This makes the two assays slow. As TBARS assay has variability in results, and both TBARS and FTC assays are slow in screening antioxidant activity, there is a need to develop a method so the assays can be rapid and more robust.

In this thesis, a rapid and robust method incorporating both FTC and TBARS assays was developed. It was carried out in several stages. In the first stage, the variability in the TBARS assay was investigated in a multi-phase oxidation system using a literature method. Steps were taken to minimise or overcome the variability. Having no or little possibility of overcoming variability, an alternative LA-emulsion system was chosen and the system was tested for variability in results, as above. The LA emulsion showed good intra-and inter-batch precision for the FTC and TBARS assays in measuring oxidation and antioxidant activity. Therefore in this oxidation system, the conditions of reaction were altered so that the assay became rapid. After optimising the method to be more rapid and robust, it was applied to screen antioxidant activity of Australian native plants used in traditional Indigenous medicine.

In the first stage of this thesis, linoleic acid (LA) was oxidised in the multi-phase system by atmospheric oxygen with vigorous shaking to promote interaction with a separate aqueous layer of Cu$^{2+}$ catalyst and Trolox as antioxidant. Initial monitoring of the results showed variability in the TBARS assay. Therefore a systematic study was conducted to identify possible sources of variability including: order of addition
of reactants; pre-formed peroxides in LA, and the effect of different concentrations of added antioxidant, Trolox. All contributed to variability for the measurement of antioxidant activity of Trolox. In this multi-phase system, breakdown of peroxides did not form TBARS consistently from one experiment to the next. Through the use of LA with pre-formed peroxides removed, the variability was not minimised. It was also found that antioxidant activities reported in other studies were most likely not due to the protective effect of antioxidant on the LA substrate, rather than the antioxidant inhibits the breakdown of pre-formed peroxides. In contrast to the multi-phase system, oxidation of LA in an emulsion showed good intra-and inter-batch precision for the FTC and TBARS assays in measuring oxidation and antioxidant activity.

In the second stage of this thesis, the oxidation of the LA emulsion system was modified by altering the reaction parameters such as temperature, catalyst, and oxygen initiator in order to make the oxidation faster. To validate the method, the order of antioxidant activity for oxidation of LA at 37 °C for 20 h, with 250 µM antioxidants (Trolox, quercetin, ascorbic acid, and gallic acid) was used. Of the altered reaction parameters, only 50 °C provided an oxidation system consistent with the validation conditions reducing the assay time from 20 hours to 5 hours. Further validation of the method was achieved with antioxidants (+)-catechin, (-)-epicatechin, caffeic acid, and α-tocopherol. Similar order of antioxidant activity was observed under accelerated conditions. It showed that the method could distinguish between different levels of protection of LA and thus could be used for screening purposes.
The optimised method was used to screen the antioxidant activity of crude extracts and fractions (n-hexane, DCM, EtOAc and EtOH) of three species of Australian native plants, *vis. Acacia implexa* bark and leaves, *Eucalyptus rossii* and *Exocarpos cupressiformis* leaves. All crude extracts of all species showed appreciable antioxidant activity against formation of both peroxides and TBARS. The non-polar n-hexane and DCM fractions showed high antioxidant activity in both FTC and TBARS assays. For comparison, the Folin-Ciocalteu and 2,2’-azinobis (3-ethyl-benzothiazolline-6-sulfonic acid) (ABTS) assays were also performed and the more hydrophilic fractions had high antioxidant activity in these assays, while the lipophilic fractions showed low activity. Thus screening with a lipid-based assay revealed antioxidants that were more potent in a lipid system, but which might have been missed if only aqueous-based tests had been used.

In order to identify potential antioxidants in the above extracts and fractions, chromatographic analysis was undertaken by liquid chromatography diode array detection quadrupole time of flight mass spectrometry (LC-DAD-qTOF-MS). Major peaks were tentatively assigned for each species. In this thesis, for the first time, several compounds such as rhamnetin, hederagenin, and quercetin diglucoside in *Acacia implexa* bark, (-)- epicatechin, rutin, and kaempferol in *Acacia implexa* leaves, ellagic acid, sideroxylin, and eucalyptin in *Eucalyptus rossii*, and (+)-catechin, and quercetrin in *Exocarpos cupressiformis* leaves have been reported. Some of these compounds are flavonoids, which are well studied antioxidants and are likely to contribute to the antioxidant activity as measured using the developed assay.
CHAPTER 1. LITERATURE REVIEW

1.1 Introduction:

Due to adverse consequences in the food industry (McClements & Decker, 2000), and more broadly detrimental effects on human health, (Frankel, 2014), lipid oxidation needs to be limited in both areas. Antioxidants play a crucial role in inhibiting lipid oxidation. Antioxidants from natural sources such as medicinal plant extracts are potentially safer than synthetic antioxidants. Indigenous people of different countries have long been using a number of traditional medicinal plants as remedies for illness. These plants may be good sources of natural antioxidants.

Antioxidant activity of medicinal plants can be measured by free radical scavenging assays such as 2,2’-azinobis (3-ethyl-benzothiazolline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH), and lipid-based assays such as ferric thiocyanate (FTC) and thiobarbituric acid reactive substances (TBARS). Free radical scavenging assays are simple, can perform high throughput screening, and they provide some information about the mechanism of antioxidant activity (e.g. electron transfer, hydrogen atom transfer, etc), however, they lack a substrate, and therefore do not provide the measure of the protection that an antioxidant affords an oxidisable molecule. On the other hand, both FTC and TBARS assays have advantages in measuring the protection of a lipid substrate in the presence of an antioxidant. The TBARS assay has been reported to have high variability in measuring antioxidant
activity and is not capable of being used in high throughput screening. The other assay, FTC is not rapid either, although it is more robust than the TBARS assay. There is therefore a need to develop a rapid and robust method, incorporating TBARS and FTC assays, for the measurement of antioxidant activity.

In this literature review, lipid oxidation, antioxidants and their activity, Australian native plants as antioxidants, and various aspects of the TBARS and FTC assays will be discussed.

1.2 Lipids

Lipids are naturally occurring substances, which have no solubility in water but are easily soluble in organic solvents such as benzene, diethyl ether and chloroform (Kates, Work, & Work, 1972). Polyunsaturated fatty acids (PUFAs), carotenoids, steroids, terpenoids, and also bile acids are common constituents of lipids. Of the constituents, fatty acids such as linoleic acid (LA) are commonly present in triglycerides and phospholipids.

1.3 Lipid oxidation

Lipid oxidation is a complex sequence of chemical reactions involving molecular oxygen and the presence of light, heat or trace amounts of metal ions (Spickett, Wiswedel, Siems, Zarkovic, & Zarkovic, 2010). Some enzymes can also accelerate
this process (Cheng & Li, 2007). All lipids may undergo oxidation, but PUFAs are particularly susceptible due to the presence of multiple double bonds. Lipid oxidation is initiated by oxygen-derived peroxyl free radicals (Fereidoon Shahidi & Ying Zhong, 2010) via peroxyl intermediate formation. During lipid oxidation, the PUFAs in lipid molecules lose at least one electron or hydrogen atom from a bisallylic position to form fatty acyl peroxyl radicals and consequently to produce peroxides such as hydroperoxides (Chen, McClements, & Decker, 2011), which in turn break down to form malondialdehyde (MDA), hexanal and 4-hydroxy-2-nonenal (4-HNE), among other compounds. This process depends on the level of unsaturation in the fatty acid moiety and the presence of free radical initiators. High unsaturation in fatty acids allows the oxygen to undergo a more complex chain reaction (McClements & Decker, 2006). Furthermore, during oxidation, less energy is needed to abstract a methylene hydrogen atom when there is more unsaturation.

Linoleic acid and its ester linoleate are the most abundant polyunsaturated lipids in vivo (Niki, 2009) so their oxidation is well studied. The oxidation process proceeds by a straightforward pathway forming conjugated diene hydroperoxides and other short chain carbonyl compounds such as MDA and hexanal. On the other hand, oxidation of higher unsaturated fatty acids and their esters such as arachidonic acid (C20:4), eicosapentaenoic acid (EPA) (C20:5) and docosahexanenoic acid (C22:6) (DHA) proceeds through complicated pathways, giving rise to numerous lipid oxidation products including complex hydroperoxides and prostaglandin-type cyclic peroxides.
1.4 Oxidisable lipid substrates

Cell membrane

The PUFAs in cell membranes are very susceptible to oxidation. Cell membrane contributes to the basic structure of the cell and 30-80% of the mass of cell membrane is made up of lipids (Catalá, 2009). In a lipid bilayer, a polyunsaturated chain of fatty acids is bonded to a phospholipid by an ester linkage. Due to lipid oxidation causing physical and chemical changes to components, the biological and physical functions of cell membrane are greatly disrupted. For example, when lipid oxidation occurs, the structures of PUFAs in the cell membranes are distorted, which imparts improper water permeability and transport of ions across membranes (Gueraud et al., 2010). As a consequence of oxidation, the permeability of cell membrane is abnormally increased and the biological function of a cell is interrupted, which eventually leads to cell death (Ilie & Margină, 2012).

Oxidation of food lipids

Food contains a complex mixture of lipid components such as triglycerides and phospholipids. PUFAs such as omega-6 (linoleic acid), omega-3 (α-linolenic acid) and arachidonic acid are common dietary components of food (Serini, Fasano, Piccioni, Cittadini, & Calviello, 2011). In the presence of heat, light or trace amounts of metal ions, these components form radicals; then react with oxygen to cause lipid oxidation in foods (Choe & Min, 2006). Some toxic volatile compounds such as hexanal are formed, and these are responsible for rancidity in food. In addition, lipid oxidation is a cause of quality deterioration in food.
1.5 Mechanism of lipid oxidation

More than half a century ago, Bergstrom (Bergstrom, 1945) outlined the mechanism of auto-oxidation of PUFAs as a radical reaction. E.N. Frankel was the pioneer in describing lipid oxidation (Frankel, 1980); in his review, along with a mechanism of lipid oxidation, he described the early studies in lipid oxidation, fatty acid oxidation by singlet oxygen, and decomposition of hydroperoxides. A comprehensive description of lipid oxidation has been depicted in the chemical review of Liu (Liu, 2010) and an excellent chemical description of the lipid oxidation mechanism is found in the review from Yin et al. (Yin, Xu, & Porter, 2011). Schneider described lipid oxidation products and a mechanism (Schneider, 2009). Min described a singlet oxygen quenching mechanism (Min & Boff, 2002). A mechanism for lipid oxidation is more broadly described in the book of Frankel (Frankel, 2014).

There are three types of mechanisms in lipid oxidation:

(i) auto-oxidation;

(ii) photo-oxidation;

(iii) enzymatic oxidation.

Auto-oxidation is mediated by free radicals and photo-oxidation proceeds with the incorporation of singlet oxygen and requires light and sensitisers such as chlorophyll, myoglobin, or riboflavin. In enzymatic oxidation, cyclooxygenase and
lipoxygenase catalyse the reactions between oxygen and PUFAs. Of the mechanisms, auto-oxidation is the most common.

**Mechanism of auto-oxidation:**

Auto-oxidation is based on a set of radical mediated chain reactions. Like other lipid oxidation mechanisms, auto-oxidation proceeds through initiation, propagation, and termination stages. A simplified mechanism is illustrated below Figure 1.1:

![Figure 1.1: Simplified mechanism of lipid oxidation, where ‘L’ stands for lipid.](image)

At the initial stage, lipid oxidation occurs at a very slow reaction rate, followed by a sudden increase in oxidation rate. The initial stage is called the induction period.
At first, a hydrogen atom from the active methylene group of a lipid (LH) is abstracted to form a lipid radical (L•). A variety of initiators including heat, light, transition metals (e.g. Cu²⁺ or Fe²⁺), reactive oxygen or nitrogen species can initiate this lipid oxidation and form a pool of radical molecules. In the propagation stage, an oxygen molecule attacks the carbon centred lipid radical ((L•) to form a peroxyl radical (LOO•). The peroxyl radical abstracts a hydrogen atom from a new lipid molecule (LH), making a new lipid radical. This lipid radical contributes to chain propagation. The propagation reaction can be repeated several thousand times until no hydrogen atom is available or the chain of oxidation is terminated by an antioxidant. In this termination stage, the reactions between radicals are quenched by forming non-radical products.

1.6 Lipid oxidation products and toxicity

Lipid oxidation results in two types of oxidation products – *vis.* primary and their breakdown products, secondary. Primary oxidation products include peroxides (e.g. hydroperoxides), while secondary products includes several aldehydes, alkanes, alkenes and conjugated dienes (Cherubini, Ruggiero, Polidori, & Mecocci, 2005).

PUFAs such as linoleic acid, linolenic acid, arachidonic acid, and their esters produce hydroperoxides, which are reactive, unstable, and easily undergo successive breakdown to yield more stable short chain compounds (Spiteller, Kern, Reiner, & Spiteller, 2001). The main breakdown products of hydroperoxides include short
chain aldehydes, such as 4-hydroxy-2-nonenal (HNE), hexanal, pentanal, and malondialdehyde (MDA), (Uchida, 2003). A flow chart of lipid oxidation product formation from PUFAs is outlined below Figure 1.2:
Of the lipid oxidation products, hydroperoxides and MDA are some of the commonly used markers to measure the degree of oxidation of a lipid substrate and hence are broadly described in this thesis.
**Hydroperoxides**

Hydroperoxides are odourless and tasteless lipid oxidation products (Papastergiadis, Mubiru, Van Langenhove, & De Meulenaer, 2012). A number of volatile aldehydes are formed upon the breakdown of hydroperoxides of linoleic acid, linolenic acid, arachidonic acid, and eicosapentaenoic acid; and their esters. Most of these aldehydic products have an unpleasant odour and are responsible for the rancidity of lipids. Hydroperoxides can interact with proteins and amino acids and make complex molecules in the body (Gardner, 1979). Hydroperoxides and their breakdown products are reported to be mutagenic and carcinogenic (Helbock, Motchnik, & Ames, 1993).

**Malondialdehyde (MDA)**

MDA is a naturally occurring aldehydic product from the lipid oxidation of PUFAs (Barriuso, Astiasaran, & Ansorena, 2013). MDA has also been extensively studied due to its many pathogenic roles in causing atherosclerosis, cancer, rheumatoid arthritis, diabetes, and neurodegenerative disorders such as Parkinson’s and Alzheimer’s diseases. MDA is mutagenic and carcinogenic (Janero, 1990); the carcinogenic effects were first tested on rats in 1972 (Marnett, 1999). MDA is highly toxic in vivo and it can perform covalent modification on DNA and proteins. This type of genotoxic activity causes mutations and can consequently lead to cancer (Del Rio, Stewart, & Pellegrini, 2005). MDA can induce chromosomal disorders, being
ten times more reactive than acetaldehyde for inducing such disorders. Another toxicity of MDA arises when it makes inter-molecular cross-linking with collagen, the most abundant protein in mammals (30% of the protein content of the human body). This cross-linking consequently contributes to the stiffening of cardiovascular tissue and is related to cardiovascular problems. MDA inhibits the genetic sequence activity of DNA and protein synthesis.

Of the lipid oxidation products, the reaction of MDA with thiobarbituric acid (TBA) is an early discovery, although some other oxidation products have been found later to be reactive with TBA. All of these compounds together are called thiobarbituric acid reactive substances (TBARS), and are described below:

**Thiobarbituric acid reactive substances (TBARS)**

A wide variety of saturated and unsaturated aldehydes, ketones, ketosteroids, acids, esters, imides and amides (urea), oxidised proteins, pyridines and pyrimidines, sucrose, fructose, and 2-deoxyribose can react with TBA to give TBA-MDA like coloured adducts (Guillen-Sans & Guzman-Chozas, 1998). Among lipid oxidation products, short chain saturated aldehydes, alka-2-enals, alka-2,4dienals can form similar types of TBA-MDA adducts. Carbonyl compounds such as hexanal are reported to be TBA reactive substances (Guzman-Chozas, Vicario-Romero, & Guillen-Sans, 1998). Many compounds can react with TBA, some of which are not aldehydes (Brownley & Lachman, 1965; Janero, 1990). Like MDA, TBARS are toxic and have been implicated in pathogenic diseases.
1.7 Lipid oxidation measurements

Lipid oxidation assays are based on an estimation of concentrations of markers formed in reaction. The amount of the markers indicates the degree of lipid oxidation. Depending on the types of markers, the assays can be categorised: measurement of primary and secondary oxidation products. For primary oxidation products, peroxides (hydroperoxides) can be determined by conjugated diene (CD) (Moon & Shibamoto, 2009), ferrous oxidation xylenol orange (FOX) (Bou, Codony, Tres, Decker, & Guardicila, 2008) or ferric thiocyanate (FTC) (Shantha & Decker, 1994). The secondary oxidation products, such as TBARS, are determined by the TBARS assay (Antolovich, Prenzler, Patsalides, McDonald, & Robards, 2002), hexanal by the hexanal assay (Brunton, Cronin, Monahan, & Durcan, 2000). Of these, FTC and TBARS assays are the focus of this thesis and are discussed extensively below:

1.7.1 Ferric thiocyanate (FTC) assay

In this assay, ferrous ions are oxidised by peroxides or hydroperoxides formed from lipid oxidation. This results in the formation of ferric ions which react with thiocyanate to form a ferric thiocyanate complex. This complex is brick-red and stable in an aqueous environment, and the complex concentration can be monitored by visible spectrometry at 500 nm (Moon & Shibamoto, 2009). The FTC assay was
developed by the International Dairy Federation (IDF) to determine peroxides in dairy products. Shantha & Decker modified the procedure to be a more rapid and sensitive method of estimating concentrations of hydroperoxides in food lipids (Shantha & Decker, 1994). In the above IDF and modified FTC method, a total volume of 10 mL CHCl$_3$/MeOH is used as a solvent. There is another very simple version of the FTC assay (Yen & Hsieh, 1998), in which a total volume of 5 mL 70% (v/v) aqueous ethanol is used. This procedure involves dissolving the oxidised lipid in the above CHCl$_3$/MeOH or 70% (v/v) solvent, adding ammonium thiocyanate solution and finally measuring absorbance after 3-5 minutes. A simple comparison of FTC methods used by different researchers is given in Table 1.1 below:
Table 1.1: Ferric thiocyanate (FTC) assay with different reagents, solvents and measurement conditions in different studies.

<table>
<thead>
<tr>
<th>Iron(II) chloride Solution</th>
<th>Ammonium thiocyanate (NH₄SCN) solution</th>
<th>Test sample preparation</th>
<th>Reaction</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4g BaCl₂·2H₂O 0.5g FeSO₄·7H₂O 2mL 10NHCl</td>
<td>3g NH₄SCN and up to 10 mL</td>
<td>0.01-0.3g in 9.8 mL CHCl₃-Methanol (7+3v/v)</td>
<td>50µL NH₄SCN and 50µL iron(II)solution, Incubated 5min in dark, absorbance at 500nm</td>
<td>(Shantha &amp; Decker, 1994)</td>
</tr>
<tr>
<td>0.132M BaCl₂ and 0.144M FeSO₄</td>
<td>3.94M NH₄SCN</td>
<td>20µL in 2.8 mL MeOH/butanol solution (2:1,v/v)</td>
<td>15µL of 3.94M NH₄SCN and 15µL of 0.072M ferrous iron solution, Absorbance taken 20min after adding the iron solution at 510nm.</td>
<td>(Kittipongpittaya, Chen, Panya, McClements, &amp; Decker)</td>
</tr>
<tr>
<td>100µL of 0.02M FeCl₂ in 3.5%HCl</td>
<td>100µL of 30% NH₄SCN in H₂O</td>
<td>25mL in hexane</td>
<td>FeCl₂ and NH₄SCN solution added Absorbance take at 490nm</td>
<td>(Rutb-Senent, Rodriguez-Gutierrez, Lama-Muñoz, &amp; Fernández-Bolaños, 2012)</td>
</tr>
<tr>
<td>0.1mL of 0.02M FeCl₂ in 3.5%HCl</td>
<td>30% NH₄SCN</td>
<td>0.006g antioxidant extract dissolved in 0.12mL ethanol and 2.88mL of a 2.51% linoleic acid solution in Ethanol 9mL of a 0.04M phosphate buffer (pH 7.0) added to this solution, mixture kept at 40°C in dark for 72h</td>
<td>0.1 mL of the sample mixture taken every 24h, diluted with 9.7 mL of 75% ethanol, followed by the addition of 0.1mL of 30% NH₄SCN. Exactly 3 min after adding 0.1 mL of 0.02M FeCl₂ in 3.5% HCl, absorbance taken at 500nm</td>
<td>(Erkan, Ayranci, &amp; Ayranci, 2008a) (Lee, Hwang, &amp; Lim, 2004) (Osawa &amp; Namiki, 1981)</td>
</tr>
<tr>
<td>0.4g BaCl₂·2H₂O 0.5g FeSO₄·7H₂O 2mL conc. HCl</td>
<td>100µL saturated NH₄SCN solution</td>
<td>0.01-0.05g in CHCl₃/acetonic acid (2:3)</td>
<td>FeCl₂ and NH₄SCN solution added After 10min absorbance take at 470nm</td>
<td>(Hornero-Méndez, Pérez-Gálvez, &amp; Mínguez-Mosquera, 2001)</td>
</tr>
<tr>
<td>4.5x10⁻³M FeSO₄·7H₂O in 0.2M HCl FeCl₂ 0.02M in 3.5%HCl</td>
<td>3% methanolic solution of KCNS</td>
<td>Extraction of lipid in CHCl₃:MeOH or CH₃Cl: MeOH (2:1v/v)</td>
<td>FeCl₂ and KCNS solution added absorbance take at 500nm</td>
<td>(Mihaljević, Katusin-Ražem, &amp; Ražem, 1996)</td>
</tr>
<tr>
<td>30% NH₄SCN solution</td>
<td>70% aqueous EtOH</td>
<td>Exactly 3 min after adding FeCl₂ absorbance taken at 500nm</td>
<td>(Yen &amp; Hsieh, 1998)</td>
<td></td>
</tr>
</tbody>
</table>
Table 1.1 shows that a CHCl₃/MeOH mixture is most often used to dissolve the lipid, although some other solvents are also used. The ferrous ion solution is prepared either from the mixture of barium chloride and ferrous sulfate or directly from ferrous chloride. A 30% ammonium thiocyanate solution is widely used and incubation time for colour stabilisation is 3-5 minutes.

The FTC assay excels in measuring lipid oxidation for many purposes, such as use of a control for oxidation in antioxidant screening and measurement of the degree of oxidation in biological samples. The assay has been re-evaluated by Mihaljevic et al. (Mihaljević et al., 1996) who found that the method is simple and very suitable to measure hydroperoxides from mono-, di-, and PUFAs. Multiple samples can be analysed with the method. Furthermore, the results with the FTC method have been found to be more reproducible than other peroxide determination methods such as iodometric and FOX (ferrous oxidation xylenol) methods (Nielsen, Timm-Heinrich, & Jacobsen, 2003).

1.7.2 Thiobarbituric acid reactive substances (TBARS) Assay

The TBARS assay has been used widely to measure lipid oxidation since the report by Kohn and Liversedge (Kohn & Liversedge, 1944) that TBA reacts with unknown carbonyl compounds in aerobic oxidation of animal tissue (lipid substrate) to form a red-pigment (pink). A few years later, Bernheim et al. (Bernheim,
Bernheim, & Wilbur, 1948) reported that the responsible carbonyl compound that reacts with TBA to form pink/red pigment was MDA. It was then established that MDA formed from lipid oxidation reacts with TBA in an acidic medium to form MDA-(TBA)$_2$, which can easily be measured using visible absorbance at 532 nm. The reaction can be illustrated in Figure 1.3 below:

![Figure 1.3: TBA reaction with MDA](image)

The pink/red colour of this adduct led to the basis of the TBARS method. This simple procedure involves adding TBA to an oxidised sample, boiling the mixture in a water bath, removing the aqueous layer and measuring the absorbance value of the pink-coloured adduct.

Criticisms of the method, such as poor reproducibility (Janero, 1990) and overestimation of results (Del Rio et al., 2005), point to its lack of specificity for accurately quantifying MDA. However, these issues have not deterred researchers from using the method, as evidenced by over 1,300 entries (using the search terms
thiobarbituric acid and malondialdehyde or variants, e.g. TBA, MDA) in the Web of Science database over the last 10 years.

The lack of specificity of the reaction involving TBA to quantify MDA has been recognised since the early 1950’s, with numerous researchers showing that TBA reacts with a variety of aldehydes and the breakdown products of proteins and carbohydrates (Guillen-Sans & Guzman-Chozas, 1998). Some of these reactions give pink-coloured compounds, while some produce yellow species which absorbances tail into the region around 530 nm and hence interfere with the measurement of MDA. Kosugi & Kikugawa has regarded the TBA method as measuring the combined effects of various lipid oxidation products including MDA, alk-2-enals, alka-2,4-dienal and hydroperoxides (Kosugi & Kikugawa, 1989).

Despite its non-specificity, the assay has great value for use in measuring lipid oxidation. The TBARS assay has been reported to be a more accurate and sensitive method to measure lipid oxidation than hexanal and p-anisidine assays (Barriuso et al., 2013). The high sensitivity and versatility of the TBARS assay to measure lipid oxidation has also been recognised by Gray & Monahan (Gray & Monahan, 1992). This assay is also very important because toxic MDA and other TBARS are measured together when formed from lipid oxidation. Considering the biological importance of this assay, a number of research groups used TBARS to measure the degree of oxidation in different lipid substrates. In the last 10 years, more than 15,000 TBARS papers have been published (Web of Science database) compared with 760 β-carotene bleaching, 30 crocin bleaching, 420 conjugated dienes, and 280
FTC publications. It clearly shows that the TBARS assay is the most widely used lipid-based assay.

1.8 Importance in inhibiting lipid oxidation

Lipid oxidation is a normal physiological process, and yet if there is excessive lipid oxidation, it poses a major threat to human health. Brain tissues of humans are mostly vulnerable to lipid oxidation because these tissues consist of PUFAs, and consequently many neurodegenerative diseases are caused by lipid oxidation. Furthermore, the products can create inflammation, atherogenesis, and cancer. In human cell, oxidation can be occurred to DNA and protein, which can cause diseases. Apart from human physiology, lipid oxidation causes deterioration of food lipids. Such deterioration causes rancidity and spoils the nutritional value of foods. Other aldehydic lipid oxidation products such as 2-alkenals, 4-hydroxy-2–alkenals, and ketoaldehydes are particularly reactive and cause toxicity in foods. For the above reasons, the food industry suffers from quality deterioration, which results in negative consumer acceptance of foods.

Lipid oxidation should be limited for the above adverse consequences in both human health and the food industry. In this issue, antioxidants can play a crucial role in limiting oxidation by inhibiting the formation of oxidation products. According to (Halliwell & Gutteridge, 1995), an antioxidant can be defined as “any substances
that, when present at low concentrations compared with that of an oxidisable substrate, significantly delay or inhibit oxidation of that substrate”. There is thus immense interest in applying antioxidants to inhibit the formation of oxidation products.

1.9 Antioxidants and their activity

Human physiology has an antioxidant network, which can serve as a natural balance between the activity of antioxidants and the formation of free radicals. In the human body, endogenous antioxidants such as glutathione, uric acid, bilirubin, coenzyme Q or melatonin protect lipids (and other substrates) from oxidation. The antioxidant network fails when excessive lipid (or other substrate) oxidation occurs. This causes oxidative stress, which has been implicated in cardiovascular and neurodegenerative diseases (Shahidi, 1997).

During oxidative stress, the endogenous antioxidants cannot work to delay the onset of lipid oxidation. In that case, the antioxidant network needs to be enriched by supplementing with exogenous antioxidants such as vitamin C, vitamin E, β-carotene, or flavonoids in the diet in order to impede lipid oxidation (Carocho & Ferreira, 2013). Exogenous antioxidants can also be incorporated into food products to protect them from quality deterioration.
Radical chain-breaking mechanism is the main pathway for an antioxidant to inhibit lipid oxidation. Phenolic antioxidants such as BHT, propyl gallate, and α-tocopherol are chain breaking or primary antioxidants in food systems. When lipid oxidation starts, the primary antioxidant (AH) donates a hydrogen atom to the peroxyl radicals (LOO•) for formation of hydroperoxides (LOOH), and also to the lipid radical (L•) for a lipid molecule (LH). The oxidised antioxidant becomes a radical species (A•), which is stabilised by resonance. Formed hydroperoxides (LOOH) are then homolytically decomposed to form alkoxy radicals (LO•) and hydroxyl radicals (•OH). Antioxidant (AH) or antioxidant radicals (A•) react with the alkoxy radicals to form stable alkoxy products and the hydroxyl radicals form water. Thus the antioxidant breaks the radical chains in the propagation step of lipid oxidation. A simplified chain-breaking mechanism is illustrated below Figure1.4:
Figure 1.4: Simplified chain-breaking antioxidant mechanism, where AH=antioxidant, LH= lipid, LOOH=lipid peroxides.

Apart from the primary reaction pathway, antioxidants can inhibit lipid oxidation by chelating metal ions, quenching singlet oxygen, or scavenging oxygen or other reactive species (Alamed, Chaiyasit, McClements, & Decker, 2009). The antioxidants acting in these pathways are generally called secondary antioxidants. Antioxidants can be sourced from synthetic or natural origins. Commercially available and synthesised compounds are often referred as synthetic antioxidants. Butylated hydroxyanisole (BHA), butylated hydroxy toluene (BHT), propyl gallate (PG) and tertiary-butylhydroquinone (TBHQ) have long been used in foods because of their low cost, mild flavour (Shahidi & Ambigaipalan, 2015) and high stability compared to those from natural sources. BHA and BHT are commonly used antioxidants for all foods whereas TBHQ is a better preservative for processed foods.
animal food products for high power of activity (Carocho & Ferreira, 2013). TBHQ is a more powerful antioxidant than BHA and BHT; and BHA is more effective than BHT (Shahidi & Ambigaipalan, 2015).

Although the above antioxidants show effective antioxidant activity, their safety has been questioned (Bera, Lahiri, & Nag, 2006). BHA and BHT have been reported to be weakly carcinogenic (Fereidoon. Shahidi & Ying. Zhong, 2010) and responsible for liver cirrhosis in animals. TBHQ and PG can form molecular complexes with nucleic acid and can also break double strands of DNA. TBHQ is the most cytotoxic among the antioxidants. BHA, BHT, and TBHQ are approved for human consumption although with increasing restriction due to the above toxicity and concerns with health effects. Therefore, the use of synthetic antioxidants is strictly regulated by governments in different countries, especially for TBHQ, which is restricted in Canada and the European Union. TBHQ has been banned for use as a food ingredient in Japan (Fereidoon. Shahidi & Ying. Zhong, 2010). Thus there is a growing negative attitude of customer preference towards synthetic antioxidants (Kalın, Gülçin, & Gören, 2015).

On the other hand, natural antioxidants are considered to be safe, are readily accepted by consumers, can already be present in foods, are capable of stabilising edible oils, and can add a nutraceutical value to oils (Bera et al., 2006). There is thus a need to find more novel natural antioxidants in order to replace synthetic antioxidants.
1.10 Roles of plant extracts as natural antioxidants

Plant extracts provide the best potential source of natural antioxidants, and should be explored for novel food grade antioxidants. Plant derived antioxidants contribute to the antioxidant activity of the extract, which can be determined by lipid-based antioxidant assays. Due to the presence of different antioxidants in different plants, the activity varies from one plant to others. The major plant-derived antioxidants are vitamin C (ascorbic acid), and vitamin E (α-tocopherol), and polyphenols (phenolic acids, flavonoids, etc.).

Antioxidant properties of plant extracts have been studied since the early 1870s (Ndhlala, Moyo, & Van Staden, 2010) and the health benefits of these antioxidants have recently been reviewed by Oroian & Escriche (Oroian & Escriche, 2015). Ascorbic acid and α-tocopherol have been reported to protect membrane lipid from oxidation (E. Choe & Min, 2009; Pisoschi & Pop, 2015). Polyphenolic compounds are the most common plant derived antioxidants and are an essential part of human diet. More than 8000 phenolic compounds have been found in the plant kingdom ranging from low molecular weight compounds such as gallic acid to large molecules such as tannins (Oroian & Escriche, 2015). These are shown in below table 1.2:
Table 1.2: Classification of plant phenolic antioxidants.

<table>
<thead>
<tr>
<th>Major class</th>
<th>Subclass</th>
<th>Polyphenolic compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenolic acids</td>
<td>Hydroxybenzoic acid derivatives</td>
<td>Gallic, ellagic acid</td>
</tr>
<tr>
<td></td>
<td>hydroxycinnamic acid derivatives</td>
<td>Caffeic, ferulic acid</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>Flavonols</td>
<td>Quercetin, kaemferol,</td>
</tr>
<tr>
<td></td>
<td>Flavanols (Catechins)</td>
<td>(+)- catechin, (-)- epicatechin, (+)- galloallocatechin, (-)- epigallocatechin</td>
</tr>
<tr>
<td></td>
<td>Flavones</td>
<td>Luteolin, rutin</td>
</tr>
<tr>
<td></td>
<td>Flavanones</td>
<td>Naringenin</td>
</tr>
<tr>
<td></td>
<td>Isoflavonoids</td>
<td>Genistein</td>
</tr>
<tr>
<td>Tannins</td>
<td>Hydrolysable</td>
<td>Ellagitannins, galloallocatechin</td>
</tr>
<tr>
<td></td>
<td>Condensed tannins</td>
<td>Mono, di, polymers</td>
</tr>
</tbody>
</table>

The phenolic acids have become integral part of human diet and hence their medicinal properties - especially antioxidant properties - have been studied (Shahidi & Ambigaipalan, 2015). Phenolic acids can show antioxidant activity as chelator or free radical quencher. Phenolic acids can inhibit oxidation in food lipids and hence are associated with the preservation of food quality including colour, flavour, and nutrition. Of phenolic acids, gallic acid is the most studied for antioxidant properties (Carocho, Barreiro, Morales, & Ferreira, 2014).

Of 8000 plant phenolics, 6000 are flavonoids (Shahidi & Ambigaipalan, 2015). Among the flavonoids, 200 flavonols and 100 flavones have been reported in plants.
According to Shahidi et al. 2015 (Shahidi & Ambigaipalan, 2015), antioxidant properties of the flavonoids are due to (i) the orientation of hydroxyl and carbonyl groups, which can act as potential metal-chelating sites, (ii) possessing hydrogen or electron-donating substituents which can neutralise free radicals, and (iii) the delocalisation of an unpaired electron through the aromatic nucleus, which can form a stable phenoxy radical. In Shahidi’s review, it is also reported that the catechol hydroxyl groups, or $o$-dihydroxylation, or $p$-quinol structure of the B ring, and 3 4-dihydroxy configuration of flavonoids, contribute to antioxidant activity.

Of flavonols, quercetin, and kaempferol are the most important antioxidants. Amongst these, quercetin is well studied and has been used as a potential antioxidant in preventing cardiovascular disease in human physiology (Oroian & Escriche, 2015).

Plant extracts contain various combinations of different phenolic compounds that may act together synergistically (Wagner & Ulrich-Merzenich, 2009). Hence there is currently much research being carried out to study plant extracts to find superior, “natural” antioxidants, either for dietary purposes, or as replacements for food additives. Traditional medicinal plants provide one avenue for locating species with bioactive compounds as Indigenous peoples have already tested these plants for efficacy treating various conditions.
1.11 Australian traditional medicinal plants as antioxidants

Two-thirds of the word’s plant species have medicinal value, including antioxidant potential, due to the presence of compounds such as those discussed, above (D. Krishnaiah, R. Sarbatly, & R. Nithyanandam, 2011). Many of these plant species have traditionally been used by the Indigenous people in different countries. Some of these traditional medicinal plants were used as food, medicines, and for other healing purposes. Australia is a country with a great diversity of plant species - 20,000 vascular and 14,000 non-vascular plants (Packer et al., 2012). A total of 85% of the vascular plants is endemic to Australia. Although the specific scientific and bioactive properties of these plants - including antioxidant ability - were not understood chemically then, the plants were traditionally used by Australian indigenous people for over 40,000 years for various general medicinal purposes including alleviating aches, soreness, and internal pains. These traditional uses were based on the requirements, convenience, and experiences of Indigenous people. The medicinal potency of a few of the plants has now been substantiated by bioactive studies including antioxidants (Sommano, Caffin, & Kerven, 2013). Some of properties of some of the plants are briefly listed in below Table 1.3:
Table 1.3: Australian traditional plants with potential antioxidant and other medicinal properties.

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Properties</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ficus macrocarpa L. Fil;</em></td>
<td>Antioxidant</td>
<td>(D. Krishnaiah et al., 2011)</td>
</tr>
<tr>
<td><em>Urtica dioica L; Leea indica</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>String orobanchioides Benth</em></td>
<td>Antioxidant</td>
<td>(Ali et al., 2008)</td>
</tr>
<tr>
<td><em>Pterocaulon sphacelatum</em></td>
<td>Antiviral</td>
<td>(S. Semple, Nobbs, Pyke, Reynolds, &amp; Flower, 1999)</td>
</tr>
<tr>
<td><em>Carymbia intermedia;</em></td>
<td>Antimicrobial</td>
<td>(Packer, Naz, Harrington, Jamie, &amp; Vemulpad, 2015)</td>
</tr>
<tr>
<td><em>Lophostemon suaveolens;</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Syncarpia glomulifera</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Acotriche agrregata;</em></td>
<td>Antibacterial</td>
<td>(Kalt &amp; Cock, 2011)</td>
</tr>
<tr>
<td><em>Leptospermum confertus;</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Marchantia polymorpha</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Alstonia scholaris;</em></td>
<td>Anticancer</td>
<td>(Tan et al., 2015)</td>
</tr>
<tr>
<td><em>Eremophila duttonii;</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Eremophila longifolia;</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Eremophila maculata</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pterocaulon sphacelatum;</em></td>
<td>Antiviral</td>
<td>(S. J. Semple, Reynolds, O'leary, &amp; Flower, 1998)</td>
</tr>
<tr>
<td><em>Dianella lingifolia;</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Euphorbia australis</em></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Studies on the medicinal plants of very few Australian plants have been reported and much of these have not been explored (Simpson, Claudie, Smith, McKinnon, & Semple, 2013). For example, the largest Indigenous group of NSW is the Wiradjuri community, yet there is no refereed publication with the medicinal properties of traditionally used plants. Wiradjuri country covers approximately 97,000 km² and includes the inland towns of Hay, Griffith, Darlington Point, Leeton, Narrandera, Wagga, Wagga, Brungle, and Tumut. Because the traditional lands cover a wider area, there is a diversity of plant species that are used for medicines. However, there
is a guide book of the Murrumbidgee Catchment Management Authority (MCMA) from the Wiradjuri (Riverina region, NSW) listing more than 80 native plants with their traditional use in medicinal purposes (Williams & Sides, 2008) while there is a little information about customary medicinal plants of the Yaegl Aboriginal community of northern New South Wales (Packer et al., 2015).

Of the Australian native plants, three genuses, i.e., Acacia, Eucalyptus and Exocarpos, were widely used by the Wiradjuri and other Australian Indigenous people and are also still easily available in Australia and hence there is an opportunity for these species to be explored as bioactive potential. The experience-based medicinal value of some of the above genuses of plants has now been supported by investigating and detecting important medicinal and bioactive properties including antioxidant; some of these properties are briefly outlined in the below table 1.4:
Table 1.4: Traditionally used of the genus Acacia, Eucalyptus and Exocarpos by Wiradjuri and other Indigenous people and some of their bioactive properties studies.

<table>
<thead>
<tr>
<th>Family</th>
<th>Botanical name</th>
<th>Wiradjuri medicinal or food use/plant part (*Ref)</th>
<th>Other Indigenous medicinal or food/plant part (Reference)</th>
<th>Bioactive properties (Reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fabaceae</td>
<td>Acacia melanoxylon</td>
<td>Aching joint, cut and sore, infection and stomach cramps/bark</td>
<td>Rheumatism/bark (Cocka, 2011)</td>
<td>Antibacterial</td>
</tr>
<tr>
<td></td>
<td>Acacia implexa</td>
<td>Medicine, /bark</td>
<td>Skin disease/bark (Cocka, 2011)</td>
<td>Anticancer (Packer et al., 2012; Tan et al., 2015)</td>
</tr>
<tr>
<td></td>
<td>Acacia auriculiformis</td>
<td>Not reported</td>
<td>Antiseptic, allergy rash/leaves (Cocka, 2011)</td>
<td>Antioxidant (D. Krishnaiah et al., 2011; Singh, Singh, Kumar, &amp; Arora, 2007a, 2007c), Antibiotic (Pennacchio, Kemp, Taylor, Wickens, &amp; Kienow, 2005)</td>
</tr>
<tr>
<td></td>
<td>Acacia bivenosa</td>
<td>Not reported</td>
<td>Colds, cough (Cocka, 2011; Packer et al., 2012)</td>
<td>Antibacterial(Packer et al., 2012), Antibiotic (Pennacchio et al., 2005)</td>
</tr>
<tr>
<td>Myrtaceae</td>
<td>Eucalyptus camaldulensis</td>
<td>Non medicinal use</td>
<td>Sore throat and skin disease, wound, sickness Food/foliage (Clarke, 2015; Packer et al., 2012)</td>
<td>Antioxidant, antibacterial, antifungal (Packer et al., 2012), Anticancer (Vuong, Chalmers, Jyoti Bhuyan, Bowyer, &amp; Scarlett, 2015), Antimicrobial (Knezevic et al., 2016)</td>
</tr>
<tr>
<td></td>
<td>Eucalyptus rossii/racemosa</td>
<td>Not reported</td>
<td>Diarrhoea, dysentery (Packer et al., 2012)</td>
<td>Not reported</td>
</tr>
<tr>
<td>Class</td>
<td>Species</td>
<td>Use/Effect</td>
<td>Notes</td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>----------------------------------</td>
<td>-----------------------------------------</td>
<td>----------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Myrtaceae</td>
<td><em>Eucalyptus pruinosa</em></td>
<td>Not reported</td>
<td>Headache, pain, rheumatism (Packer et al., 2012)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Eucalyptus resinifera</em></td>
<td>Not reported</td>
<td>Syphilitic sores (Packer et al., 2012),</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>syphilis, diarrhoea, dysentery/bark, leaves (Cocka, 2011)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Eucalyptus gomphocephala</em></td>
<td>Not reported</td>
<td>Not reported</td>
<td></td>
</tr>
<tr>
<td>Santalaceae</td>
<td><em>Exocarpos cupressiformis</em></td>
<td>Food/fruit</td>
<td>Food/fruit (Clarke, 2015)</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Exocarpos aphyllus</em></td>
<td>Medicine as snake bite/sap</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Not reported</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Exocarpos latifolius</em></td>
<td>Not reported</td>
<td>Medicine/bark and leaves (<a href="http://anpsa.org.au">http://anpsa.org.au</a>)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Antimycobacterial(anti-tuberculosis) (Koch et al., 2009)</td>
<td></td>
</tr>
</tbody>
</table>

*Ref- The guide book of the Murrumbidgee Catchment Management Authority (MCMA) from the Wiradjuri (Riverina region, NSW) (Williams & Sides, 2008).
The above table shows that some of the *Acacia, Eucalyptus, and Exocarpos* species used by the Wiradjuri or other Indigenous people possess bioactivity. *Acacia auriculiformis, Eucalyptus camaldulensis, Eucalyptus gomphoceph* exhibit antioxidant properties while some other species of *Acacia and Eucalyptus* show other bioactive properties. Antioxidant properties of *Exocarpos* have not been reported yet.

*Acacia implexa, Eucalyptus rossii, and Exocarpos cupressiformis* are still widely available in eastern Australian Riverina region (Wiradjuri country) of NSW. The above table shows that *Acacia implexa, Eucalyptus rossii and Exocarpos cupressiformis* were used by Wiradjuri people for medicinal purposes, but their antioxidant activity was not studied. In addition, there are very few phytochemical reports of these species. The report of Maiden et al. (Maiden, 1904) shows that two *Acacia implexa* bark samples, one was found to have 7.82% and other 14.16% of tannic acids. Tindale et al. (Tindale & Roux, 1969) reported the presence of some phenolic compounds including catechins, melacacidin, and dihydroflavonol in *Acacia implexa*. The phytochemical study of some of the other species of *Acacia* has been reviewed (Seigler, 2003) although no report is included in the review for *Acacia implexa*. Similarly two phytochemical compounds of *Eucalyptus rossii* have been reported (Tucker et al., 2010) while phytochemical study of some of the other species of *Eucalyptus* has been reviewed (Vuong et al., 2015). On the other hand, there is one phytochemical report (Cooke & Haynes, 1960) for *Exocarpos cupressiformis*, in which kaempferol-7-rhamnoside, dihydrokaempferol-7-rhamnoside and quercetin-3- have been identified. In other species of *Exocarpos*, an
astringent bioactive ingredient *vis.* exocarpic acid was extracted from the plant (Koch et al., 2009).

The traditional medicinal use of *Acacia implexa, Eucalyptus rossii, and Exocarpos cupressiformis* can be supported by the presence of the phytochemical components in the species. In addition, the parent genuses *Acacia, Eucalyptus,* and *Exocarpos* possess bioactive properties. Therefore the above three species may have antioxidant properties. Considering the above information, the three species was chosen for antioxidant activity study in this PhD project. The author’s research institute Charles Sturt University, Wagga Wagga is located in the Wiradjuri country boundary and hence it was convenient to collect samples of these species and studied by the optimised method.

**1.12 Assays for screening antioxidant activity:**

In order to explore the potency of plant extracts as antioxidants, the activity should be screened. Various assays have been developed to screen for antioxidant activity (Ndhlala et al., 2010). Based on the reaction mechanism and substrate involved, the assays comprise four groups: free radical scavenging, non-radical reduction-oxidation (redox) potential, non-lipid substrate based, and lipid substrate based. A simplified summary of commonly used assays is outlined in the below Figure 1.5:
Figure 1.5: Simplified category of antioxidant screening assays.

Of antioxidant mechanisms - single electron transfer (SET) and hydrogen atom transfer (HAT) are frequently involved in the above without substrate and non-lipid substrate based assays. ABTS, DPPH, FRAP assays are based on SET mechanisms while ORAC is based on HAT mechanism. On the other hand, in FTC, TBARS, CD and hexanal assays, more complex mechanisms are involved.

The ABTS / Trolox equivalent antioxidant capacity (TEAC) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) tests are widely used free radical scavenging assays to screen antioxidant activity of plant extracts (Shahidi & Zhong, 2015). In these assays, an electron is donated from the antioxidant to the oxidants being probed (i.e. ABTS radical cation for ABTS; DPPH radical for DPPH assays) as shown below:

\[
\text{Probe (oxidant) + e (from antioxidant) } \rightarrow \text{ reduced probe + oxidised antioxidant}
\]
When the reaction occurs, the colour of the probe changes, the intensity of the change becomes proportional to the concentration of antioxidant present in the reaction mixture. The colour change stops when the reaction is finished. This colour change is measured spectrophotometrically (ABTS at 734 and DPPH at 515 nm). In the redox potential-based ferric reducing antioxidant power (FRAP) assay, the antioxidant donates an electron to the probe ferricyanide to yield ferrocyanide, which in turn binds Fe$^{3+}$ to form Prussian blue (ferric ferrocyanide). Antioxidant activity is determined by the absorbance of Prussian blue at 593 nm.

In Folin-Ciocalteu assay (total phenol assay), electron transfer occurs between antioxidant (or other reductants) and the metal ion, Mo (VI), used in the reagent (Huang, Ou, & Prior, 2005). Although this assay is not actually an antioxidant assay because it does not measure antioxidant activity (and so is not included in the Figure above), it is necessary to obtain a preliminary indication of the presence of total phenolic content in an extract before the exploration of antioxidant ability.

The other reaction mechanism - hydrogen transfer (HAT) - is involved in the Oxygen Radical Absorbance Capacity (ORAC) assay, in which the antioxidant donates a hydrogen atom to peroxyl radicals. In this assay, peroxyl radicals are generated by azo compounds (e.g. AAPH-2, 2′-azobis (2-amidinopropane) dihydrochloride). The radicals react with the probe (i.e. fluorescein) and the fluorescence of the probe is lost. By transferring a hydrogen atom and subsequent
quenching peroxyl radicals, the antioxidant slows the loss of fluorescence. Activity of the antioxidant is thus determined from the changing fluorescence.

The above chemical assays are widely used for antioxidant screening because of their simplicity. However, this advantage can be diminished with the lack of a substrate (for ABTS/TEAC and DPPH) or a physiologically relevant substrate (for ORAC). In human physiology, oxidation occurs on a lipid as substrate and hence the antioxidant activity measured by the above assays may not be consistent with those that occur in the human body. Furthermore, a number of limitations of the assays have recently been reviewed (Schaich, Tian, & Xie, 2015; Shahidi & Zhong, 2015).

For lipid-based assays, lipid substrates are mandatory because the assays are based on the oxidation of a lipid substrate and measuring the inhibition of formation of oxidation products. Using these assays, some of the physiological conditions can be preserved in the screening antioxidant activity. The lipid oxidation measurement assays of peroxides (FTC) and TBARS discussed in sections 1.7.1 and 1.7.2 can be used in antioxidant assays by adding antioxidant (for example plant extracts) to the systems. As the thesis focuses on peroxides (FTC) and TBARS antioxidant assays, these are discussed in sections 1.12.1 and 1.12.2.
1.12.1 Ferric thiocyanate (FTC) assay to measure antioxidant activity in lipid systems

When the FTC assay is used for the measurement of antioxidant activity, a suitable lipid substrate such as linoleic acid (LA) needs to be oxidised to form peroxides. Then, as noted in section 1.7.1, FTC can be used to measure the inhibition of peroxides formation by a decreased absorbance at 500 nm. A simplified pathway of the assay is outlined in below Figure 1.6:

![Simplified pathway of the FTC antioxidant assay](image)

Figure 1.6: Simplified pathway of the FTC antioxidant assay.

The modified FTC assay of Shantha and Decker et al. (Shantha & Decker, 1994), developed to measure peroxides in oils and food lipids, can be used in antioxidant assay. In Shantha and Decker’s method, lipid and antioxidant can be mixed together in a lipid system and oxidation can be conducted for a particular time; the inhibition of formation of peroxides can then be measured. In this method, the oxidation
involves a multi-phase system, in which lipid substrate lies over the aqueous system. As discussed in section 1.7.1, there is another version of the FTC assay for measuring peroxides, used by Mistuda et al. (Mistuda, Yuasumoto, & Iwami, 1996) and later on by Yen and Hsieh (Yen & Hsieh, 1998). In this method, oxidation is conducted in an emulsion. A number of groups (shown in Table 1.5 below) used the method of Mistuda et al. (1966) in antioxidant studies although with a variety of oxidation conditions and systems.
Table 1.5: Ferric thiocyanate assay and antioxidant activity of plant extracts or standard antioxidants.

<table>
<thead>
<tr>
<th>Oxidation system</th>
<th>Oxidation state reagents</th>
<th>Oxidation Temperature</th>
<th>Oxidation Time</th>
<th>Peroxides measurement reagents</th>
<th>Antioxidant/plant extract</th>
<th>Reference antioxidant as positive control</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>LA emulsion</td>
<td>phosphate buffer (pH 7), Tween 20</td>
<td>37–40 °C</td>
<td>40h–7 days</td>
<td>75% ethanol, 30% ammonium thiocyanate, ferrous chloride in 3.5% HCL, 3 time stand, measured at 500 nm (Mistuda et al., 1996)</td>
<td>Plant extract, sweet potato, ellagic acid, cove extract, L-carnitine, caffeic acid, peptide.</td>
<td>α–tocopherol, ascorbic acid, BHA, BHT, Trolox, EDTA</td>
<td>(Das, Das, Miyaji, &amp; Deka, 2016; Gülçin, 2006a, 2006b; Gülçin, Elmastas, &amp; Aboul-Enein, 2012; Hu et al., 2016; Je, Park, &amp; Kim, 2005; Kilic, Yeşiloğlu, &amp; Bayrak, 2014; Yen &amp; Hsieh, 1998)</td>
</tr>
<tr>
<td>LA, ethanol solution (pH 7)</td>
<td>phosphate buffer (pH 7), Tween 20</td>
<td>40°C</td>
<td>40 h–11 days</td>
<td>As group of Mitsuda et. al. (Mistuda et al., 1996).</td>
<td>Grape, rosemary extract, plant extract</td>
<td>BHT, Carnosic acid, rosmarinic acid, α-tocopherol, BHA</td>
<td>(Y. Chen et al., 2011; Erkan, Ayranci, &amp; Ayanci, 2008b; Lee et al., 2004; Rahmat, Edrini, Ismail, Hin, &amp; Bakar, 2006; Sofi, Raju, Lakshmisha, &amp; Singh, 2016).</td>
</tr>
</tbody>
</table>
The above table shows that the antioxidant activity of varieties of plant extracts and standard antioxidants have been readily measured by the FTC assay with LA as the lipid substrate. Oxidation of substrate can be done in either LA in ethanol mixture or LA emulsion. Aqueous phosphate buffer (pH 7) has commonly been used as the medium. For the emulsion, emulsifier Tween 20 has been used. Physiological temperature 37°C (or closer temperature 40°C) has been used as oxidation temperature. Antioxidants such as ascorbic acid, α-tocopherol and rosmarinic acid, which are commonly available in nature, have been used as positive control. Some synthetic antioxidants such as BHA and BHT have also been used. BHA and BHT possess high antioxidant activity, and have been most often used for comparison with unknown potency of plant extracts or other samples.

The main drawback of the assay revealed by the above reports is that time of oxidation is very long – ranging from 40 hours to 11 days, and much longer than ABTS (7 min) (Re et al., 1999), FRAP (30 min) (Pulido, Bravo, & Saura-Calixto, 2000), ORAC (30-40 min) (B. Ou, Hampsch-Woodill, & Prior, 2001) and DPPH (1 h) (Sánchez-Moreno, Larrauri, & Saura-Calixto, 1998). The long oxidation time is not suitable for routine analysis of antioxidant screening. Therefore, there is a need to develop a high throughput FTC assay in which oxidation time can be shortened.
1.12.2 Thiobarbituric acid reactive substances (TBARS) assay to measure antioxidant activity in lipid systems

As peroxides are formed first and then break down to TBARS, the production of TBARS comes at the end of the various stages of lipid oxidation (Figure 1.7). However, as highlighted in Figure 1.7, an antioxidant may act at any steps of the process prior to the development of the coloured TBARS adducts. Therefore, the measurement of TBARS gives no indication of the mechanism of action of the antioxidant, that is, whether it is able to interact with oxygen or metal ions, react directly with hydroperoxides, or intercept the free radicals involved in the breakdown of primary to secondary oxidation products. However, it is worth mentioning that antioxidants may generally act to scavenge free radicals formed during lipid oxidation and thereby interrupt the oxidation reaction, or alternatively, react with peroxides so that they cannot be further degraded into TBARS.

Figure 1.7: Stages of lipid oxidation and antioxidant action in the TBARS antioxidant activity assay
TBARS assay is extensively used in measuring antioxidant activity of plant extracts, pure compounds, pulses, milk protein hydrolysates in different lipid substrate and conditions. Table 1.6 shows some of the studies of TBARS assay:
Table 1.6: TBARS assay to measure antioxidant activity in different lipid substrates with various conditions.

<table>
<thead>
<tr>
<th>Antioxidants</th>
<th>Substrate</th>
<th>Reaction conditions &amp; quantification method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure compounds &amp; olive fruit extracts</td>
<td>LA</td>
<td>2 phases, Cu²⁺ catalyst, 37 °C, 20 h, BHT added to quench oxidation, 10 min colour development, extract pink layer into n-butanol, 532 nm; % inhibition at 20 h</td>
<td>(McDonald, Prenzler, Antolovich, &amp; Robards, 2001)</td>
</tr>
<tr>
<td>Extracts of <em>Penstemon. gentianoides</em> &amp; <em>Barkleyanthus salicifolius</em>, BHT, quercetin, tocopherol</td>
<td>rat brain homogenate</td>
<td>1 phase, Fe²⁺ catalyst, 37 °C, 1 h, 30 min colour development in SDS emulsion, 532 nm; standard curve from tetramethoxypropane (TMP) expressed as nanomoles TBARS/mg protein; % inhibition; EC₅₀</td>
<td>(Dominguez et al., 2005)</td>
</tr>
<tr>
<td>Methanol extracts of black caraway, cranberry, carrot &amp; hemp seed oils</td>
<td>LDL</td>
<td>emulsion, Cu²⁺ catalyst, ambient temperature, 60 min, 30 min colour development, 532 nm; standard curve from TMP</td>
<td>(Yu, Zhou, &amp; Parry, 2005a)</td>
</tr>
<tr>
<td>Wheat bran extracts from 2 varieties, Akron &amp; Trego, grown in 3 locations in Colorado</td>
<td>LDL</td>
<td>same as Yu et al. (2005a)</td>
<td>(Yu, Zhou, &amp; Parry, 2005b)</td>
</tr>
<tr>
<td>Pure compounds (12) – data aggregated from various other studies</td>
<td>liposomes; rat liver microsome homogenate; human blood plasma; LDL + VLDL</td>
<td>various</td>
<td>(Roginsky &amp; Lissi, 2005)</td>
</tr>
<tr>
<td>Ascorbic acid, BHT, 4-MBC (4-methylbenzylidene camphor), tocopherol, Trolox, Lipochroman-6</td>
<td>liposome prepared from L-α-lecithin</td>
<td>liposome (emulsion), Fe²⁺/ascorbate catalyst, 37 °C, 1 h, BHT added, 15 min colour development, absorbance at 540 nm; not mentioned, but presumably % inhibition</td>
<td>(Buenger et al., 2006)</td>
</tr>
<tr>
<td>Various pulses</td>
<td>LDL</td>
<td>LDL “solution”, Cu²⁺ catalyst, 37 °C, 3 h, EDTA added, 30 min colour development, 532 nm; % inhibition converted to Trolox equivalents</td>
<td>(Xu, Yuan, &amp; Chang, 2007)</td>
</tr>
<tr>
<td>Extracts of <em>Salvia virgata</em> Jacq.</td>
<td>LA (in ethanol)</td>
<td>1 phase, AAPH catalyst/initiator, 50 °C, 10 h, 20 min colour</td>
<td>(Kosar, Goger, &amp;</td>
</tr>
<tr>
<td>Antioxidants</td>
<td>Substrate</td>
<td>Reaction conditions &amp; quantification method</td>
<td>Reference</td>
</tr>
<tr>
<td>-----------------------------------------------------------</td>
<td>----------------------------------</td>
<td>---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Pure compounds - polar: ferulic acid, coumaric acid, propyl gallate, gallic acid, ascorbic acid; non-polar: BHT, rosmarinic acid, tert-butylhydroquinone (TBHQ), tocoferol</td>
<td>cooked ground beef</td>
<td>2 phases, no catalyst, heating until 77 °C internal temperature, propyl gallate added, colour development 15 min, 533 nm; extinction coefficient of TBA-MDA used to convert absorbance measurements to mg TBARS/kg muscle</td>
<td>(Baser, 2008)</td>
</tr>
<tr>
<td>Pure compounds (22)</td>
<td>cooked, ground, poultry meat</td>
<td>2 phases, no catalyst, heating until 70 °C internal temperature, BHT added, 20 h colour development room temperature, 530 nm; Trolox equivalents</td>
<td>(Capitani, Carvalho, Rivelli, Barros, &amp; Castro, 2009)</td>
</tr>
<tr>
<td>Milk protein hydrolysates</td>
<td>cooked ground beef</td>
<td>2 phases, no catalyst, heating until 71 °C internal temperature, then stored at 4 °C for 1, 8 &amp; 15 days post cooking, propyl gallate &amp; EDTA added, 60 min colour development in emulsion, extraction into pyridine/n-butanol, 532 nm; “standard curve” presumably TMP</td>
<td>(Hogan, Zhang, Li, Wang, &amp; Zhou, 2009)</td>
</tr>
<tr>
<td>Chilean wild black-berry fruits, <em>Aristotelia chilensis</em> (Elaeocarpaceae), EtOH &amp; water extracts &amp; fractions</td>
<td>rat brain homogenate</td>
<td>As Dominguez et al. (2005)</td>
<td>(Céspedes et al., 2010)</td>
</tr>
<tr>
<td>Methanol extracts of 8 medicinal plants grown on a mine site</td>
<td>linolenic acid in SDS emulsion</td>
<td>emulsion, Fe^{2+} catalyst, 37 °C, 16 h, BHT added, 60 min colour development, 532 nm; IC_{50} values calculated from % inhibition</td>
<td>(Dutta &amp; Maharia, 2012)</td>
</tr>
<tr>
<td>Extracts of <em>Lavandula pedunculata</em> subsp. <em>lusitanica</em> (Chaytor) Franco</td>
<td>mouse brain homogenate</td>
<td>2 phases, Fe^{2+} catalyst, 37 °C, 1 h, colour development in emulsion, 532 nm; standard curve from tetramethoxypropane</td>
<td>(Costa et al., 2013)</td>
</tr>
<tr>
<td>Ginger extract</td>
<td>LA emulsion</td>
<td>emulsion, 37 °C, 12 days, TBA-TCA solution, 15 min colour development, 532 nm; standard curve from tetramethoxypropane and TBRAS expressed as mg of MDA/ kg dry matter</td>
<td>(Stoilova, Krastanov, Stoyanova, Denev, &amp; Gargova, 2007)</td>
</tr>
</tbody>
</table>
One use of the TBARS assay is in antioxidant screening of plant extracts (e.g. medicinal plant extracts) in order to find the best antioxidant or fraction of plant materials. This quantification of antioxidant activity provides the opportunity for research to rank various antioxidants or plant fractions tested so that new or novel antioxidant can be explored. In this thesis, antioxidant activity of some selected Australian medicinal plant extracts and fractions have been screened with the TBARS assay.

1.13 Current applications of Thiobarbituric acid reactive substances (TBARS) and Ferric thiocyanate (FTC) assays in antioxidant screening studies

Antioxidant screening studies are sought to find the “best” antioxidant from a range of candidates, whether they may be plant extracts from different species (Xu et al., 2007); different extracts from the same species (Céspedes et al., 2010); protein hydrolysates (Hogan et al., 2009); or pure compounds (Alamed et al., 2009). These studies typically employ a number of different assays designed to assess various types of antioxidant activities (single electron transfer, hydrogen atom transfer, and lipid oxidation inhibition). Not only is it important to identify whether the extract/compound is having an antioxidant effect (and what that effect might be), but quantification of that effect so as to rank the samples is also important. Ideally, standardised assays should be developed so that results from one laboratory can be compared to those from another (Frankel & Finley, 2008).
Two main types of antioxidant screening studies incorporating the TBARS and FTC assay can be explained. The first are those that screen for antioxidant activity using rapid, radical-scavenging assays such as ABTS or DPPH, then, having found the most effective antioxidants, undertake further testing with a “real” substrate and measure antioxidant activity using TBARS or FTC assay. For example, Yu et al. (Yu et al., 2005a) first screened methanolic extracts of cold-pressed seed oils using DPPH, ABTS, ORAC and FC assays, and based on the results for ABTS and DPPH, extracts from cranberry and black caraway seed oils were further tested for antioxidant activity by oxidising LDL and monitoring TBARS formation. Although both extracts showed significantly different ($p < 0.05$) ABTS activity and total phenols, they exhibited the same (non-significantly different $p > 0.05$) antioxidant activity in the TBARS assay. Similarly, Barpatre et al. screened chloroform fraction of *Acacia nilotica* lignin using ABTS, DPPH and then FTC (LA emulsion) assay, and found good antioxidant activity in FTC assay (Barapatre, Meena, Mekala, Das, & Jha, 2016).

Challenges may arise when trying to select the most effective antioxidant. For example, there may be very little correlation between some measures of antioxidant activity and total phenols, as was observed in Yu et al.’s (Yu et al., 2005b) report, and also noted by McDonald et al. (McDonald et al., 2001). In both cases, total phenols did not correlate with antioxidant activity in the TBARS assay. Further challenges may arise where one type of antioxidant assay (e.g. DPPH) is used to select an antioxidant for further testing using a different type of assay (e.g. TBARS). For example, in the study conducted by Hogan et al. (Hogan et al., 2009), using
DPPH or ORAC assays did not reveal the most effective antioxidant in a different system where the TBARS assay was employed. Similarly, the chloroform fraction of *Acacia nilotica* lignin studied by Anand Barpatre et al. exhibited high antioxidant activity in FTC assay and had lowest activity in ABTS assay (Barapatre et al., 2016).

The second main types of screening studies involving the TBARS or FTC assay are those where these assays are used along with all other assays to assess antioxidant activity. In other words, all potential antioxidants are screened by all assays, and any correlations (or contradictions) among assays may be revealed. For example, Xu et al. (Xu et al., 2007) found significant correlations between TBARS and DPPH ($r = 0.97, p < 0.01$) and TBARS and ORAC ($r = 0.81, p < 0.001$) for acetone/water extracts of various legumes. On the other hand, McDonald et al. (McDonald et al., 2001) found that the compounds that were the most effective in the TBARS assay (caffeic acid, tyrosol, and naringin), had minimal activity in protecting phycoerythrin from 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH)-initiated oxidative damage. Similarly, Mo et al. found week correlation between FTC assay and total phenol found for antioxidant activity of extract of medicinal plant (Mo, Kim, Yang, Oh, & Sung, 2011). Also antioxidant activity was higher in FTC assay and not in ABTS or DPPH assay. The lack of a 100% consistency between the assays does raise concerns about studies where results from one type of assay are used to select antioxidants for further testing based on TBARS or FTC assay.
The potential lack of correlation between results of antioxidant activity from free radical scavenging assays and real life systems such as foods was explicitly investigated by Alamed et al. (Alamed et al., 2009). In this study, 9 compounds were evaluated for antioxidant activity, either as polar compounds in a modified ORAC assay, or as non-polar compounds in the DPPH assay. A statistically significant ($p \leq 0.05$) rank for the polar antioxidants was achieved: ascorbic acid < gallic acid < propyl gallate < coumaric acid < ferulic acid. Similarly, the non-polar antioxidants were also ranked: $\alpha$-tocopherol < tert-butylhydroquinone (TBHQ) $\approx$ butylated hydroxytoluene < rosmarinic acid. However, in cooked ground beef, only propyl gallate and TBHQ were effective in inhibiting oxidation as measured by the TBARS assay. In addition, polar antioxidants ranked as ferulic acid < gallic acid < propyl gallate in FTC assay. With non-polar compounds, TBHQ were more effective than rosmarinic acid. Various explanations were offered by Alamed et al. (Alamed et al., 2009) in order to explain the results. One is that in complex food systems, the ability to chelate iron (a pro-oxidant) becomes as important as, or more important than, the ability to scavenge free radicals. This may explain why possible chelators such as propyl gallate and TBHQ are more effective than compounds such as ferulic acid, which is not able to chelate metal ions. Another possible explanation is that some compounds, e.g. ascorbic acid, may reduce ferric ions to ferrous ions, which are more effective as pro-oxidants. A further explanation may relate to the physical properties of the antioxidants in that those that are more hydrophobic may partition more effectively into the lipid phase of heterogeneous systems such as ground beef or corn-oil emulsion, and therefore offer more protection at the site of oxidation. Regardless of the explanation, the results of Alamed et al. (Alamed et al., 2009) suggest that care must be exercised when using one type of assay to select
antioxidants for use in a different system. In addition, suitable versions of oxidation systems such as multi-phase or emulsion system should be sought for consistent results.

In contrast to FTC, TBARS assay is less precise (Bakır, Sönmezoglu, İmer, & Apak, 2013). Hence the variability of TBARS assay should be investigated first. However, when considering the TBARS assay as a potential candidate for an improved screening assay, some challenges need to be overcome for development. One is the choice of substrate, which can be same for both FTC and TBARS assay: a substrate that is chemically consistent is essential in order for the results from one research group to be comparable to those of another. In TBARS assay, as shown in Table 1.6, the substrates used are: LA (McDonald et al., 2001); SDS emulsion of LA (or another polyunsaturated fatty acid, e.g. arachidonic acid (Dutta & Maharia, 2012; Moon & Shibamoto, 2009); rat brain homogenate (Dominguez et al., 2005); LDL (Yu et al., 2005a); rat liver microsome homogenate; liposomes; human blood plasma (Roginsky & Lissi, 2005); liposome prepared from L-α-lecithin (Buenger et al., 2006), ground beef (Alamed et al., 2009); and ground poultry meat (Capitani et al., 2009). Many of these substrates, such as LDL, liver/brain homogenates, or beef or poultry, cannot be guaranteed to be chemically similar from one sample to the next. For example, increases in polyunsaturated fatty acids across these substrates may result in significant differences in susceptibility to oxidation. Of the above substrates, LA has the potential to be chemically consistent from one study to the next, since it is a pure compound (available in ≥ 99% purity). The use of LA in antioxidant assays has been both advocated (Moon & Shibamoto, 2009; Roginsky &
Lissi, 2005) and criticised (Frankel, 2014). It is generally regarded in the literature that LA is a poor source of MDA, but Jardine et al. (Jardine, Antolovich, Prenzler, & Robards, 2002) have proven that under certain conditions (and in fact those used in this thesis (Chapter 2)) MDA is the main TBARS formed. In this thesis, the advantages of using LA as a substrate outweigh its disadvantages vis-à-vis: it is a fatty acid of relevance to both food and physiological systems; it is readily available commercially in high purity; and it is at least as relevant as other popular lipid substrates such as crocin or β-carotene.

Another challenge to be addressed for TBARS to be used as an improved assay is that of reaction conditions. The reaction conditions for the TBARS reaction are many and varied. To be utilised as an antioxidant assay, the reaction conditions for both the oxidation step and the colour development step need to be consistent across studies. Studies with TBARS assay utilising LDL, concentrations of LDL and reaction media vary from 50 μg/mL in PBS (Xu et al., 2007), to 100 μg/mL in PBS (Yu et al., 2005a), to 200 μg/mL in HEPES (Roginsky & Lissi, 2005). Oxidation times vary from 30 min (Domínguez et al., 2005) to 20 h (McDonald et al., 2001). Also, some authors used n-butanol to extract TBARS (McDonald et al., 2001), whereas others did not (Li et al., 2015; Silambarasan et al., 2014). Therefore it is evident that, there has been a wide variety of reaction conditions utilised in the TBARS assay in screening studies, making it very difficult to compare results from one study to the other.
Even when conditions are standardised, there is still no guarantee that an assay will be sufficiently robust to give comparable results across different laboratories as shown by Buenger et al. (Buenger et al., 2006). In this study, six laboratories evaluated four antioxidant assays, namely, DPPH, TEAC, “lipid assay” (i.e., conjugated diene assay, LA as substrate), and “TBA” (i.e., TBARS assay, liposomes from alpha-lecithin as substrate). Variability differed according to the assay, within laboratory: TEAC (5.7–7.7%), DPPH (2–18%), conjugated dienes (2.6–14%) and TBARS (18–102%); and among laboratories: TEAC (9–40%); DPPH (6–57%); conjugated dienes (10–67%); TBARS (78–154%). It was noted that the TBARS assay was especially poor in terms of reproducibility, but the cause was not investigated further. Assays were found to be more or less variable according to the antioxidant tested. For example, BHT exhibited a CV of 18% in the DPPH test, whereas other antioxidants showed CVs of 2–8%. In the TBARS assay, tocopherol was found to be particularly problematic with a CV of 102%. These results for the TBARS assay would again suggest that caution is needed in interpreting data from other studies where the TBARS assay has been used. There is a need to standardise for the operation of this assay.

The other FTC assay has much lower variability than TBARS assay because the reaction mechanism for peroxides formation is less complex than TBARS formation. In addition, the red-colour development is more specific than TBARS as non-responsive species like TBARS are not involved in FTC assay. However, some drawbacks have been explained by Branka Mihaljevic et al. (Mihaljević et al., 1996). In this study, it has been reported that ferrous ions can be oxidised by amino acid and
H₂O₂ in biological materials and superoxide driven Fenton’s reaction may produce additional Fe³⁺. In another study (Kolthoff & Medalia, 1951), it has been reported that both Fe²⁺ and Fe³⁺ ions can be present in the test system in a soluble form, which can interfere with the colour measurement. Nevertheless, the above problems can easily be overcome. The main problem with the FTC assay is that the oxidation stage of this method requires much more time than TBARS assay, which has been reviewed in section 1.12.1. Similar to TBARS assay, the oxidation system of this assay needs to be made faster.

1.14 Summary of literature review

Due to adverse effects of peroxides and TBARS formed from lipid oxidation, the FTC and TBARS assays are of great interest in inhibiting these toxic oxidation products using antioxidants. In this literature review, various aspects of the FTC and TBARS assay have been discussed. Among the assays, there are many variations in the reaction conditions used to measure TBARS compared to peroxides. Such differences make it difficult to compare results across studies using the TBARS assay. These issues are compounded when the TBARS assay is used to monitor lipid oxidation in the presence of an antioxidant, as further variations in methodology arise around parameters such as: mode of delivery of antioxidant, time of oxidation, and choice of substrate.
Despite the challenges, the TBARS assay has potential to be further developed to be a useful assay in antioxidant screening studies especially with medicinal plants. This literature review has identified that TBARS assay is not rapid and no research has been performed to improve the TBARS method making it faster. Research is required to find ways to increase the rate of lipid oxidation so that the overall assay can be made faster. This is a key prerequisite for use of an assay in antioxidant screening studies while maintaining the intrinsic lipid oxidation pathways. Following on from the work of Buenger et al. (Buenger et al., 2006), another key area of research should be to thoroughly investigate the sources of variability in the TBARS assay, such that low coefficients of variation can be consistently obtained.

The other assay FTC is much slower than TBARS assay. Once the variability of TBARS assay is understood in the above research, an oxidation system can be developed so that it becomes more rapid in screening antioxidant activity against formation of both peroxides and TBARS.

Due to versatile uses, TBARS and FTC assays are well accepted in many fields of study, and if a rapid and robust antioxidant assay based on TBARS and peroxides was developed, it would likely be utilised in diverse areas where lipid oxidation is an issue. This can be extended to study the antioxidant effectiveness of medicinal plants (e.g. traditionally used plants) and explore phytochemical components of the plants.
1.15 Research Question and Objectives

Through a critical and extensive review of the literature, a primary research question can be proposed:

“Is it realistic to develop a rapid and robust method for the measurement of antioxidant activity in a lipid system?”

In order to answer the primary research question a number of research objectives will be addressed. They are to:

(1) Investigate the sources of variability in the TBARS assay as conducted by this laboratory and as reported in the literature;

(2) If possible, minimise the variability in the system;

(3) If this is not possible, investigate an alternative oxidation system (e.g. oil-in-water emulsion) to measure antioxidant activity;

(4) If acceptable variability in the alternative system is found, apply accelerated conditions in order to make the method faster while preserving the oxidation mechanism under the original conditions (such as at physiological temperature, 37 °C);

(5) Demonstrate the application of the developed method to screen antioxidant activity of Australian native plants, including identification of potential antioxidant compounds by LC-DAD-qTOF-MS.
This thesis addresses the above research question and objectives in five chapters. Chapter 2 investigates the variability of TBARS assay in a version of multi-phase oxidation system. Chapter 3 presents the optimisation of a rapid and robust method to measure antioxidant activity in LA emulsion. Chapter 4 describes the screening of antioxidant activity of selected Australian native plants using the developed method and also the identification of active compounds. Finally, Chapter 5 summarises the main findings of the study. Based on the findings, recommendations for future research are also delineated in the Chapter.
CHAPTER 2. SUBSTRATE AND TBARS VARIABILITY
IN A MULTI-PHASE OXIDATION SYSTEM

2.1 Introduction

As highlighted in Chapter 1, the TBARS assay is widely used in the study of antioxidant activity, yet is not suitable to be implemented in screening studies due mainly to the long time involved in oxidation of a lipid substrate. The initial stage of the thesis sought to replicate the test as reported from this laboratory by McDonald et al. (McDonald et al., 2001) and Inayatullah et al. (Inayatullah, Prenzler, Obied, Rehman, & Mirza, 2012), then modify reaction parameters so that a rapid assay could be developed for screening purposes. However, on commencing this project, significant problems with variability were encountered, which were not reported in the original work by McDonald (2001) and Inayatullah et al. (2012).

As discussed in Section 1.13, variability in the TBARS assay has been previously documented. Buenger et al. (2006) found that the TBARS method gave the highest intra- and inter-laboratory variability compared to three other antioxidant assays (i.e., DPPH, lipid assay-, and Trolox equivalent antioxidant capacity (TEAC)) (Buenger et al., 2006). In addition, the authors observed highest variability with the antioxidant tocopherol compared to four other antioxidants (ascorbic acid, butylated hydroxytoluene (BHT), 4-methylbrenzcatechin (4-MBC), and Trolox).
The variability in the assay could arise from the lipid oxidation stage and/or from the colour development stage, yet there is no study assessing the sources of variability of this assay. Therefore, the aim of this Chapter was to revisit the TBARS assay (McDonald et al., 2001) and conduct a systematic investigation to determine possible sources of variability such as: reaction conditions; different batches and suppliers of substrate; and different concentrations of antioxidant. In this version of assay, LA was oxidized with and without antioxidant in a multi-phase system in which LA was layered on the surface of an aqueous solution of catalyst and antioxidant, with dissolved and atmospheric oxygen as the oxidant. During the course of these investigations it became necessary to undertake the FTC assay so that results from TBARS assay could be checked for reliability, i.e. comparing outcomes for primary and secondary oxidation products.

2.2 Materials and methods

2.2.1 Chemicals and reagents

Different batches of linoleic acid (>99%) were obtained from Sigma-Aldrich, USA (1, 5 and 10 g amber coloured sealed ampoule) and Nu-Chek Prep Inc., Elysian, MIN, USA (10 g glass screw capped ampoule). Sunflower oil was purchased from a local supermarket. Magnesol-R60 was obtained from Dallas group, USA. (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) (97%), 2,6-di-tert-butyl-4-methylphenol (butylated hydroxytoluene, BHT) (99%), thiobarbituric acid (TBA) (>98%), iron(II) sulfate heptahydrate (>99%), ammonium thiocyanate
(>97.5%), 1-butanol (>99.7%), and absolute ethanol (99.8%) from Sigma-Aldrich (USA), barium chloride dihydrate (99%) from Biolab (Australia), methanol (99.99%) from Merck Chemicals (Germany), chloroform (>99%, HPLC grade) from Fisher Scientific (UK), n-hexane (96%, HPLC grade) from Scharlab S.L (Spain), hydrochloric acid (32%, v/v) from Ajax Finechem Pty Ltd (Australia), copper(II) chloride dihydrate (>99%) and activated charcoal from Chem-Supply (Australia). Ultra pure water (Edwards Instruments Co, Australia) was used throughout and glassware was soaked overnight in 10% (v/v) aqueous nitric acid (prepared from 70%, v/v, RCI Labscan Ltd, Thailand).

2.2.2 Experimental design

TBARS and peroxides were measured in fresh linoleic acid (pre-formed TBARS and peroxides) or upon oxidation (with or without antioxidant).

2.2.2.1 Preparation of lipid substrate

For measurements on fresh linoleic acid (LA), freshly opened LA (100 µL), 50 µL 50% (v/v) aqueous methanol and 300 µL water were mixed together.

Alternatively, LA was oxidised following the method of McDonald et al. (McDonald et al., 2001). Briefly, 100 µL LA, 50 µL of 50% (v/v) aqueous methanol or antioxidant solution (250, 1000, 2500 and 5000 µM Trolox in 50% (v/v) aqueous
methanol) and 300 µL 0.05 mM aqueous copper(II) chloride were sequentially added in a 10 mL centrifuge tube. The above order of addition of reagents was established as discussed in the results and discussion below (section 2.4.2.1). The mixture was vortexed for 5 s and incubated in a shaking water bath (Ratek Instrument, Australia) at 37 °C for 20 h under reduced light.

2.2.2.2 Measurement of thiobarbituric acid reactive substances (TBARS)

The oxidation was quenched with 20 µL 10 mM ethanolic BHT (for pre-formed TBARS, 20 µL ethanol was added to the mixture of fresh LA instead), 3 mL freshly prepared TBA solution (0.67 g TBA in 100 mL 0.1 M HCl sonicated for 30 min) was added, and the mixture vortexed for 5 s and heated in a boiling water bath for 10 min. After cooling the mixture to room temperature, a 2 mL aliquot of the aqueous layer was carefully transferred to a test tube containing 2.5 mL 1-butanol. The test tube was vortexed for 5 s and the absorbance of the upper 1-butanol layer was measured at 532 nm against a blank of 1-butanol (Bio-Rad Smartspectro™ plus spectrophotometer (Australia)).

When using an antioxidant, the percentage inhibition of TBARS formation was calculated using equation (1).

\[
\text{\% Inhibition of TBARS formation} = \frac{(A_0 - A_4)}{A_0} \times 100 \quad (1)
\]

where \(A_0\): absorbance value without antioxidant, \(A_4\): absorbance value with antioxidant.
2.2.2.3 Measurement of peroxides

Peroxides were measured following the ferric thiocyanate (FTC) assay of Shantha (Shantha & Decker, 1994). First, a 39 mM aqueous iron(II) solution was prepared by adding 50 mL 33 mM barium(II) chloride to 50 mL 39 mM iron(II) sulfate, followed by 2 mL 10.2 M hydrochloric acid. The solution was filtered to remove the white barium sulfate precipitate. Then, the LA mixture (fresh or after oxidation) was diluted in a chloroform-methanol (7: 3 (v/v)) solution (9 mL) in a 10 mL volumetric flask, to which 50 µL 30% (w/v) aqueous ammonium thiocyanate and 50 µL 39 mM aqueous iron(II) were added and the volume was made up to 10 mL with the chloroform-methanol solution. The mixture was vortexed for 5 s, and the volumetric flask wrapped with aluminium foil. After exactly 5 min incubation, the absorbance of the mixture was measured at 500 nm against a blank containing all reagents except LA. The percentage inhibition of peroxide formation in the presence of Trolox was calculated using the above equation (1).

2.2.2.4 Removal of pre-formed peroxides and thiobarbituric acid reactive substances (TBARS)

Pre-treatment of adsorbents

Pre-treatment of the adsorbents, activated charcoal and Magnesol, was conducted to remove water soluble impurities in the adsorbents, as adapted from Ferreira-Dias et
al. (Suzana Ferreira-Dias, Monteiro, & Ribeiro, 2002). The adsorbents were washed with water three times using 10% (w/v) adsorbent: water. The washed adsorbents were dried in an oven at 105 °C for 24 h and stored in an air tight container. Before use, the adsorbents were re-heated at 105 °C for 1 h and then cooled to room temperature in a desiccator.

**Choice of adsorbent and optimisation of adsorption**

Mixtures of 30, 40, 50 and 60% (w/w) activated charcoal or Magnesol in 10% (w/v) commercial sunflower oil or LA and n-hexane were prepared to determine the adsorbent efficiency in removing pre-formed peroxides and TBARS. The methods of Ferreira-Dias et al. (S Ferreira-Dias, Ribeiro, & Lourenço, 2000) and Ribeiro et al. (Ribeiro, Lourenço, Monteiro, & Ferreira-Dias, 2001) were adapted as follows: the lipid substrate-hexane-adsorbent mixture (in a capped scintillation vial) was stirred on a magnetic stirrer for 1 h at room temperature, centrifuged at 3000 rpm for 20 min, the supernatant decanted and the solvent removed by rotary evaporator (Buchi Rotavap). For sequential adsorbent treatments, the above cleaning process was repeated 3 times. The percentage of removal of peroxides and TBARS was calculated by the FTC and TBARS assays, respectively, using equation (2) below.

\[
\% \text{ Removal of peroxides or TBARS} = \left[ \frac{(A_{ba} - A_{aa})}{A_{ba}} \right] \times 100 \quad (2)
\]

where \( A_{ba} \): absorbance value before adsorption; \( A_{aa} \): absorbance value after adsorption.
Magnesol was selected as the most efficient adsorbent, and a further trial was performed on sunflower oil and LA (10% (w/v) in n-hexane) at a ratio of 60% (w/w) Magnesol: lipid.

2.3 Statistical analysis

All samples were prepared in triplicate (analytical replicates) and results are expressed as mean ± standard deviation. The statistical significance of differences was determined by one way ANOVA and posthoc least significant difference (LSD) using the SPSS software, version 20 (SPSS Inc., USA). Results were considered to be statistically significant at \( p < 0.05 \).

2.4 Results and discussion

2.4.1 Inter- and intra- batch variability of Linoleic acid (LA) in the thiobarbituric acid reactive substances (TBARS) assay:

Antioxidant activity for the TBARS assay is calculated according to equation 1, and involves measurement of \( A_{532} \) values with and without antioxidant (control). The robustness of the assay depends on obtaining consistent values for \( A_{532} \) both within replicate measurements (i.e. low intra-batch variability) and between different batches of LA (low inter-batch variability). The author’s initial measurements of
antioxidant activity using LA as the substrate revealed high intra- and inter-batch variability (Figure 2.1).

![Figure 2.1: Absorbance values at 532 nm (TBARS assay, LA batches I-III) for control (without Trolox) and with 250 and 1000 µM Trolox (in batch II, 1000 µM Trolox was not conducted). Different letters mean significant differences ($p < 0.05$). Absorbance values are presented as mean ± standard deviation (n=3), and statistically significant difference was determined by one way ANOVA.](image)

Several aspects of Figure 1 should be noted. Firstly, the presence of the antioxidant, Trolox, seems to affect the reliability of replicate measurements within a batch of LA. For example in Batch I, reproducibility (as measured by %CV) ranged from 13% (control) to 40% (250 µM Trolox). In Batch III, %CV ranged from 7% (control) to 29% (1000 µM Trolox). Notably, %CVs are reasonable for the control samples in all the batches, whereas they are quite inconsistent and generally high for the samples containing Trolox.
Secondly, inter-batch variability in TBARS formation is evident from comparing $A_{532}$ values among the control samples from Batches I-III. There is a statistically significant difference ($p<0.05$) among these samples indicating that each different batch has produced different amounts of TBARS under ostensibly the same oxidation conditions. Furthermore, the presence of 250 μM Trolox yields statistically significantly ($p<0.05$) different amounts of TBARS indicating different antioxidant activities for the same concentration of Trolox.

The above observations show that there is a problem with the TBARS assay in assessing antioxidant activity using LA as a substrate. In order to determine the sources of variability, a systematic investigation was conducted by looking at various aspects of the assay, including the methodology, the substrate, and the concentration of antioxidant. In line with the potential variability in measuring antioxidant activity found in batches I-III, it was necessary to keep track of the batches of LA used, hence the data and results of this chapter are presented in “batches” with numbers IV-XIII.

2.4.2 Sources of variability:

2.4.2.1 Effect of order of addition of reagents on variability:

The first source of variability the author investigated was the order of addition of reagents. It was important to first establish whether this parameter had an effect on
variability, so that conditions could be chosen to minimise variability in experiments involving the substrate and different concentrations of antioxidant. Three different addition orders were investigated according to Scheme 1.

\[
\begin{align*}
\text{Cu}^{2+} & \rightarrow \text{LA} \rightarrow \text{Control/Trolox} \ (i) \\
\text{Cu}^{2+} & \rightarrow \text{Control/Trolox} \rightarrow \text{LA} \ (ii) \\
\text{LA} & \rightarrow \text{Control/Trolox} \rightarrow \text{Cu}^{2+} \ (iii)
\end{align*}
\]

Scheme 1

Reaction conditions (i) were used for experiments on batches of LA (I-III) as reported in 2.4.1, above.

In testing reaction conditions (ii) and (iii), it was found that 1000 μM Trolox continued to give high %CVs (ranging from 10 to 25%), whereas with 5000 μM Trolox, %CVs (ranging from 6 to 13%) were lower. Hence, results for reaction conditions (ii) and (iii), using 5000 μM Trolox, are presented in Figure 2.2A for LA batch (IV). These results show that reaction conditions (iii) give lower %CVs for the control (2%) and Trolox (2%) illustrating consistent antioxidant activity measurement. Using reaction conditions (iii), two new batches of LA (V-VI) were tested in order to confirm the results shown in Figure 2.2 A. In Figure 2.2 B both the control and Trolox systems show very low % CVs (ranging for control, 0.1-4% and Trolox, 2-6%).
Figure 2.2: Absorbance values at 532 nm (TBARS assay) for control (without Trolox) and 5000 µM Trolox, A: different order of addition of reagents (LA batch IV) and B: using the LA, solvent/Trolox, Cu2+ order of addition of reagents (LA batches (V-VI)). Different letters mean significant differences ($p < 0.05$). Absorbance values are presented as mean ± standard deviation ($n=3$), and statistically significant difference was determined by one way ANOVA.

The above results reveal that order of addition of reagents contributed to the variability of oxidation of the LA substrate in the presence and absence of the antioxidant Trolox. Since this is a multi-phase system – air (oxidant), aqueous (catalyst/antioxidant), lipid (substrate) – variability in mixing of reagents may contribute to difference in oxidation. In reaction conditions (i) and (ii), the Cu$^{2+}$ catalyst is present in the system at the beginning, whereas in (iii) it is added last. It is possible that initial exposure of LA (i) or Trolox (ii) with the catalyst, combined with differences in amount of air mixing into the system, may result in inconsistent oxidation of the substrate.
Having established that reaction conditions (iii) gave consistent results (i.e. low intra-batch variability), the author exclusively followed these conditions for the remaining experiments exploring the causes of inter-batch variability in the LA substrate, and further experiments investigating variability induced by Trolox.

2.4.2.2 Effect of batch to batch Linoleic acid (LA) variability:

The second source of variability the author investigated was the inter-batch variability in LA substrate, as measured by $A_{532}$ values. Figure 2.3A shows the results of testing a further four batches of LA (VII-X). These four batches of LA gave significantly different absorbance values ($p < 0.05$) ranging from 0.937 to 1.424 (AU). These results may be evaluated together with the results from batches (V-VI) (Figure 2.2B), where significant differences ($p < 0.05$) were found in $A_{532}$ ranging from 0.648 to 1.437 (AU), since all batches of LA (V-X) were reacted under the same conditions (iii) above. These data show that different batches of LA gave significantly different amounts of oxidation products when using the reaction conditions (iii) that minimise intra-batch variability.
Figure 2.3: TBARS and peroxides in batches VII-X of LA at opening; and after 20 h oxidation without (control) and with 250 µM Trolox. A: Absorbance values at 532 nm (TBARS assay) B: Absorbance values at 500 nm (FTC assay). Different letters mean significant differences ($p < 0.05$). Absorbance values are presented as mean ± standard deviation ($n=3$), and statistically significant difference was determined by one way ANOVA.

The extent of oxidation in different batches of LA may be influenced by the presence of TBARS or peroxides already present in the LA prior to opening. These pre-formed oxidation products may be present in different initial concentrations, and/or there may be other differences that affect the extent of oxidation e.g. multi-phase system with potentially differing amounts of oxygen interacting with the catalyst and substrate. Further experiments were therefore conducted to measure pre-formed peroxides and TBARS, and to determine if they contributed to the observed batch-to-batch variability of the $A_{532}$ values, reported above.
The absorbance values in Figure 2.3 A showed that pre-formed TBARS were present in the four batches of LA (VII-X), but that there was no statistically significant difference in the amounts of pre-formed TBARS ($p > 0.05$) among the batches. Therefore pre-formed TBARS are not a source of the batch to batch variability in the $A_{532}$ values after 20 h oxidation. On the other hand, variation in pre-formed peroxides and/or variability in their breakdown to secondary oxidations products, may contribute to the variability in TBARS formation.

Peroxides were determined by FTC assay in batches (VII-X) of LA, both before and after 20 h oxidation, and the results are presented in Figure 2.3 B. The absorbance values showed that there were substantial and significantly different ($p < 0.05$) amounts of pre-formed peroxides in the different batches of LA (average 1.346 AU, range 1.317 to 1.425 (AU)). After 20 h oxidation, the peroxide values tended to a consistent value of around 1.35 (AU). It is also noteworthy that the amount of peroxides does not increase after 20 h oxidation. This suggests that the level found in the LA at opening is near the steady-state level and that during oxidation the rate of formation of peroxides is approximately equal to the rate of breakdown to secondary oxidation products.

The results illustrate that the substantial and variable amounts of pre-formed peroxides in different batches of LA is a possible source of variability for the batch to batch variation of TBARS formation. While there is not a direct correspondence between the amounts of pre-formed peroxides and TBARS measured after 20 h, it is
possible that erratic breakdown of peroxides occurs to give variable amounts of TBARS or some other secondary oxidation products.

2.4.2.3 Effect of different concentrations of Trolox:

The third source of variability the author investigated was the variability induced by the presence of Trolox. As shown in Figure 2.1, the %CVs for A$_{532}$ values in presence of Trolox (range 3-40%) were generally much higher than in the controls (7-13%). However, order of addition was subsequently shown to be an important factor in affecting variability, leaving presence of Trolox to be investigated separately. Using reaction sequence (iii) (Scheme-1), the effect of 250 and 5000 μM Trolox on intra batch variability was examined. Figures 2.2 and 2.3A show the %CVs for A$_{532}$ values in presence of 5000 and 250 μM Trolox respectively. Clearly, the %CVs are much improved by using reaction sequence (iii), with ranges of 4-11% for 250 μM and 2-6% for 5000 μM Trolox. Although it appears that the reaction sequence was the major contributor to the variability shown in Figure 2.1, it should be noted that previous research has shown that different antioxidants do affect the precision of the TBARS assay and further research, beyond the scope of this study, would be warranted to investigate the findings of Buenger et al. (Buenger et al., 2006) in the multi-phase system used in this study.

Since the investigations led the author to include peroxide measurements, the effect of Trolox on the intra-batch variability of A$_{500}$ values may also be reported. The A$_{500}$
values in the presence of 250 μM Trolox for batches VII-X of LA show very low %CVs ranging 0.2-1% (Figure-2.3B). This result illustrates that the measurement of peroxides (A₅₃₀) is more precise than the measurement of TBARS (A₅₃₂). This is supported by another study (Bakır et al., 2013) on the concentration dependent response of oxidative inhibition measured by FTC and TBARS assays although under a different oxidation system (LA emulsion).

As sequence (iii) has minimised intra-batch variability in the presence of antioxidant, antioxidant activity may be reported as % inhibition with acceptable precision. For TBARS, the values for % inhibition of Trolox at 5000 μM in batches (IV-VI) of LA were: 42 ± 1%, 77 ± 1% and 57 ± 1%, respectively. These values are significantly different (p < 0.05), which shows that Trolox has different antioxidant activity in different batches of LA. When the concentration of Trolox was 250 μM, % inhibition values were 33 ± 6%, 35 ± 2%, 26 ± 8% and 29 ± 3% in batches (VII-X) of LA. While the variation in % inhibition from batch to batch is lower at this concentration of Trolox, there is still significant difference (p < 0.05) among some values. The lack of reproducibility in the % inhibition values is extremely problematic for the use of this multi-phase system as an antioxidant assay. While variability in absolute formation of TBARS might be tolerated if % inhibition values were consistent, the lack of consistency would preclude the use of this system for measurement of antioxidant activity.
Looking at the peroxide levels in systems containing Trolox, further issues are evident as to the suitability of the multi-phase LA system to measure antioxidant activity. As observed above for the control samples, in the presence of 250 µM Trolox no net change in $A_{500}$ values was evident ($p > 0.05$, Figure 2.3B). In other words, the presence of antioxidant has had no apparent effect on the amounts of peroxides in LA over 20 h oxidation. To investigate this phenomenon further several other concentrations of Trolox were trialed, viz. 1000, 2500 and 5000 µM, with the same result – no measurable antioxidant activity. The implication of these results is that in the current multi-phase system, antioxidant activity of Trolox in preventing primary oxidation products cannot be detected. Therefore, it would appear meaningless to try and draw conclusions about antioxidant activity in the subsequent formation of secondary oxidation products, including TBARS. We can speculate that any antioxidant activity that has been reported by this method here and elsewhere (Inayatullah et al., 2012; Kishida et al., 1993) is due to the interaction of the antioxidant with the pre-formed peroxides as they break down, which may randomly inhibit TBARS formation.

It is clear from the above observations that the presence of the pre-formed peroxides and the lack of detectable antioxidant activity of Trolox against primary oxidation products are problematic in developing a valid antioxidant assay in a multi-phase system. Therefore, as a way to further investigate the suitability of the multi-phase method, the author set about to remove the peroxides to obtain a “clean” sample of LA. With such a sample, it should be possible to observe the de novo formation of
peroxides in control samples and any antioxidant activity of Trolox against primary and secondary oxidation products.

### 2.4.2.4 Method development to remove pre-formed peroxides

As revealed in the previous section, pre-formed peroxides were needed to be removed from LA for use in an antioxidant assay. In the current literature, there is no directly applicable method for removal of peroxides from LA; thus a method needed to be developed for purposes of this study. However, the expense of LA led to the selection of sunflower oil for method development, as sunflower oil generally contains 70% LA. A bottle of 1-year old sunflower oil was found to have an $A_{500}$ value of $1.37 \pm 0.02$ AU similar to $A_{500}$ values ($1.37 \pm 0.01$ AU) in LA and hence was deemed suitable for method development.

Removal of contaminants in oil is commonly achieved by using adsorbents such as activated charcoal (Bhattacharya, Sajilata, Tiwari, & Singhal, 2008) and more recently Magnesol (Turan & Yalcuk, 2013). Percentage of removal of peroxides calculated as per equation-2 and ratio of adsorbent to oil were considered as the parameters for optimising adsorbent efficiency. The initial experiment with 20% (w/w) activated charcoal in sunflower oil gave only $36 \pm 0.7$ % removal of pre-formed peroxides. This is lower than the reported removal of 50-60% conjugated hydroperoxides from olive residue oil using 30% w/w activated charcoal (Suzana Ferreira-Dias et al., 2002). Due to the low affinity of activated charcoal for polar
hydroperoxides, Magnesol was trialled as an adsorbent with better properties for removing polar compounds (Farag & Basuny, 2009). The initial trial of 30% (w/w) Magnesol in oil gave 78 ± 0.7 % removal of peroxides, and being lower than expected, led us to consider treatment of the adsorbents prior to addition to oil. The ratio of absorbents to oil was gradually increased (i.e., 40% - 60% (w/w)) to obtain more removal of peroxides.

Table 2.1 shows that 30, 40 and 50% (w/w) activated charcoal in sunflower oil showed 62, 73 and 78% removal of peroxides while the same ratio of Magnesol in sunflower oil showed 78, 80 and 87% removal. Clearly, Magnesol has a higher efficiency in removing pre-formed peroxides from sunflower oil. Considering the increasing efficiency of Magnesol, 60% (w/w) Magnesol in sunflower oil was found to remove 94% of the peroxides from sunflower oil (Table 2.1). Alternatively, 30% (w/w) Magnesol in sunflower oil provided 95 ± 1.3 % removals of peroxides (data not shown) upon three sequential treatments of adsorbent.

Table 2.1: Removal of peroxides from Sunflower oil using different percentage (w/w) of activated charcoal and Magnesol in 10% (w/v) in oil and n-hexane.

<table>
<thead>
<tr>
<th>% (w/w) absorbent in 10% (w/v) oil and n-hexane</th>
<th>% Removal of peroxides from Sunflower oil</th>
<th>Activated Charcoal</th>
<th>Magnesol</th>
</tr>
</thead>
<tbody>
<tr>
<td>30%</td>
<td>62 ± 1.2</td>
<td>78 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>40%</td>
<td>73 ± 0.7</td>
<td>80 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>50%</td>
<td>78 ± 1.9</td>
<td>87 ± 3.4</td>
<td></td>
</tr>
<tr>
<td>60%</td>
<td>-</td>
<td>94 ± 2.2</td>
<td></td>
</tr>
</tbody>
</table>
As 60% (w/w) Magnesol in sunflower oil gave 94% removal of pre-formed peroxides, this ratio was kept constant for Magnesol to remove pre-formed peroxides from LA. However, 60% (w/w) Magnesol in LA gave only 36 ± 1 % removal of pre-formed peroxides with 67% recovery of LA (data not shown). Various other values of % Magnesol were trialed, resulting in an optimum 94% removal of peroxides from LA using three sequential treatments of 60% (w/w) Magnesol. Although the recovery of LA (39%) was low, the nearly 100% removal of peroxides resulted in LA suitable for use in an antioxidant assay (Table 2.2).

Table 2.2: Removal of peroxides from LA by 3 sequential treatments of adsorbent using 30 and 60% (w/w) Magnesol in 10% (w/v) LA and n-hexane.

<table>
<thead>
<tr>
<th>% (w/w) Magnesol in 10% (w/v) LA and n-hexane (*3 sequential treatments)</th>
<th>% Removal of peroxides from LA</th>
<th>Recovery of LA after adsorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>30%</td>
<td>84 ± 2.0</td>
<td>51%</td>
</tr>
<tr>
<td>60%</td>
<td>94 ± 2.3</td>
<td>39%</td>
</tr>
</tbody>
</table>

Having optimised the method, the batches of LA (XI, XIII-XV) from Sigma Aldrich and the batch XII from Nu-Chek Prep Inc. were cleaned by removal of pre-formed peroxides. 90-98% pre-formed peroxides were removed from the above five batches of LA. The optimised method was also investigated to remove secondary oxidation product TBARS from the XI and XII batches of LA. The method successfully removed 59-89% TBARS from two batches of LA. Immediately after removal of the pre-formed oxidation products, the cleaned batches of LA were used for subsequent antioxidant activity measurement.
2.4.2.5 Outcomes of oxidation and antioxidant activity using treated Linoleic acid (LA)

For experiments involving the oxidation of LA and the observation of \textit{de novo} formation of peroxides and TBARS, five batches of LA (XI–XV) were treated with Magnesol as above resulting in removal of 96 ± 3\% pre-formed peroxides. Oxidation of LA using reaction sequence (iii), in the absence of Trolox, was then conducted for 20 h and the results are presented in Figure 2.4A. The formation of peroxides was not consistent from batch to batch with $A_{500}$ values ranging from 0.59 to 1.35 AU (data not shown, for batches XIII-XV). Clearly the removal of pre-formed peroxides has not improved batch to batch variability in subsequent peroxide formation as reported above. The conclusions reached above regarding the unpredictable nature of the conditions of oxidation in the multi-phase system, are borne out by the result here. In addition, it can now be shown that \textit{de novo} formation of peroxides is not reproducible in the multi-phase system.
Figure 2.4: Absorbance values for cleaned LA after 20 h oxidation without Trolox (control) and with 250, 1000, 2500 and 5000 µM Trolox. A: for peroxides at 500 nm; B: for TBARS at 532 nm. Different letters mean significant differences (p < 0.05). Absorbance values are presented as mean ± standard deviation (n=3), and statistically significant difference was determined by one way ANOVA.

Despite this lack of reproducibility, a suitable antioxidant assay may be developed if the % inhibition of oxidation is consistent from batch to batch (as noted above). In Section 2.4.2.3, % inhibition was shown to not be consistent, but the presence of pre-formed peroxides was suspected to be problematic. With pre-formed peroxides removed, no antioxidant activity at all was observed when 250 µM Trolox was added to the system. In fact in two of the batches (XII and XIII) significant pro-oxidant activity was detected (results not shown). Similarly for systems containing 1000, 2500 and 5000 µM Trolox, no antioxidant activity was observed and in some cases pro-oxidant was evident.
Thus neither the overall formation of peroxides nor the % inhibition of peroxides was found to be consistent from batch to batch of LA. Such lack of reproducibility suggests that the multi-phase system reported in the literature (Antolovich et al., 2004; Kishida et al., 1993; McDonald et al., 2001), is in fact not suitable to reliably measure antioxidant activity.

In terms of TBARS formation, little helpful information can be gained from experiments conducted with LA where peroxides were removed (Figure 2.4B). One point to note is that TBARS formation in the control samples is much reduced compared to results reported above (Figures 2.1–2.3). In the earlier experiments the $A_{532}$ values ranged from 0.648 to 1.895 AU (batches LA, I-X), whereas in experiments where peroxides have been removed they are <0.05 AU. This strongly suggests that the origin of the TBARS in previous experiments is from the pre-formed peroxides in LA. It is also consistent with reports in the literature that LA is not a good source of TBARS (Laguerre, Lecomte, & Villeneuve, 2007). Figure 2.4B also shows that the presence of Trolox results in either no antioxidant activity or pro-oxidant activity. The latter is most evident in the sample containing 5000 μM Trolox, although it is evident from the error bars that considerable intra-batch variability has returned at this concentration of antioxidant.

2.5 Conclusions

The Chapter concludes that oxidation of LA in a multi-phase system exhibits variability in the TBARS and FTC assays. The variability is associated with the methodology of the reaction, the presence of pre-formed peroxides in LA, and the
concentration of the antioxidant Trolox used. Of the two types of LA variability, intra-batch variability can be minimised by the order in which reagents are mixed; specifically, limiting the time that LA is exposed to oxygen and/or the catalyst. On the other hand, inter-batch variability seems to be related to the presence of pre-formed peroxides, which break down erratically to give TBARS and other secondary oxidation products. One of the results of this Chapter suggests that any TBARS formed and any antioxidant activity measured is due to pre-formed peroxides and not due to oxidation products formed de novo from LA.

Thus the multi-phase system is problematic in assessing antioxidant activity. Firstly, the use of cleaned LA undergoes very slow oxidation to peroxides and TBARS. This is not suitable for an antioxidant assay where high throughput is required. Secondly, even if a slow reaction was acceptable, the breakdown of peroxides giving inconsistent levels of TBARS precludes the use of this methodology as an antioxidant assay. This finding may be extended to the common use of the TBARS assay to measure lipid oxidation and antioxidant activity in other systems (e.g. meat products (Papastergiadis et al., 2012)). If pre-formed peroxides are not measured, and/or they break down erratically, then the findings of antioxidant activity may not be reproducible from one study to the next. Further research is needed to investigate the likelihood of this issue and to standardise a suitable alternative oxidation system in which antioxidant activity can be reliably measured against both TBARS and peroxides.
CHAPTER 3. DEVELOPMENT OF A RAPID AND
ROBUST METHOD TO MEASURE ANTIOXIDANT
ACTIVITY IN A LINOLEIC ACID EMULSION

3.1 Introduction

The potential variability in substrate and TBARS with multi-phase oxidation system discussed in Chapter 2 indicates the method was not robust. For a robust method, an alternative oxygen system of LA was therefore needed. One of these systems would be LA emulsion (oil-in-water). In emulsion system, LA is uniformly dispersed in water phase with small droplets. The droplet of LA can be stabilised by an emulsifiers such as Tween 20. Each droplet is surrounded by an interfacial region, which is surrounded by aqueous phase. Interfacial region consists of some LA, antioxidant, and emulsifier (Berton-Carabin, Ropers, & Genot, 2014). Antioxidant can uniformly partition in interface region. Therefore measuring antioxidant activity in the emulsion system may be robust.

For example, Stoilova el al. (Stoilova et al., 2007) and Kljak & Grbesa (Kljak & Grbeša, 2015) used LA emulsion for TBARS assay to measure antioxidant activity of plant extracts and their results showed good precision as standard deviation (SD) was low. In emulsion, LA gets more opportunity to be mixed well through homogenisation relative to other systems, such as multi-phase. This mixing of LA might provide consistent oxidation and antioxidant activity with the above report.
In addition, Yen and Hsieh (Yen & Hsieh, 1998) and Gulcin et al. (2012) (Gülçin et al., 2012) also used LA emulsion, for FTC assay to measure antioxidant activity of plant extracts and lower standard deviation indicated that the results were consistent. Furthermore, as shown in their reported figures initial peroxides for FTC assay were very low. This indicates that pre-formed peroxides may much lower in emulsion compared to other systems. Additionally, using LA emulsion, other groups have reported good precision in both TBARS and FTC tests (Erkan, 2012; Rubio-Senent et al., 2012; Yen, Chang, & Su, 2003).

Similar to multi-phase system, physiologically relevant temperature, 37 °C is used in LA emulsion with either FTC (Yen & Hsieh, 1998), or TBARS (Stoilova et al., 2007), or both assays (Bakir et al., 2013). However, when conducted at 37 °C in LA emulsion, it takes a long time to be oxidised for a substrate, and times (i.e., end points of oxidation) of this oxidation ranging from 16 hours to 12 days have been reported. For such long oxidations, the above assays are not suitable to high throughput screening of antioxidants, where rapid measurement of antioxidant activity was aimed for this study.

Increase in temperature, use of a catalyst, or use of an oxygen initiator such as transition metal ions in the presence of hydrogen peroxide can accelerate the oxidation of LA emulsion used in FTC and TBARS tests. However, all these approaches may lead to a change in the mechanism of lipid oxidation (Frankel,
1993), which may not give results for antioxidant activity that are valid compared to those at 37 °C. In order to assess antioxidant activity under accelerated conditions, validation parameters are needed, one of which would be order of antioxidant activity, which can be established using several antioxidants with a definite concentration at 37 °C and for definite oxidation time. As it is known that order of antioxidant activity can change under accelerated conditions (Frankel, 2014; Frankel, Huang, Kanner, & German, 1994), attempts to speed up the oxidation system of the FTC or TBARS tests should need to be performed in such a way that can preserve the order of antioxidant activity at 37 °C.

The objective of this Chapter was therefore to develop a method in LA emulsion, based on the FTC and TBARS tests, by altering various parameters, so that it becomes faster, and its validity can be checked through order of antioxidant activity.

### 3.2 Materials and methods

#### 3.2.1 Chemicals and reagents

Linoleic acid (>99%) was purchased from Nu-Chek Prep InC., Elysian, MIN, USA. (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) (97%), gallic acid (97.5%), (+)- catechin hydrate (>98%), (−)- epicatechin (>90%), caffeic acid (>98%), α-Tocopherol (>96%), quercetin (>95%), trichloroacetic acid (TCA) (>99%), thiobarbituric acid (TBA) (>98%), ammonium thiocyanate (>97.5%),
polyoxyethylene sorbitan monolaurate (Tween 20) (97%) and iron(II) chloride (98%) were obtained from Sigma-Aldrich (USA). L-ascorbic acid (>99%) was purchased from Sigma-Aldrich (China), copper(II) chloride dihydrate (>99%), hydrogen peroxide (30%, v/v) and anhydrous sodium dihydrogen orthophosphate (sodium phosphate monobasic) (99%) from Chem-Supply (Australia), absolute ethanol (99.97%) from VWR Internationals (France), methanol (99.99%) from Merck Chemicals (Germany), nitric acid (70%, v/v) from Ajax FineChem Pty Ltd (New-Zealand), hydrochloric acid (32%, v/v) from Ajax Finechem Pty Ltd (Australia), chloroform (>99%, HPLC grade) from Fisher Chemical (UK), cobalt(II) chloride hexahydrate (>98%) from M & B Laboratory Chemicals (UK) and anhydrous di-sodium hydrogen phosphate (sodium phosphate dibasic) (99.7%) from VWR International BVBA (Belgium). Ultra pure water (Edwards Instruments Co, Australia) was used throughout and glassware was soaked overnight in 10% (v/v) aqueous nitric acid (prepared from 70%, v/v, RCI, Labscan Ltd, Thailand).

3.2.2 Experimental design

In this study, a LA nano-emulsion was first prepared, and then oxidised under a range of conditions: absence or presence of antioxidants; physiological temperature (37 °C) or accelerated conditions (50 and 60 °C); absence or presence of catalysts (copper(II) or cobalt(II) and oxygen initiator (iron(II) + hydrogen peroxide)). After oxidation, peroxides and TBARS were measured. All solutions, including LA emulsions, were freshly prepared before conducting the experiments.
3.2.2.1 Preparation of solutions

Antioxidants

A range of antioxidants including Trolox, gallic acid, ascorbic acid, quercetin, (+)-catechin, (-)-epicatechin, caffeic acid and α-tocopherol were used. Quercetin and α-tocopherol were dissolved in methanol while other antioxidants were dissolved in 50% (v/v) aqueous methanol. Trolox was used at the final concentrations of 25, 250 or 400 µM and other antioxidants at 250 µM in oxidation samples consisting of a total 5 mL of LA emulsion, phosphate buffer solution (PBS) and antioxidant solution. A final concentration of 250 µM of Trolox, gallic acid, ascorbic acid or quercetin was used for the added catalysts and added oxygen initiator experiments. A 50% (v/v) aqueous methanol without added antioxidant was used as control solvent.

Catalysts and oxygen initiator

Aqueous solutions (0.05 M) of copper(II) chloride and cobalt(II) chloride were prepared and 1 mM was used as the final concentration in the oxidation mixture (Bakır et al., 2013). For the oxygen initiator (iron(II) + hydrogen peroxide), 3 µM iron(II) chloride and 2 µM hydrogen peroxide solutions were prepared separately.
from which 0.04 µM iron(II) and 0.01 µM hydrogen peroxide were used as the final concentrations (Dutta & Maharia, 2012).

**Phosphate Buffer**

A 0.2 M aqueous sodium phosphate buffer solution (PBS) was prepared at pH 7. First, 0.2 M solutions of sodium phosphate monobasic (NaH₂PO₄) (solution A) and sodium phosphate dibasic (Na₂HPO₄) (solution B) were prepared in water. Then, 19.5 mL of A and 30.5 mL of B were mixed together in a volumetric flask and the volume made to 100 mL with water. The resulting pH was adjusted to pH 7 (John Morris Scientific Pty limited, Australia) by dropwise additions of solution A or B. Solution A, being acidic, was used to decrease and B, being basic, to increase pH compared to pH 7.

**Linoleic acid (LA) nano-emulsion (oil-in-water)**

A 0.02 M LA (pH 7) nano-emulsion (droplet size < 250 nm in diameter, range 94-243 nm) was prepared in the above PBS following the method of Yen and Hsieh (Yen & Hsieh, 1998). Briefly, 0.2804 g of LA and 0.2804 g Tween 20 were mixed in 50 mL 0.2 M (pH 7) PBS and vortexed for 5 s. A milky mixture was produced, and the resulting pH was adjusted to pH 7 as above. The container of LA mixture was
kept on ice until nano-emulsion preparation using the homogenisation procedure below:

**Homogenisation and nano-emulsion formation**

Coarse pre-emulsions of LA were first prepared using an Ultra Turax homogeniser (T25 basic, Janka & Kunkel IKA Labortechnik, Malaysia) at speed 4 (19,000 rpm) for 6 min. Nanoemulsions were then formed by passing the coarse mixture through a pneumatically driven high pressure valve homogeniser (Emulsiflex™-C5, AVESTIN Inc., Ottowa, ON, Canada) for 3 times at pressures between 70-140 ×10³ kPa. While collecting the emulsion, the container was also kept on ice to avoid oxidation. After homogenisation, a clear solution with no creaming or no separation of layers was obtained. Creaming was visually examined to see whether there was a change in turbidity between the top and bottom layers of the emulsion.

**Droplet size (zeta-average[d]) and poly-dispersity index (PDI) measurements**

The mean droplet size (diameter) and poly-dispersity index (PDI) of the nano-emulsions (50 µL diluted in 4950 µL water, 25 °C) were measured in duplicate using a dynamic light scattering Zetasizer (Model ZEN 3600, Nano-ZS, Malvern, ATA Scientific, UK) (Adjonu, Doran, Torley, & Agboola, 2014) . Droplet size and PDI were automatically calculated by the Zetasizer nano-ZS dynamic light scattering
(DLS) software (version 6.2). PDI is a dimensionless measure of the size distribution of oil droplets, and is calculated using equation $\sigma^2/Z_p$, where $\sigma$=standard deviation and $Z_p$=average diameter of droplets. A high value (maximum 1) shows a polydisperse system and a low value (minimum 0) denotes a monodisperse system.

### 3.2.2.2 Oxidation

Three types of oxidation samples with or without antioxidants, catalysts, and oxygen initiator were prepared in 2.5 mL LA emulsion (0.02 M pH 7) and 2 mL PBS (0.2 M pH 7) for oxidative studies at 37 °C or accelerated temperatures:

1. Antioxidant oxidation sample at 37, 50 or 60 °C: added 0.5 mL 25, 250 or 400 µM antioxidant/control solvent,

2. Antioxidant with added catalysts sample at 37 °C: 0.25 mL 250 µM antioxidant solution/control solvent + 0.1 mL catalyst solution + 0.15 mL methanol,

3. Antioxidant with added oxygen initiator sample at 37 °C: 0.25 mL 250 µM antioxidant solution/control solvent, 0.07 mL iron(II), 0.03 mL hydrogen peroxide solution and 0.15 mL methanol.

All solutions were mixed together in a screw-capped 50 mL centrifuge tube and vortexed for 5s. The cap was then put loosely in order to allow sufficient oxygen ingress and the mixture was oxidised in a water bath (Thermoline Scientific,
Australia). Sample 1 was oxidised in between 10 to 96 h while samples (2-3) were oxidised for 10 h. Two 0.1 mL aliquots of the oxidation samples were taken at different intervals of oxidation, and peroxides and TBARS were measured using procedures below.

3.2.2.3 Measurement of peroxides

Peroxides were measured following the ferric thiocyanate method by Yen and Hsieh (Yen & Hsieh, 1998). Briefly, an aqueous solution of ethanol (4.7 mL, 75% (v/v)), aqueous ammonium thiocyanate (0.1 mL, 30% (w/v)), the above oxidation sample (0.1 mL) and aqueous iron(II) chloride (0.1 mL, 0.02 M in 3.2% (v/v) hydrochloric acid) were sequentially added in a 10 mL volumetric flask and vortexed for 5 s. After exactly 3 min incubation, absorbance was measured at 500 nm against a blank containing all reagents except LA (Bio-Rad Smartspectro™ plus spectrophotometer (Australia)). A control was performed with the LA sample but without the antioxidant. The antioxidant activity (% inhibition of peroxides formation) was calculated using equation 1.

\[
\% \text{ inhibition of peroxides} = \left( \frac{A_0 - A_s}{A_0} \right) \times 100 \tag{1}
\]

where \( A_0 = \) Absorbance in absence of antioxidant, \( A_s = \) Absorbance in presence of antioxidant.
3.2.2.4 Measurement of thiobarbituric acid reactive substances (TBARS)

TBARS were measured following the TBARS method by Stoilova (Stoilova et al., 2007). Briefly, an aliquot of 0.1 mL oxidation sample was added to 2 mL TBA-TCA solution (20 mM TBA in 15% (w/v) aqueous TCA) in a 10 mL centrifuge tube and vortexed for 5 s. The mixture was heated in a water bath (100 °C) for 15 min and cooled to room temperature. Then, 2 mL chloroform was added and vortexed for 5 s. The mixture was centrifuged (Eppendorf Centrifuge 5810) at 2000 rpm for 15 min. The supernatant was carefully transferred to a plastic cuvette (10 mm) and absorbance was measured at 532 nm against a blank using all reagents except LA. Control sample preparation and measurement of antioxidant activity (% inhibition of TBARS formation) were performed as per above ferric thiocyanate method.

3.3 Statistical analysis

All samples were prepared in triplicate (analytical replicates) and results are expressed as mean ± standard deviation. The statistical significance of differences was determined by one way ANOVA and posthoc least significant difference (LSD) using the SPSS software, version 20 (SPSS Inc., USA). Results were considered to be statistically significant at $p < 0.05$. 
3.4 Results and Discussion

3.4.1 Effect of Linoleic acid (LA) nano-emulsion

For reproducible formation of peroxides and TBARS in a robust FTC and TBARS assay, an emulsion needs to be consistently prepared from different batches of LA. It is evident that oxidation of a lipid substrate is related to the size of the lipid droplets in an emulsion (Berton-Carabin et al., 2014). In addition, PDI (polydispersity index) is a measure of the degree of homogeneity (PDI for efficient emulsion is <0.7) of the lipid substrate in the emulsion. Therefore characterisation of the LA nano-emulsions was carried out by measuring the above two parameters: droplet size and polydispersity index (PDI). For initial experiments to determine the reproducibility of peroxide and TBARS formed from different batches of LA, two emulsions were prepared with droplet size of a nano emulsion and PDI < (0.3-0.4); vis. emulsion from batch I, 185.1 ± 0.2 nm, PDI 0.3 ± 0.0; and emulsion from batch II, 243 ± 0.4 nm and PDI 0.4 ± 0.0.

With these emulsions, oxidation was conducted at 37 °C over 96 h, monitoring $A_{500}$ values for peroxides and $A_{532}$ for TBARS. Several aspects of reproducibility were evaluated such as consistency of time to obtain maximum amount of peroxides and TBARS, intra-batch precision, and inter-batch variability in LA oxidation. In Figure 3.1(A and B), the two batches of LA emulsions showed the same values of $A_{500}$ maximum, both at 24 h. As shown in Figure 3.1, intra-batch precision was good. In
Chapter 2, there was some batch-to-batch variability in pre-formed peroxides in the multi-phase system. However, here no statistically significant difference ($p>0.05$) was found, and essentially any pre-formed peroxides are either diluted or removed during the preparation of the emulsion ($A_{500}$ values are near 0). Thus oxidation in LA emulsion for peroxides is consistent compared to a multi-phase system.
Figure 3.1: Absorbance values (n=3) for peroxides (A: LA emulsion batch 1) and (B: LA emulsion batch 2) measured by FTC assay, for TBARS (C: LA emulsion batch 1) and (D: LA emulsion batch 2) measured by TBARS assay (Oxidation at 37 °C). Error bars are based on standard deviation.
Maximum TBARS formation occurred at 48 h ($A_{532}$ values ~0.2 A for both batches) (Figure 3.1(C and D)). As expected, this is later than for peroxides since TBARS are secondary oxidation products formed from the breakdown of peroxides. In Chapter 2, the intra and inter-batch LA oxidation variability ($A_{532}$ values) were high at 20 h. The Figure 3.1 (C and D) shows that using LA emulsion, $A_{532}$ values for TBARS formation were consistent within each batch of LA at 20 h. There are no statistically significant inter-batch differences ($p>0.05$) in the $A_{532}$ values for TBARS formation at this time. There are some inter-batch differences in the $A_{532}$ values for TBARS formation after 48 h (maximum TBARS formation), which was due to the unavailability of peroxides for consistent TBARS formation for such a long time. However, monitoring TBARS after 48 h was not important for method optimisation as a rapid test (i.e. completed before 48 h) was desired. Contrary to Chapter 2, oxidation of LA in an emulsion is much more consistent for TBARS measurement in terms of intra- and inter-batch variability.

Having established good precision for the control system, it was also necessary to establish precision of the assays in the presence of an antioxidant. This is because it has been found, particularly for TBARS (Buenger et al., 2006), that lower precision can occur when an antioxidant is present in the system. Furthermore, in Chapter 2, it was found that precision can vary depending on the concentration of antioxidant for both FTC and TBARS. Therefore in this work, three concentrations of Trolox were tested: 25, 250 and 400 µM. Figure 3.1(A-D) shows that the intra-batch precision for $A_{500}$ and $A_{532}$ values in presence of all of the concentrations of Trolox were consistently high (i.e., low standard deviation). For batch to batch consistency,
emulsions with 250 and 400 µM Trolox showed good reproducibility over 96 h whereas, the emulsion with 25 µM Trolox did not show the consistency after 48 h for both FTC and TBARS assay. 25 µM Trolox is much less concentrated compared to 250 and 400 µM and hence might not be present sufficiently after 48 h and so might show inconsistency. Furthermore, as the variability occurs after the maximum amount of peroxides have formed (24 h), it is possible that when peroxides breakdown, they do so unpredictably in terms of how much TBARS are generated, and for this, TBARS formation might be variable as discussed in Chapter 2.

Although the oxidation of LA emulsion showed intra and inter- batch consistency in the absence or presence of Trolox at 24 h (for maximum peroxides) and 48 h (for maximum TBARS), these times of oxidation are too long to be of use in antioxidant screening studies. Likewise, it is more convenient in a screening application to measure antioxidant activity at the same oxidation time, rather than two different times for two different tests. Therefore, as a starting point for further studies to reduce oxidation time (below), 20 h was chosen as the common time at which to measure peroxides and TBARS. As can be seen from Figure 3.1, 20 h gives good reproducibility, both in the control and with Trolox at all concentrations tested.
3.4.2 Method optimisation

A total of 9 emulsions (mean droplet size, 110 ± 2 nm and mean PDI 0.3 ± 0.03) were prepared using the above procedure. With the emulsion, validation factors vis. order of antioxidant activity of several antioxidants were determined at 20 h for 37 °C. The concentration of antioxidant was 250 µM. The determined order of antioxidant activity was used as a common factor for the whole optimisation process in order to monitor oxidation at accelerated conditions. The accelerated conditions such as temperature, catalyst, or oxygen initiator used to speed up the oxidation might change the mechanism of oxidation from that at 37 °C and hence can change the order of antioxidant activity. Frankel (Frankel, 2014) described that ascorbic acid was less effective than ascorbyl palmitate at 45 °C in soybean oil while it becomes more effective than ascorbyl palmitate and even than BHA and BHT at 98 °C. Consequently order of antioxidant activity was needed as a validation factor for justification of oxidation system at accelerated conditions compared to that at 37 °C. On the basis of the validation process and using various accelerated conditions, the 20 h oxidation time was shortened in order to obtain a rapid method for the measurement of antioxidant activity.
3.4.3 Oxidation stage at 37 °C

3.4.3.1 Optimising the concentration of antioxidant

Trolox, ascorbic acid, gallic acid, and quercetin as antioxidant standards were investigated to determine the order of antioxidant activity. Trolox was chosen as a reference as it was used during characterisation of the emulsion in section 3.4.1 and also it is a powerful antioxidant and water soluble analogue of α-tocopherol. Ascorbic acid, gallic acid and quercetin were chosen as they are commonly used in antioxidant activity studies as references (Nenadis, Lazaridou, & Tsimidou, 2007; Yen & Hsieh, 1998) and also commonly available in nature. Additionally, the antioxidants cover a range of polarities. Two concentrations (i.e., 25 and 250 µM) of the antioxidants were investigated. From these, a suitable concentration was selected to establish the order of antioxidant activity.

Figure 3.2 (A and B) shows the time course oxidation of LA at 0, 5, 10 and 20 h in absence or presence of 25 µM antioxidants. Of the oxidation times, 20 h was used as it was the starting point of optimisation of the method. At 25 µM antioxidants and 20 h oxidation, Trolox and quercetin showed antioxidant activity in both FTC and TBARS assays. Gallic acid and ascorbic acid showed little or no antioxidant activity in comparison with the control. However, at 0 h, they were prooxidant, which is defined as causing or accelerating oxidation. Thus half of the antioxidants tested were not effective at this concentration. For an effective order of antioxidant activity
a majority of the used antioxidants should show effectiveness. Therefore 25 µM was not a useful concentration for optimisation of the method.
Figure 3.2: Absorbance values (n=3) for (A) peroxides and (B) TBARS at 25 µM, for (C) peroxides and (D) TBARS at 250 µM antioxidants (Oxidation at 37 °C). Error bars are based on standard deviation.
On the other hand, 250 µM of all tested antioxidants at 20 h showed appreciable antioxidant activity in FTC (Figure 3.2 C) and 3 antioxidants in TBARS assay (3.2 D) compared to control. Only ascorbic acid showed no antioxidant activity in TBARS assay. Thus 250 µM was an effective concentration compared to 25 µM and hence was chosen to optimise the order of antioxidant activity. It can be noted that Trolox has been reported to be more active than ascorbic acid (Yen & Hsieh, 1998) in LA emulsion while quercetin has been reported be more active than gallic acid although the emulsion was in Camelia oil (Zhu, Long, Zhou, Prenzler, & Zhong, 2013).

In Figure 3.2 D, for TBARS, at the same oxidation time, Trolox, quercetin and gallic acid showed same trend of antioxidant activity as peroxides (FTC assay), i.e., Trolox > quercetin > gallic acid > ascorbic acid. Thus the above order of antioxidant activity was consistent for both peroxides-FTC and TBARS assays for oxidation at 37 °C and will be used to validate the results obtained below under conditions of accelerated oxidation.
3.4.4 Oxidation at accelerated conditions

3.4.4.1 Effect of temperature

Temperature is one of the parameters to accelerate the oxidation reaction. As a 10 °C increase in temperature can double the oxidation rate (Johnson & Decker, 2015), this amount of increase in temperature can halve the oxidation time in an assay. Therefore 20 h oxidation can be reduced to 10 h or possibly 5 h by gradually raising temperature about 10 °C from 37 °C. Thus 50 and 60 °C were systematically trialled to find an optimum oxidation temperature. In Figure 3.3 (A-D), oxidation at these accelerated temperatures was monitored at 5 and 10 h; 20 h was not monitored because the Figures 3.2 (C-D) showed oxidation at 10 and 20 h having the same order of antioxidant activity.

For peroxides, in Figure 3.3 A, the $A_{500}$ values of the control increased at 50 °C for 5 and 10 h increased compared to those at 37 °C (Figure 3.2 C). In Figure 3.3 C, the above values increased only at 60 °C for 5 h compared to those at 37 °C (Figure 3.2 C). For TBARS, in Figure 3.3B, only the $A_{532}$ values of the control at 50 °C for 5 h increased compared to those at 37 °C (Figure 3.2 D). The results with 50 °C and 60 °C revealed that only at 50 °C for 5 h was the oxidation rate increased for the formation of both peroxides and TBARS compared to that for 37 °C at 5 h. Thus oxidation at 50 °C could be one of the effective parameters for making a faster antioxidant assay.
Figure 3.3: Absorbance values (n=3) for (A) peroxides and (B) TBARS at 50 °C, for (C) peroxides and (D) TBARS at 60 °C and 250 µM antioxidants. Error bars are based on standard deviation.
Validation of temperature effect by order of antioxidant activity at 37 °C

Figure 3.3 A demonstrates that for peroxides, oxidation for 5 h and 10 h at 50 °C showed the same order of antioxidant activity as being Trolox > quercetin > gallic acid ≈ ascorbic acid. Similarly, in Figure 3.3 B, for TBARS, the same order of antioxidant activity was observed for 5 h and 10 h at 50 °C. This same order for both peroxides and TBARS at 50 °C and 37 °C demonstrates the applied accelerated temperature provided a similar oxidation as at 37 °C.

On the other hand, Figure 3.3 C shows that at 60 °C, the time course of oxidation reactions and the order of antioxidant activity were changed for both for 5 and 10 h. The order of antioxidant activity was not consistent with that at 37 °C for 20 h, demonstrating oxidations at 60 °C for 5 and 10 h were not valid in developing a faster assay. Similar results were found with 60 °C in TBARS assay (Figure 3.3 D) and even a more severe change in time course of oxidation and order of antioxidant activity could be observed at this assay compared to FTC assay.

Based on the effect of temperature in accelerating oxidation, it is evident that the order of antioxidant activity and the time course of oxidation can only be preserved at 50 °C for 5 and 10 h compared to those at 37 °C for 20 h oxidation. Thus the 50 °C for 5 h oxidation can be reliably used in an assay for the measurement of antioxidant activity. Additionally, this temperature can reduce the assay time from 20 h to at most 10 h and possibly less. Five (5) h oxidation time, as per absorbance
values (Figure 3.3 (A and B)) and 3.2 (C and D), appears to give faster oxidation and good results compared to that at 37 °C.

In addition, antioxidant activity (% inhibition) for both peroxides and TBARS was found to be higher for all antioxidants at 50 °C for 5 h compared to that for 10 h. For peroxides, the percentage (%) inhibition by Trolox, ascorbic acid, gallic acid and quercetin were 83, 37, 43, and 72 % for 5 h, and 78, 32, 36, and 69% respectively for 10 h. For TBARS, antioxidant activity for 5 h was higher or closer than that at 10 h. The percentage inhibitions of TBARS for the above antioxidants were 76, 28, 42, and 68 % at 5 h, and were 81, 25, 36, and 71% respectively at 10 h.

3.4.4.2 Effect of catalysts

The Cu$^{2+}$ and Co$^{2+}$ ions at 37 °C were used during oxidation to determine if the catalysts could accelerate oxidation compared to 37 °C with no added metal ions. The Cu$^{2+}$ ion was chosen because of its strong prooxidant effect (Bakır et al., 2013) and it is also present in biological systems, while Co$^{2+}$ ion for being itself a powerful oxidising agent (Ragnarsson & Labuza, 1977).
Figure 3.4: Absorbance values (n=3) of (A) peroxides and (B) TBARS for 1 mM Cu^{2+}, (C) peroxides and (D) TBARS for 1 mM Co^{2+}, (E) peroxides and (F) TBARS for (0.04 µM Fe^{2+} + 0.01 µM H_2O_2) at 37 °C and 250 µM antioxidants. Error bars are based on standard deviation.
Figure 3.4 A shows that the $A_{500}$ values using Cu$^{2+}$ at both 5 and 10 h did not increase compared to those of the control values at 37 °C with added metal ions (Figure 3.2 C). On the other hand, in Figure 3.4 B, only the $A_{532}$ value using Cu$^{2+}$ for 5 h increased compared to that at 37 °C with no added metal ions (Figure 3.2 D). Similarly, Figure 3.4 C shows that with Co$^{2+}$, the $A_{500}$ values for both 5 and 10 h did not increase compared to those at 37 °C with no added metal ions (Figure 3.2 C). It is notable that at 0 h, the $A_{500}$ values for Co$^{2+}$ were high. On the other hand, in Figure 3.4 D, the $A_{532}$ values for using Co$^{2+}$ at 5 and at 10 h increased compared to those at 37 °C with no added metal ions (Figure 3.2 D). Similar to peroxides, the $A_{532}$ values for Co$^{2+}$ at 0 h were high.

Based on the effect in accelerating oxidation using catalysts Cu$^{2+}$ and Co$^{2+}$, it can be noted that speeding up the FTC assay is not possible due to the breakdown of peroxides compared to at 37 °C with no added metal ions. This breakdown of peroxides provides the increased formation of TBARS, which could be effective in speeding up the TBARS assay if the order of activity showed consistency with the validation parameter.

**Validation of catalysts effects by order of antioxidant activity at 37 °C**

Figure 3.4 (A and B) demonstrates that in the presence of Cu$^{2+}$ ion at 5 h, for peroxides, the antioxidants showed order of activity as quercetin > gallic acid > ascorbic acid > Trolox (no antioxidant activity), nearly the same as at 10 h,
quercetin $> \text{ascorbic acid} \approx \text{gallic acid} > \text{Trolox (no antioxidant activity)}$. For TBARS, at 5 h, the order was quercetin $> \text{ascorbic acid} \approx \text{gallic acid} > \text{Trolox (no antioxidant activity)}$, and at 10 h, quercetin $> \text{gallic acid} > \text{ascorbic acid} > \text{Trolox (no antioxidant activity)}$. None of these orders of activity were the same as those at $37^\circ C$ for 20 h and also revealed that the powerful antioxidant Trolox was less effective in presence of the Cu$^{2+}$ ion for both peroxides and TBARS (Figure 3.4 A and B). Even Trolox was pro-oxidant for peroxides (at 10 h) (Figure 3.4 A) and for TBARS (at 5 and 10 h) (Figure 3.4 B), because the $A_{532}$ values were not statistically different ($p > 0.05$) to those of $A_{532}$ values of the respective controls.

Similarly in presence of Co$^{2+}$ ion for 5 and 10 h, for peroxides (FTC assay) (Figure 3.4 C), the order of antioxidant activity and the oxidation reactions in course of time were severely changed compared to $37^\circ C$ for 20 h. Similar change was observed for TBARS (Figure 3.4 D), at 5 and 10 h compared to that at $37^\circ C$ for 20 h.

Based on the above results, for both peroxides and TBARS, oxidation including Cu$^{2+}$ or Co$^{2+}$ ions for 5 or 10 h is not valid for use in a faster antioxidant assay. Therefore the oxidation at $50^\circ C$ for 5 h with no added catalyst remains valid in providing accelerated rate of reaction with the same order of activity as established at $37^\circ C$ for 20 h.
### 3.4.4.3 Effect of oxygen initiator

In this study, an oxygen initiator, FeCl$_2$ + H$_2$O$_2$ at 37 °C for 5 or 10 h, was used to try to accelerate the oxidation with and without the antioxidants above. This oxygen initiator at 37 °C was also used by Dutta and Maharia (Dutta & Maharia, 2012) to accelerate oxidation in measuring antioxidant activity.

In Figure 3.4 E, the use of Fe$^{2+}$ + H$_2$O$_2$ conducted breakdown of peroxides for both 5 and 10 h compared to the 37 °C because the A$_{500}$ values were lower than those of 37 °C for 20 h oxidation (Figure 3.2 C). Similarly in Figure 3.4 F, oxidation with Fe$^{2+}$ + H$_2$O$_2$ did not speed up the formation of TBARS compared to at 37 °C for 5 and 10 h oxidation as the A$_{532}$ values were lower than those of 37 °C (Figure 3.2 D).

The use of Fe$^{2+}$ + H$_2$O$_2$ provided no acceleration in oxidation in either FTC or TBARS assay compared to 37 °C. Additionally, in the presence of the oxygen initiator, the oxidation was very complex in which ascorbic acid and gallic acid showed prooxidant activity at both 5 and 10 h compared to their control ($p>$0.05) (Figure 3.4 E and F).
Validation of oxygen initiator effects by order of antioxidant activity at 37 °C

In the presence of Fe^{2+} + H_{2}O_{2} for 5 h at 37 °C, for peroxides, the order of antioxidant activity was quercetin \approx Trolox > ascorbic acid (no antioxidant activity) \approx gallic acid (no antioxidant activity); the same order was observed for 10 h oxidation (Figure 3.4 E). For TBARS, at 5 h, the order was the same as for peroxides, Trolox \approx quercetin > gallic acid (no antioxidant activity) \approx ascorbic acid (no antioxidant activity), and essentially the same for 10 h (Figure 3.4 F). Similar to Cu^{2+} and Co^{2+} ions, the above orders of activity were not consistent with those at 37 °C for 20 h although the order for TBARS at 5 h showed some similarity, but this order of activity could reasonably be excluded due to high abnormal prooxidant properties for both gallic and ascorbic acids.

Based on these overall effects, some accelerated oxidation parameters – the increase of temperature to 60°C, the use of Cu^{2+} and Co^{2+} catalysts, and the use of an oxygen initiator, can be excluded for optimising faster FTC and TBARS assays because these parameters cannot provide the oxidation and order of antioxidant activity similar to that established at 37 °C for 20 h. On the other hand, order of antioxidant activity and time course of oxidation reactions are preserved at both 5 h and 10 h for 50 °C. Thus 5 h for 50 °C was exclusively selected as the valid accelerated oxidation time.
3.5 Method performance

Having established the above accelerated conditions, further performance evaluation was required to measure activity for other antioxidants - especially those that are commonly available in plant extracts. Such antioxidants were chosen so that the developed method could be applied to measure antioxidant activity of plant extracts. Trolox was used as a reference, and (+)-catechin, (-)-epicatechin, caffeic acid and α-tocopherol were trialled as representative of plant extract antioxidants to measure activity and test the method performance. Catechin and epicatechin can be sourced from tea plants (Rice-evans, Miller, Bolwell, Bramley, & Pridham, 1995) or other plants (Tsanova-Savova, Ribarova, & Gerova, 2005) whereas caffeic acid and α-tocopherol are common antioxidants present in a variety of plants such as coffee beans, olives and other plants (Saini & Keum, 2016; Son & Lewis, 2002).

With this new group of antioxidants, the accelerated oxidation conditions at 50 °C for both peroxides and TBARS (Figure 3.5, C and D) were replicated using the same parameters used in the optimisation process. The order of antioxidant activity of these oxidations was compared to that at 37 °C for 20 h for both peroxides and TBARS (Figure 3.5 A and B). A consistent order of antioxidant activity was found, demonstrating the method can show reliable performance for measuring antioxidant activity with different antioxidants.
Figure 3.5: Absorbance values (n=3) for (A) peroxides and (B) TBARS at 37 °C, for (C) peroxides and (D) TBARS at 50 °C and 250 µM antioxidants. Error bars are based on standard deviation.

Using these new oxidation conditions allowed the determination of antioxidant activity against both peroxides and TBARS. Even the antioxidant activity was the same or close to that at 37 °C for 20 h oxidation (Table 3.1). Therefore the method can be used reliably for high throughput measurement of antioxidant activity of new compounds or plant extracts.
Table 3.1: Antioxidant activity (% inhibition) of 250 µM Trolox, ascorbic acid, gallic acid, quercetin, (+)-catechin hydrate, (-)-epicatechin, caffeic acid and α-Tocopherol at 20 h + 37 °C and 5 h + 50 °C.

<table>
<thead>
<tr>
<th>Antioxidant 250 µM</th>
<th>FTC 20 h + 37 °C Antioxidant activity (%)</th>
<th>5 h + 50 °C Antioxidant activity (%)</th>
<th>TBARS 20 h + 37 °C Antioxidant activity (%)</th>
<th>5 h + 50 °C Antioxidant activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trolox</td>
<td>86±0</td>
<td>83±1</td>
<td>89±3</td>
<td>76±9</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>25±2</td>
<td>37±8</td>
<td>-5±10</td>
<td>28±5</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>29±2</td>
<td>43±8</td>
<td>32±3</td>
<td>42±4</td>
</tr>
<tr>
<td>Quercetin</td>
<td>80±0</td>
<td>72±0</td>
<td>81±1</td>
<td>68±2</td>
</tr>
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<td>Trolox (+)-Catechin hydrate</td>
<td>90±1</td>
<td>76±4</td>
<td>78±14</td>
<td>53±12</td>
</tr>
<tr>
<td>(-)-Epicatechin</td>
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<td>68±3</td>
<td>78±22</td>
<td>58±2</td>
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<td>α-Tocopherol</td>
<td>87±1</td>
<td>86±1</td>
<td>94±5</td>
<td>89±6</td>
</tr>
</tbody>
</table>

3.6 Conclusions

Various ways of accelerating oxidation including increase of temperature, use of catalysts and an oxygen initiator have been adapted in the oxidation stage of FTC and TBARS assay in order to speed up the method. An accelerated method is important for use of FTC and TBARS assay in high throughput screening of antioxidant activity for plant extracts. In this Chapter, the acceleration of oxidation generated a number of complexities for both oxidation and the behaviour of the antioxidants used. These complexities include: some of the antioxidants behaving as a pro-oxidant at some oxidation times, or, acting as powerful antioxidants depending on the accelerated conditions. Therefore the activity was changeable compared to the oxidation at 37 °C. As lipid oxidation occurs in human physiology at 37 °C, the antioxidant activity measured at that temperature will provide comparable activity to
the physiological system. Preserving these physiological conditions for determining the antioxidant activity of samples such as plant extracts is important for relating activity to human physiology when the extract is taken through food. Therefore, while optimising the method, a comparable oxidation at 37 °C was preserved by the increase of temperature to 50 °C; this temperature, provided consistent oxidation in only 5 h. Thus the oxidation time has consistently been shortened from 24 h (maximum peroxides formation) and 48 h (maximum TBARS formation) and 20 h (both peroxides and TBARS) to 5 h. Therefore, this new method is rapid, robust and the conditions used are comparable to those at 37 °C. The method was applied to use a range of standard antioxidants, which are commonly present in plant extracts. This method should have wide application to real plant samples for screening antioxidant activity. With this method, a follow up study was conducted examining the antioxidant properties of Australian native plants used by Indigenous peoples (e.g., Wiradjuri people) for medicinal purposes, as described in the following Chapter.
CHAPTER 4. SCREENING ANTIOXIDANT ACTIVITY OF SELECTED AUSTRALIAN NATIVE PLANTS AND IDENTIFICATION OF ACTIVE COMPOUNDS.

4.1 Introduction

In Chapter 3, a rapid and robust FTC and TBARS method to screen antioxidant activity was developed. The method was validated against a range of antioxidants of different structures, polarity, and chemical classes. The method was able to differentiate antioxidant activity among these various compounds. This was important because it allowed ranking of antioxidants, which could be extended to a screening study. The ability of ranking of antioxidants by the method could allow some compounds, fractions, or extracts of plants to be selected for further studies. In order to demonstrate the applicability of the method, a study was conducted in this Chapter 4 on extracts from Australian native plants that have been used by Indigenous people for traditional medicines. In addition, the free radical scavenging assay, ABTS and total phenolic content measuring assay FC, discussed in Chapter 1, have been used to compare the results with FTC and TBARS assays.

The medicinal plants have numerous biologically active compounds. Hence the use of these plants has been substantially increased for either as traditional medicines or
dietary supplements (Sponchiado et al., 2016). Thus the issue is important for better understanding of “diet and health” for human. Traditional medicinal plants had also long been used by Indigenous people in different countries for either food or medicine (Duduk Krishnaiah, Rosalam Sarbatly, & Rajesh Nithyanandam, 2011). In Chapter 1, it has been discussed that Australia is a country with a great diversity of plant species. Australian Indigenous people had an immense knowledge about the diverse of the plants and they used these plants as either an agent of medicine or a source material of food (Simpson et al., 2013).

Of Australian Indigenous communities, Wiradjuri community is the largest group of Indigenous people in New South Wales (NSW). Wiradjuri people had a diverse field of various plants. Hence they used numerous plants for medicinal purposes (Williams & Sides, 2008). However, there is very little information about the bioactive properties of these plants and even there is no refereed publication with these plants.

Wiradjuri people used different species of Acacia, Eucalyptus and Exocarpos. Of the plants, Acacia implexa, Eucalyptus rossii, and Exocarpos cupressiformis are readily available near Wagga Wagga and hence were chosen for ease of sampling for this study. Wiradjuri community and also other groups (Packer et al., 2012) used the bark of Acacia implexa for skin disease and sores complaints, Eucalyptus rossii alternatively Eucalyptus racemosa for diarrhoea and dysentery and sap of Exocarpos cupressiformis for snake bite. In addition, fruits of Acacia implexa and Exocarpos
*cupressiformis* were eaten as foods. There are very little reports on the phytochemistry for the species. Some of phytochemical components including catechins, melacacidin and dihydroflavonol have been reported in *Acacia implexa* (Tindale & Roux, 1969). Some polyphenolic compounds including kaempferol-3-rhamnabiosides and quercetin-3-rhamnabiosides have been explored in *Exocarpos cupressiformis* (Cooke & Haynes, 1960). There is very little information on the phytochemistry of *Eucalyptus rossii*. However, there is one study by Tucker et al. (Tucker et al., 2010), in which 2,5-dihydroxy-7-methoxy-6,8-dimethylflavanone and 2,5,7-trihydroxy-6,8-dimethylflavanone have been identified from *Eucalyptus rossii*. The phytochemistry of some of the other species of *Acacia* (Seigler, 2003) and *Eucalyptus* (Vuong et al., 2015) genuses has been reported. As the three species or their parent genuses have medicinal value, they might have antioxidant properties. To date, there is no report with the species for antioxidant activity and even for other bioactive properties.

The objective of this study was to apply the developed FTC and TBARS assay (discussed in Chapter 3) to screen antioxidant activity of bark or leaves of the species and identify some of the potentially bioactive compounds in the plant materials. In addition, Folin-Ciocalteu test and ABTS assays were also used to screen the plant materials and to compare the antioxidant activity with those of FTC and TBARS assay.
4.2 Materials and methods

4.2.1 Chemicals and Reagents

The same chemicals and reagents purchased for LA emulsion preparation, FTC and TBARS assays described in the Chapter 3 were used. In addition, dichloromethane (DCM) (≥99.5%) and anhydrous sodium sulfate (≥95%) were obtained from Fisher Scientific (UK), ethyl acetate (EtOAc) (99.8%) from Sharlau, S.L (Spain), Folin-Ciocalteu reagent (≥97%), caffeine (≥98%), formic acid (~98%) and ABTS (≥98%) from Sigma-Aldrich (USA), anhydrous sodium carbonate (99.5%) from Biolab (Australia), potassium persulfate (99%) from Ajax FineChem (Australia) and acetonitrile (≥99.9%) from VWR International, France.

4.2.2 Plant species and materials

Australian native plants, *Acacia implexa* (bark and leaves) (AIB and AIL), *Eucalyptus rossii* (leaves) (EUR) and *Exocarpos cupressiformis* (leaves and stems) (EXOC) were chosen for screening antioxidant activity. Before collecting the plant materials, a scientific licence (SL 101521, dated 02 April 2015) was approved from the office of Environment & Heritage, NSW National Parks, & Wildlife Service. The plant materials of AI was collected from the Rocky Hill, Wagga, Wagga, NSW, EUR from Livingstone National park (state forest), Big Springs, NSW, and EXOC from near the Blowering dam access road, NSW. The three places are located within Wiradjuri country. Samples of the plant species were identified, vouched (AI-NSW
4.2.3 Preparation and fractionation of plant extracts

An amount of 2 kg AIB, 1 kg AIL, 2 kg EUR and 3 kg EXOC was collected. The plant materials were separated from foreign materials and air-dried at ambient temperature in the fume cupboard with fan running until constant weigh was attained for each species. The plant materials were then cut into smaller pieces and ground into fine power using blender (Breville, Australia). An amount of 1000 g of ground AIB was suspended in 1700 mL 70% (v/v) aqueous ethanol or a 500 g each of AIL, EUR and EXOC in 1600 mL of same solvent in conical flask, covered by aluminium foil and shaken for 15 h in a shaker incubator at 150 rpm at room temperature. The plant materials were centrifuged (Eppendorf centrifuge 5810) at 4000 rpm for 20 min using 400 mL wide-mouth plastic bottle (Eppendorf, Germany). The residue was extracted twice. For each extraction, the residue of AIB was suspended in 1000 mL and the residue of EUR or EXOC in 600 mL 70% (v/v) aqueous ethanol. Three aqueous-ethanolic extracts were combined together and dried by removing solvent using rotary evaporation (Buchi) under vacuum at 37 °C. Then the aqueous residue was freeze dried (76 h) using Alpha 2-4 LD plus freeze dryer (John Morris Scientific, Australia) to afford the crude extracts.
A 50 g of the crude extract was suspended in 200 mL water, placed in a separatory funnel, and successively partitioned with n-hexane (3 x 100 mL), dichloromethane (DCM) (3 x 100 mL) and ethyl acetate (EtOAc) (3 x 100 mL). The combined similar solvent filtrates were first dried by adding pre-heated (2 g) anhydrous sodium sulfate and then by rotary evaporator under vacuum at 37 °C to afford the n-hexane, DCM and EtOAc lipid fractions. After successively partitioning the above fractions of crude extract, the aqueous layer was discarded through filtration or centrifuge and the residue was extracted with absolute ethanol (EtOH) (3 x 100 mL). The solid EtOH fraction was obtained by removing solvent by rotary evaporator as above. For convenient, the crude extract and n-hexane, DCM, EtOAc and EtOH fractions were denoted as for *A. implexa*, AIB crude, AIB H, AIB DCM, AIB EtOH, for *A. implexa* leaves, AIL crude, AIL H, AIL DCM, AIL EtOH, for *E. rossii*, EUR crude, EUR H, EUR DCM, EUR EtOH and for *E. cupressiformis* EXOC crude, EXOC H, EXOC DCM and EXOC EtOH. The extraction yield was calculated from \[
\frac{\text{obtained extract or fractions after extraction or fraction}}{\text{total used fine plant particle or crude extract}} \times 100
\]. A simplified flow chart of the extraction and fractionation is outlined below:
Figure 4.1: A simplified flow chart of the extraction and fractionation of crude extracts and fractions (A.implexa, E.rossii, and E.cupressiformis), (s=solid, l=liquid, and s, l=solid suspended in liquid).
4.2.4 Determination of total phenolic contents

Total phenolic contents of crude extracts and fractions were determined using Folin-Ciocalteu test (FC) in accordance with the method of Qawasmeh et al. (Qawasmeh, Obied, Raman, & Wheatley, 2012). Briefly, an aliquot of 100 µL crude extract or fraction (dissolved in absolute EtOH, 1 mg/mL, w/v) was added to 10 mL volumetric flask containing 5 mL water. Five hundred (500) µL Folin-Ciocalteu reagent was added and after 1 minute, 1.5 mL 20% (w/v) aqueous sodium carbonate was added. The mixture was shaken well and the total volume was made up to 10 mL with water. The total contents were mixed thoroughly and incubated for 1 h at ambient temperature. The absorbance was read at 760 nm (A760 value) (UV-visible spectrophotometer, Agilent Technologies, Cary-4000). The measurement was conducted in triplicate. Results were expressed as milligrams of gallic acid equivalent per gram dry weight of extract or fraction, which were calculated from a standard calibration curve. The calibration curve was constructed in 70% (v/v) aqueous ethanol using 0.0, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mg/mL gallic acid (7 concentration points) vs. A760 values for a linear regression with r² 0.999.

4.2.5 Free radical scavenging 2,2'-azinobis (3-ethyl-benzothiazolline-6-sulfonic acid) (ABTS) assay

The percentage of scavenging activity of the crude extracts and fractions was determined following the method Qawasmeh et al. (Qawasmeh et al., 2012). Briefly,
a stock solution of ABTS radical (ABTS•+) was prepared adding aqueous ABTS solution (7.0 mM) and aqueous potassium persulfate (2.45 mM) in a stoppered schott bottle with wrapping by aluminium foil. The bottle was incubated in darkness overnight (15 h) at room temperature. The working solution of ABT•+ was prepared by diluting an aliquot (1mL) of (ABTS•+) stock solution in 37 mL water to a final absorbance of 0.720 ± 0.003 at 734 nm. A fifty (50) µL of crude extract or fraction (dissolved in absolute EtOH, 1 mg/mL, w/v) was added to the 3 mL working solution of ABT•+ in a 1cm plastic cuvette. The cuvette was covered, shaken well, and incubated at room temperature for 30 min. The percentage of scavenging of ABT•+ for the crude extracts and fractions were calculated at the measured absorbance at 734 nm (A_sample) (Agilent Technologies, Cary-4000) compared to that of control (A_control) in accordance with the formula:

\[
\text{\% scavenging} = 100 \times \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}}
\]

4.2.6 Developed lipid-based ferric thiocyanate (FTC) and thiobarbituric acid reactive substances (TBARS) assay

4.2.6.1 Linoleic acid (LA) nano-emulsion and oxidation

LA nano-emulsion and oxidation mixture were prepared following the developed FTC and TBARS assay discussed in Chapter 3. Briefly, a 2.5 mL homogenised (nano droplet size <250 nm and PDI <0.7), 0.02 M LA emulsion (pH 7), 0.5 mL (1 mg/mL) crude extract or fraction and 2 mL 0.2 M aqueous phosphate buffer solution (PBS) (pH 7) were mixed together in a 50 mL centrifuge tube. For control sample,
0.5 mL PBS and for positive control (as reference antioxidant), 0.5 mL Trolox (1 mg/mL) were used. After the mixture had been mixed thoroughly using vortex, it was oxidised at 50 °C for 5 h in a water bath. Two aliquots of each of 0.1 mL (100 µL) were taken from the oxidation mixture for the measurement of peroxides used by FTC or TBARS by TBARS assay as below:

4.2.6.2 Ferric thiocyanate (FTC) assay

Peroxides were measured following the FTC method discussed in Chapter 3. Briefly, an aqueous solution of ethanol (4.7 mL, 75%), aqueous ammonium thiocyanate (0.1 mL, 30%), the above oxidation sample (0.1 mL) and iron(II) chloride (0.1 mL, 0.02 M in 3.2% hydrochloric acid) were sequentially added in a 10 mL volumetric flask and the peroxides were determined at 500 nm against a blank containing all reagents except LA. The antioxidant activity (% inhibition of peroxides formation) was calculated using equation 1.

\[
\% \text{ inhibition of peroxides formation} = 100 \times \frac{A_{\text{control}}-A_{\text{sample}}}{A_{\text{control}}} \quad (1)
\]

4.2.6.3 Thiobarbituric acid reactive substances (TBARS) assay

TBARS were measured following the TBARS method discussed in Chapter 3. Briefly, an aliquot of 0.1 mL oxidation sample was added to 2 mL TBA-TCA solution (20 mM TBA in 15% TCA) in a 10 mL centrifuge tube and after 15 min
boiling in water bath, cooling and separating the aqueous supernatant, the absorbance of the supernatant was measured at 532 nm against a blank using all reagents except LA. The antioxidant activity (% inhibition of TBARS formation) was calculated using the above equation as for the FTC assay.

4.2.7 Ultra High Pressure Liquid Chromatography-Mass Spectrometry (UHPLC-MS) analysis

4.2.7.1 Crude extracts and fractions sample preparation:

A 1 mg/mL solution for each of crude extract (70% aqueous ethanol) and its DCM, EtOAc and EtOH residues were prepared in 50% aqueous (v/v) MeOH. The n-hexane residue was dissolved in absolute ethanol to prepare the same concentration of fraction solution. The samples were filtered through 0.22 µm syringe filter before injecting in the UHPLC-MS.

4.2.7.2 Ultra High Pressure Liquid Chromatography-quadrupole Time of Flight-Mass Spectrometry (UHPLC-qTOF-MS) analysis

The crude extracts and fractions of all species were analysed by a UHPLC-qTOF-MS (Agilent Technologies, USA) using a Poroshell 120 SB-C-18 column with 2.1 x 100 mm (width and length) and 2.7 µm (particle size) (Agilent Technologies, USA).
The UHPLC-qTOF-MS (Agilent Technologies, USA) was equipped with a quaternary pump (1260 series), a diode-array detector (DAD) (1200 series), a 6530 accurate mass analyser (Agilent Technologies, USA), and an autosampler (1200 series). Data acquisition of UHPLC-qTOF-MS was as following conditions: mass range (m/z): 100-1600, nozzle voltage (V): 1000, fragmentor voltage (V): 150, gas temperature 300 °C, gas flow: 10 L/min, nebulizer (psig): 45 and electron spray ionisation mode: negative. DAD was set at 280 and 380 nm and column compartment was kept at 40 °C. Injection volume was 2 µL. The flow rate was 0.6 mL/min and maximum column pressure was set at 550 bars. The 0.2% formic acid in water (solvent A) and 0.2% formic acid in acetonitrile (solvent B) were prepared.

The sample was run with the gradient below Figure 4.1:

Table 4.1: Gradient for UHPLC-qTOF analysis.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Solvent A (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>95</td>
</tr>
<tr>
<td>1</td>
<td>95</td>
</tr>
<tr>
<td>4</td>
<td>90</td>
</tr>
<tr>
<td>10</td>
<td>60</td>
</tr>
<tr>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>21</td>
<td>95</td>
</tr>
<tr>
<td>26</td>
<td>95</td>
</tr>
</tbody>
</table>

UHPLC-qTOF data was analysed using MassHunter workstation, Qualitative Analysis Software (version B. 07. 00, 2014) (Agilent Technologies, USA).
4.2.7.3 Quality control

A mixed standard consisting of gallic acid, caffeine, (+)- catechin, (-)- epicatechin, and quercetin was prepared at a final concentration of 0.3 mg/mL for each antioxidant in either 50% (v/v) aqueous methanol or in 50% (v/v) aqueous acetonitrile. Gallic acid, caffeine, (+)- catechin were dissolved in 50% (v/v) aqueous solution and (-)- epicatechin in 50% (v/v) aqueous acetonitrile and prepared a mixed solution (mixed standard A) at final concentration of 0.3 mg/mL. A methanolic solution (mixed standard B) of 0.3 mg/mL quercetin was prepared. Equal portion of mixed standard A and B were mixed to obtain the final mixed standard. This standard was used as quality control and run before every species of plant materials in UHPLC-qTOF-MS analyses.

4.3 Statistical analysis

All samples were prepared in triplicate (analytical replicates). Result presentation (mean ± SD) and statistical significant difference (one way ANOVA) analysis were conducted as discussed in Chapter 3.
4.4 Results and discussion

4.4.1 Extraction yield

Percentage yield of 70% aqueous ethanol crude extracts for *Acacia implexa* (A. *implexa*) was appreciably dependent on the parts of the plant. Table 4.2 shows that the yield of *A. implexa* leaves (28 %) were higher than bark (11%). The yield of the leaves of *E. rossii* was 30%, which was followed by the leaves of *E. cupressiformis* with 28%. The Table also shows that the yields of extractions were also dependent on various solvents due to the degree of solubility of the compounds present in the crude extracts and the fractions. The results illustrated that ethyl acetate (EtOAc) contributed to the high yield of extraction. Among the species, the yields of EtOAc fractions ranged from 3 to 30, EtOH from 0.8 to 34, DCM from 0.3 to 5 and *n*-hexane from 0.08 to 3%. As the crude extracts were in a 70% aqueous EtOH solution and hence gathered higher the polar compounds than non polar ones. In addition, fractionations were conducted from the crude extracts, and therefore the above trends of yields of the fractions based on polarity of solvents can be considered as consistent.
Table 4.2: Extraction yield and total phenolic contents of crude extracts and fractions of crude extracts. Different letters in the same column show significant difference at \( p < 0.05 \)

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Crude extracts and fractions, reference antioxidant Trolox</th>
<th>Extraction yield (% yield)</th>
<th>Total phenolic content (mg GAE/g dry extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. impplexa</em> bark (AIB)</td>
<td>Crude (AIB)</td>
<td>11</td>
<td>439.5 ± 19.7&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>AIB H&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.08</td>
<td>53.2 ± 2.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>AIB DCM&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.5</td>
<td>149.1 ± 2.1&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>AIB EtOAc&lt;sup&gt;*&lt;/sup&gt;</td>
<td>3</td>
<td>328.5 ± 11.8&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>AIB EtOH&lt;sup&gt;*&lt;/sup&gt;</td>
<td>34</td>
<td>408.8 ± 21.6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>A. impplexa</em> leaves (AIL)</td>
<td>Crude (AIL)</td>
<td>28</td>
<td>195.2 ± 10.1&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>AIL H</td>
<td>0.5</td>
<td>42.4 ± 2.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>AIL DCM</td>
<td>0.3</td>
<td>98.6 ± 5.0&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>AIL EtOAc</td>
<td>3</td>
<td>583.2 ± 32.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>AIL EtOH</td>
<td>1</td>
<td>118.7 ± 8.5&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>E. rossii</em> (EUR) (leaves)</td>
<td>Crude (EUR)</td>
<td>30</td>
<td>419.2 ± 15.7&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>EUR H</td>
<td>3</td>
<td>106.4 ± 6.2&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>EUR DCM</td>
<td>15</td>
<td>159.9 ± 4.4&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>EUR EtOAc</td>
<td>11</td>
<td>530.5 ± 30.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>EUR EtOH</td>
<td>0.8</td>
<td>439.7 ± 21.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>E. cupressiformis</em> (EXOC)</td>
<td>Crude (EXOC)</td>
<td>28</td>
<td>437.6 ± 15.5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>EXOC H</td>
<td>1</td>
<td>59.6 ± 4.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>EXOC DCM</td>
<td>5</td>
<td>185.8 ± 21.5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>EXOC EtOAc</td>
<td>30</td>
<td>413.8 ± 20.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>EXOC EtOH</td>
<td>3</td>
<td>417.5 ± 6.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>*H=</sup>n-hexane, DCM=Dichloromethane, EtOAc=Ethyl acetate, EtOH=Ethanol fractions, GAE=Gallic acid equivalents.

4.4.2 Determination of total phenolic contents (TPC)

Phenolic compounds are important plant secondary metabolites (Yasir, Sultana, Nigam, & Owusu-Apenten, 2016), which are responsible for antioxidant activity of plant extracts, and therefore before screening antioxidant activity of the extracts it is
important to estimate the total phenolic contents (TPC) in various plant extracts. Although the popularly used Folin-Ciocalteu method to measure TPC is not specific (Zhang, Chen, Wang, Wu, & Xu, 2010) and its results mostly correlate to the free radical scavenging assays such as ABTS or DPPH, the method was used to obtain preliminary assessment of the presence of phenolic compounds in the crude extracts and fractions.

Table 4.2 outlines that crude extracts of all species showed appreciable amounts of total phenolic contents ranging from 195.2 to 439.5 GAE/g dry extract. Of the crude extracts, A. implexa bark contained highest TPCs with 439.5 GAE/g dry extract, followed by E. cupressiformis 437.6, E. rossii 419.2 and A. implexa leaves 195.2 GAE/g dry extract. The highest TPC in A. implexa bark may be due to high content of tannin in bark (Tran, McRae, Lynch, & Palombo, 2010). In bark, the different simple phenolic acids such as caffeic acid, ferulic acid are generally condensed to flavonoids, tannin, and lignin in course of time. In addition, bark being more matured than other part -accumulates higher phenolic compounds than other younger parts such as leaves and stem of plants (Sultana, Anwar, & Przybylski, 2007; Wei, Zhou, Lin, Liao, & Chai, 2010) and hence the high yield of TPC in A. implexa bark was consistent with the above literature.

When the extracts were fractionated with n-hexane, DCM, EtOAc and EtOH, TPCs were varied depending on the solvent polarity. The TPCs of EtOAc fractions of the species ranged from 328.5 to 583.2 GAE/g dry extract, followed by EtOH from 118.7 to 439.7 GAE/g dry extract. The DCM fractions ranged from 98.6 to 185.8, followed by n-hexane fractions from 42.4 to 106.4 GAE/g dry extract. The results
demonstrate that EtOAc fractions possessed highest TPC while the least polar n-hexane had the lowest contents. These results of TPCs for EtOAc and n-hexane fractions were consistent with the report in one of species of Acacia (Tung, Wu, Kuo, & Chang, 2007) and in other medicinal plant (Chua, Tung, & Chang, 2008).

Based on the results, the crude extracts and polar fractions do have the high contents of total phenolic compounds whereas the non-polar fractions low contents. It can be noted that for the antioxidant activity of these crude extracts, polar and non-polar fractions may not be same in free radical scavenging assay or lipid-based assay. In order to investigate these, the plant materials were screened using free radical scavenging ABTS assay, and the developed rapid and robust lipid-based FTC and TBARS assay.
4.4.3 Screening antioxidant activity

4.4.3.1 2,2’-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) assay

Plant phenolics are highly effective as free radical scavengers and antioxidants (Tung et al., 2007). The crude extracts and the fractions of all the species were found to contain high amount of TPC discussed in section 4.4.2, and hence the free radical scavenging capability of the crude extracts and fractions were investigated by ATBS assay.

Figures 4.2(A-D) illustrate that all crude extracts and some of the fractions of all species show free radical scavenging activity as antioxidant. Crude extracts of all species showed high percentage of activity ranging from 81 to 100 %, which constituted a decreasing order of activity following as AIB (100%)= EUR (100) > EXOC (96) > AIL (81%). The order of activity revealed that crude extracts of *A. implexa* bark and *E. rossii* possessed high free radical scavenging activity, which was statistically similar (*p* >0.05) with Trolox. This result highly correlates to the total phenolic contents of the crude extracts of *A. implexa* bark and *E. rossii* (Table 4.2).
Figure 4.2: Percentage of scavenging activity for ATBS assay: A (A. implexa bark), B (A. implexa leaves), C (E. rossii leaves) and D (E. cupressiformis leaves). Different letters (a-f) in crude extract and fractions of same species show significant difference at \( p < 0.05 \). Different small Roman numbers (i-iii) in crude extracts of different species show significant difference at \( p < 0.05 \). Results are presented as mean ± standard deviation (n=3), and statistically significant difference was determined by one way ANOVA.

Among the fractions, EtOAc fractions showed the higher range of activity (100-84%). The order of activity followed as EUR (100) > AIB (99) = AIL (99) > EXOC (84%). This result highly correlates to the TPCs of the EtOAc fractions (Table 4.2).
Using ABTS assay, EtOAc fractions showed high TPC and antioxidant activity in few species of *Acacia* (Barapatre et al., 2016; Tung, Wu, Huang, Kuo, & Chang, 2009). High antioxidant activity and TPC were also reported in one of the species of *Eucalyptus* (Miranda, Lima, Quilhó, Knapic, & Pereira, 2016). Similar to EtOAc fractions of tested plant materials, high scavenging activity was found with EtOH fractions ranging 100-55% with the order of activity as EUR (100) > EXOC (98) > AIB (97) > AIL (55%). This range of activity was followed by DCM fractions 33-67% with an order of activity followed as EUR (67) > AIB (49) > EXOC (48) > AIL (33%). The *n-*hexane fraction showed the lowest activity ranging from 12-38% and order of activity was as follows: EUR (38) > AIL (16) > EXOC (15) > AIB (12%). Among all the crudes and fractions of all species, EtOAc fractions of AIB and EUR showed the highest antioxidant activity (100%), which were statistically equivalent (*p >* 0.05) to Trolox (100%). The high ABTS scavenging activity and TPCs of plant EtOAc fractions is consistent with the literature report (Ardestani & Yazdanparast, 2007). On the other hand, *n-*hexane fractions showed the lowest antioxidant activity (ranged from 12-38%) followed by DCM fractions (67-33%). These fractions consistently showed lowest TPCs among the tested fractions (Table 4.2) for all species. The lowest ABTS scavenging activity and low TPCs of the plant extracts in *n-*hexane were in conformity with the reports in either *n-*hexane or other non-polar fractions (Ardestani & Yazdanparast, 2007; Tiwari & Tripathi, 2007).

Using ABTS assay, for all species, *n-*hexane fractions showed least antioxidant activity followed by DCM. The lowest activity of *n-*hexane is in agreement with the report by Tachakittirungrod et al. (Tachakittirungrod, Okonogi, &
Chowwanapoonpohn, 2007). The low activity of \( n \)-hexane and DCM fractions can be attributed to the fact that being non-polar, the fractions may possess mostly lipophilic compounds; some of them may be other bioactive compounds rather than polyphenolics. These compounds could not respond to ABTS scavenging. Therefore, antioxidant activity of the plant extracts and fractions was conducted by using developed lipid-based FTC and TBARS assay.

4.4.3.2 Ferric thiocyanate (FTC) assay

Figure 4.3 (A, C, E and G) show that using FTC assay, the crude extracts of all species showed appreciable amount of antioxidant activity against peroxides ranging from 55 to 81% . The decreasing order of activity follows as: EUR (81) > EXOC (75) > AIL (56) > AIB (55%). The order of activity indicated that crude extract of \( E. \) rossii showed significantly \((p<0.05)\) higher activity in suppressing lipid oxidation than \( A.\)implexa even that of \( E.\)cupressiformis. The other species \( E.\) cupressiformis showed 75% antioxidant activity, which is also significantly \((p <0.05)\) higher than \( Acacia \) species. There is no statistically significant difference \((p>0.05)\) of the antioxidant activity in \( A.\)implexa bark and leaves revealing that both parts of the same \( Acacia \) species show similar antioxidant activity. Some of the fractions of the crude extracts (especially \( n \)-hexane and DCM fractions) showed higher antioxidant activity compared to the crude extracts. This is consistent with the work of Singh et al. (Singh, Singh, Kumar, & Arora, 2007b), who found that fractions were comparatively more effective antioxidant than that of crude extracts in one species of \( Acacia \). For \( A.\)implexa bark, \( n \)-hexane, DCM and EtOAc fractions (Figure 4.3A); for
A. implexa leaves, above fractions and EtOH fraction (Figure 4.3C); and for E. cupressiformis n-hexane fraction (Figure 4.3G) showed significantly higher ($p<0.05$) antioxidant activity than that of crude extracts. This may be attributed to the possibility of antagonistic effects of the compounds present in the complex mixture of crude extracts compared to fractions (Zin, Hamid, Osman, & Saari, 2006), which might provide net lower activity in crude extracts compared to the fractions.
Figure 4.3: Antioxidant activity (% inhibition) for FTC (peroxides) assay: A (A. implexa bark), C (A. implexa leaves), E (E. rossii leaves) and G (E. cupressiformis leaves), and for TBARS assay: B (A. implexa bark), D (A. implexa leaves), F (E. rossii leaves) and H (E. cupressiformis leaves). Different letters (a-d) in crude extract and fractions of same species show significant difference; different numbers (1-3) for FTC and different capital Roman numbers (I-III) in crude extracts of the different species show significant difference at \( p < 0.05 \). Results are presented as mean ± standard deviation (n=3), and statistically significant difference was determined by one way ANOVA.
Among the fractions, \( n \)-hexane showed the higher range of activity (89-80\%, order AIB (89) > EUR (81) > EXOC (83) > AIL (80\%)). Similar high antioxidant activity was found with DCM fractions ranging 87-77\% with order of activity as AIB (87) > AIL (79) = EUR (79) > EXOC (77\%). The non-polar or organic fraction of \( n \)-hexane and DCM fractions showed the highest antioxidant activity although they did not correlate to the TPC (Table 4.2). This is consistent with the report by Atmani et al. (Atmani et al., 2011). This can be attributed to the fact that non polar phenolic or other compounds with diverse molecular structures can play role for high antioxidant activity in these factions rather than the highly polar phenolic compounds (Zin et al., 2006). The range of activity of \( n \)-hexane and DCM fractions was followed by EtOH fractions 81-61\% with an order of activity as EUR (81) > EXOC (74) > AIB (65) > AIL (61\%), and then EtOH 81-20\% with an order of activity as EUR (81) > AIL (73) > EXOC (70) > AIB (20\%). Among all the crudes and fractions of all species, \( n \)– hexane fraction of AIB showed the highest antioxidant activity (89\%) followed by DCM fraction of AIB (87\%), which are closer to the average activity (89.5\%) of Trolox (averaged in 4 sets of experiments). This result is consistent with the report of Sultana et al. (Sultana et al., 2007), who found high antioxidant activity of bark in one species of \textit{Acacia} using FTC assay in LA emulsion. Using FTC assay, the high activity of \( n \)-hexane fraction was also found in other plants (Mo et al., 2011).

Apart from common antioxidant activity among the all species, the extracts and fractions showed significantly different \((p<0.05)\) antioxidant activity compared to Trolox in their respective species. For \textit{A. implexa} bark (Figure 4.3A), and for \textit{A. implexa} leaves (Figure 4.3C), the activity of crudes and all fractions was
significantly different ($p<0.05$) from that of Trolox. Of these, $n$-hexane and DCM fractions showed closer activity to Trolox, illustrating that the fractions are likely as active as Trolox.

For *A. implexa* bark, the order of antioxidant activity of extracts and fractions can be noted as AIB H (89) > AIB DCM (87) > AIB EtOAc (65) > AIB Crude (55) > AIB EtOH (20%) (Figure 4.3 A). Similarly for *A. implexa* leaves, the order of activity is as follows: AIL H (80) > AIL DCM (79) > AIL EtOH (73) > AIL Crude (61%) (Figure 4.3C). The different orders of activity indicated that different compounds in the crude extracts were partitioned in different fractions for bark and leaves, and hence showed different activity.

In Figure 4.3 E, for *E. rossii*, crude extract and all fractions showed the statistically similar ($p>0.05$) activity ranging 79-81%, which was close to Trolox (85%). Thus crude extracts and all fractions might have similar antioxidant potent compounds as Trolox. On the other hand, in Figure 4.3 G, for *E. cupressiformis* activity of crude extracts and all fractions was significantly ($p<0.05$) different activity compared to Trolox. The crude extract and fractions of *E. cupressiformis* showed high antioxidant activity ranging from 70-83%, which reveals that this species was potent source of antioxidant compounds.

Based on the FTC results, fractions were generally more active antioxidants than crude extracts. All fractions of all species showed antioxidant activity. Of these, $n$-hexane and DCM fractions were more active antioxidants compared to other
fractions. These two fractions in all species provided consistently high antioxidant activity comparable to Trolox.

**4.4.3.3 Thiobarbituric acid reactive substances (TBARS) assay**

As evidenced in the previous section, the formation of peroxides from lipid oxidation was inhibited by the crude extracts and fractions of all tested plant species; the inhibition of the breakdown products of peroxides, *vis.* TBARS were investigated by the developed TBARS assay.

Figure 4.3 (B, D, F, and H) shows that crude extracts of all species showed modest antioxidant activity ranging from 50-13%. The crude extracts ranked in accordance with order of activity as EXOC (50) > AIB (33) > AIL (26) > EUR (13%). The order of activity indicates that crude extract of *E. cupressiformis* processed significantly high capability (*p*<0.05) in inhibiting TBARS formation compared to other species.

Of the fractions, AIB EtOAc, AIB EtOH, AIL EtOAc and EUR ETOAc fractions showed no antioxidant activity compared to other fractions. This might be because of the chemical composition of the mixture in the fractions. Due to different mechanism, some constituents of the fractions might prevent peroxides formation, but are not effective in preventing the formation of secondary oxidation products, TBARS.
Additionally, due to the complexity of the composition of compounds, the fractions might have antagonistic effect in TBARS assay system. According to Koolen et al. (Koolen, da Silva, Gozzo, de Souza, & de Souza, 2013), antioxidant activity in plant extracts may be effected by negative interaction between the compounds (polyphenolics), which may generate antagonistic effects. This might be what happened in the inhibition of TBARS formation. The net result thus might provide no antioxidant activity.

Other fractions showed antioxidant activity ranging from 12-67%. The n-hexane and DCM fractions of AIB and AIL possessed significantly ($p<0.05$) higher antioxidant activity compared to Trolox. The two fractions of EUR and EXOC showed statistically ($p>0.05$) similar activity with Trolox. Of all fractions, EXOC n-hexane fraction showed the highest activity (67%) followed by EXOC DCM fractions (52%). The high antioxidant activity of n-hexane and DCM fractions is consistent with the report by Mo et al. (Mo et al., 2011) and Chua et al. (Chua et al., 2008) who found high antioxidant activity of n-hexane or other non-polar fractions of plant extracts using TBARS assay. Due to the non polar characteristic of these n-hexane and DCM fractions, they might contain lipophilic compounds, which might contribute to the high antioxidant activity of these fractions. The results of these fractions were similar to FTC assay, where lipophilic fractions showed high activity.

In the case of individual species, for -A. implexa bark, the order of antioxidant activity of the extracts and fractions -is as follows: AIB H (42) = AIB DCM (42)> AIB Crude (33) > AIB EtOAc (0) = AIB EtOH (0%) (Figure 4.3 B). The order was different from A. implexa leaves, which is as follows: AIL H (28) > AIL Crude (26)
> AIL DCM (12) = AIL EtOH (12%) (Figure-4.3 D). These results demonstrate that the fractions of *A. implexa* bark and leaves responded differently against TBARS. Similar to FTC assay, except EtOAc fraction, crude extract and other fractions of *E. rossii* showed statistically similar (*p*>0.05) activity with Trolox (Figure-4.3F). Of the species, *E. cupressiformis* showed the higher activity against TBARS ranging from 19-67%, which follows an order of activity as: EXOC H (67) > Trolox (54) > EUR DCM (52) > EXOC crude (50) > EXOC EtOAc (43) > EXOC EtOH (19%) (Figure 4.3 H). This is first finding of antioxidant activity against TBARS for this *E. cupressiformis* species. The aforesaid activity is an evidence of *Exocarpos* species to be a potential source of bioactive compounds including new antioxidant.

Based on the overall screening antioxidant activity of the extracts and fractions, it can be mentioned that antioxidant activity of crude extracts and polar fractions could be effectively measured by the ABTS assay whereas this assay could not effectively determine the activity of the non polar *n*-hexane and DCM fractions. The activity of these fractions could be very effectively screened by the lipid-based FTC and TBARS assay. Therefore with the fractions, opposite results were obtained using free radical scavenging and lipid-based assay. In addition, some of the polar fractions (EtOAc and EtOH), which showed high activity in ABTS assay and even modest activity in FTC assay, showed no activity in TBARS assay.

Based on the overall results for screening antioxidant activity, lipophilic compounds present in the non polar fractions of the plants are more effective in lipid oxidation based FTC and TBARS assay rather than SET (single electron transfer) mechanism in ABTS assay. In Chapter 1, it has been discussed, for antioxidant screening, ABTS
is used first, and on the basis on the result lipid-based assays are used. The results of this Chapter 4 indicate that if only ABTS assay is used for screening antioxidant, the activity of non-polar fractions cannot be revealed.

The other finding is that using FTC and TBARS assays, the developed method was able to differentiate the antioxidant activity of different crude extracts and fractions. Bases on these, the highest activity of the tested crude extracts (E. rossii) and fractions (n-hexane) was found. Furthermore, the 5 h oxidation of the method provided rapid and convenient analysis of 20 testes samples (crude extracts and fractions), compared to the 20 h oxidation used in the literature method of McDonald et al. (McDonald et al., 2001).

Irrespective of the mechanisms of tested assays, all the extracts and fractions showed antioxidant activity in either lipid-based FTC and TBARS or ATBS assay. Hence the used plant extracts and fractions may contain some compound having bioactive properties. Some of the compounds were tentatively identified by LC-DAD--qTOF-MS.
4.4.4 Identification of compounds

Qualitative analysis of *Acacia implexa* bark and leaves, *Eucalyptus rossii* and *Exocarpos cupressiformis* leaves were conducted on the basis of the major peaks found at 280 (for general polyphenolic) and 380 nm (for flavonoids) using LC-ESI-qTOF-MS. The qTOF-MS provides accurate mass of peaks for m/z (mass to charge ratio). In TICs (total ion chromatograms), a number of peaks are observed. The major peaks of the 70% aqueous ethanol crude extracts of all species were analysed first and then for other fractions. The presence of the major peaks acquired at 280 and 380 nm were checked by using extracted ion chromatograms (EIC) at the m/z value obtained from TICs. In Appendices (1-9), the negative mode total ion chromatograms (TIC) (marked as A), and chromatograms detected at 280 nm (B), and 380 nm (C) for all crude extracts are shown. As non-polar *n*-hexane or dichloromethane (DCM) fractions showed very high antioxidant activity using the developed FTC and TBARS assays, the chromatograms with the highest antioxidant activity of these fractions are shown in Appendices (6-9), along with extracted ion chromatograms for the m/z value obtained from TICs. Tables (4.3-4.6) show the assignment of compounds on the basis of the chromatograms in the Appendices (1-9). Among the compounds, – gallic acid, (+)- catechin, and (-)- epicatechin – were identified using standards (see Appendix 10 for structures). The other twenty five (25) compounds were tentatively identified based on literature.

In the literature, there are very few reports on the phytochemistry of the three species. There is only one report *A. implexa* (Tindale & Roux, 1969). The
phytochemistry of some of the other species of the *Acacia* has been reviewed (Seigler, 2003). There is one report for *E. cupressiformis* (Cooke & Haynes, 1960) and one for *E. rossii* (Tucker et al., 2010). Phytochemistry of some of the other species of *Eucalyptus* has been reported (Vuong et al., 2015). In this thesis, tentative identification was based on the above reports.

**4.4.4.1 Acacia implexa bark**

In Table 4.3 and Appendix 2, Compound 1 was assigned as gallic acid. The retention time and m/z matched with those of gallic acid in the quality control sample (RT=0.713 and m/z=169.0149). Assignment of gallic acid was also considered on the basis of the literature. Gallic acid was reported in other *Acacia* species (Feregrino-Pérez et al., 2011; Rather & Mohammad, 2015) as well as in other plant species (Kumar et al., 2015). Compound 2 was assigned as pyrocatechuic acid (protocatechuric acid), which was also reported in *Acacia nilotica* species (Rather & Mohammad, 2015) and other plant species (Khadem & Marles, 2010; Kumar et al., 2015). Compounds 3-7 were assigned as gallocatechin/ Melacacudin, isomer of gallocatechin/ Melacacudin, rhamnetin and 3,3’-dimethyquercetin and isomer of rhamnetin respectively, which were characterised in either *A. implexa* (Tindale & Roux, 1969) or other *Acacia* species (Seigler, 2003). Compound 8 was characterized as hederagenin, which is a plant triterpenoid. As bark of *Acacia* species is rich in saponin (Tran et al., 2010), the compound 8 might be present in the *Acacia implexa*. Hederagenin was identified and characterised in a medicinal plant, conducted by Xin et al. (Xin, Yang, Zhong, Aisa, & Wang, 2009).
Compounds (1-4) were prominent in either EtOAc fraction or crude extract followed by EtOH fraction because of polar characteristics of the assigned compounds. The presence of the polar compounds in the above fractions and crude extracts might contribute to the modest antioxidant activity in lipid-based FTC (Figure 4.3 A) and high antioxidant activity in aqueous-based ABTS assay (Figure 4.2 A). In other lipid-based TBARS assay, the compounds (1-4) might contribute to modest antioxidant activity. Compounds (5-8), being less polar in nature, were prominent in n-hexane or DCM fractions and hence likely contributed to the high antioxidant activity in both FTC and TBARS assay (Figures 4.3 A-4.3 B). Compound 8 was shown in Appendix 6 for EIC chromatogram (DCM fraction).
Table 4.3: Tentative assignment of compounds in *Acacia implexa* bark (crude extract).

<table>
<thead>
<tr>
<th><em>Comp</em></th>
<th><em>RT</em></th>
<th><em>m/z</em> expt.</th>
<th><em>m/z</em> calc</th>
<th><em>MF</em></th>
<th><em>δ in ppm</em></th>
<th>Tentatively indentified compound</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.714</td>
<td>169.0146</td>
<td>169.0142</td>
<td>C_{7}H_{6}O_{5}</td>
<td>2.08</td>
<td>Gallic acid</td>
<td><em>ST</em></td>
</tr>
<tr>
<td>2</td>
<td>1.014</td>
<td>153.0192</td>
<td>153.093</td>
<td>C_{7}H_{6}O_{4}</td>
<td>0.86</td>
<td>Pyrocatechuric acid (2,3-dihydroxybenzoic acid)</td>
<td>(Khadem &amp; Marles, 2010)</td>
</tr>
<tr>
<td>3</td>
<td>1.147</td>
<td>305.0671</td>
<td>305.0667</td>
<td>C_{13}H_{14}O_{7}</td>
<td>0.08</td>
<td>Gallocatechin/Melacacidin</td>
<td>(Seigler, 2003; Tindale &amp; Roux, 1969)</td>
</tr>
<tr>
<td>4</td>
<td>2.629</td>
<td>305.0670</td>
<td>305.0667</td>
<td>C_{13}H_{14}O_{7}</td>
<td>1.06</td>
<td>Isomer of gallocatechin/melacacidin</td>
<td>(Seigler, 2003; Tindale &amp; Roux, 1969)</td>
</tr>
<tr>
<td>5</td>
<td>7.418</td>
<td>315.0512</td>
<td>315.0510</td>
<td>C_{16}H_{12}O_{7}</td>
<td>0.55</td>
<td>Rhamnetin</td>
<td>(Seigler, 2003)</td>
</tr>
<tr>
<td>6</td>
<td>8.117</td>
<td>329.0665</td>
<td>329.0667</td>
<td>C_{17}H_{14}O_{7}</td>
<td>0.53</td>
<td>3,3′-Di-methylquercetin</td>
<td>(Seigler, 2003)</td>
</tr>
<tr>
<td>7</td>
<td>9.524</td>
<td>315.0518</td>
<td>315.0510</td>
<td>C_{16}H_{12}O_{7}</td>
<td>2.45</td>
<td>Isomer of Rhamnetin</td>
<td>(Seigler, 2003)</td>
</tr>
<tr>
<td>8</td>
<td>12.980</td>
<td>471.3483</td>
<td>471.3480</td>
<td>C_{30}H_{48}O_{4}</td>
<td>0.67</td>
<td>Hederagenin (steroid)</td>
<td>(Xin et al., 2009)</td>
</tr>
</tbody>
</table>

4.4.4.2 *Acacia implexa* leaves

In Table 4.4 and Appendix 3, Compound (4) was also assigned as an isomer of gallicatechin. Compound (9) was assigned as procyanidin B2. Procyanidin dimers were found in *Acacia catechu* (Shen et al., 2006). Compounds (10-15) were tentatively assigned as (-)-epicatechin, quercetin diglucoside, epicatechin gallate, rutin, quercetin-3-galactoside/myricitrin and kaempferol, because these compounds were reported in either *A. implexa* (Tindale & Roux, 1969) or other *Acacia* species (Seigler, 2003). Of these compounds, the assignment of compound (10) as (-)-epicatechin was also supported by matching the RT and m/z with those of quality control sample (RT 5.068 min and m/z 289.0724).

Similar to *A. implexa* bark, compounds (4 and 9-12) were prominent in either EtOAc fraction or crude extract followed by EtOH fraction whereas compounds (13-15) were in *n*-hexane or DCM fractions. Antioxidant activity of crude extract, *n*-hexane, DCM fractions, EtOAc and EtOH of *A.implexa* leaves in Figure 4.3 (C and D) might be due to the presence of compounds (4 and 9-15). Compound 15 was shown in Appendix 7 for EIC chromatogram (*n*-hexane fraction).
Table 4.4: Tentative assignment of compounds in *Acacia implexa* leaves (crude extract).

<table>
<thead>
<tr>
<th><em>Comp</em></th>
<th><em>RT</em></th>
<th><em>m/z</em> expt</th>
<th><em>m/z</em> calc</th>
<th><em>MF</em></th>
<th>δ in ppm</th>
<th>Tentatively indentified compound</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>2.629</td>
<td>305.0669</td>
<td>305.0667</td>
<td>C\textsubscript{15}H\textsubscript{14}O\textsubscript{7}</td>
<td>0.73</td>
<td>Isomer of gallocatechin/melacacidin</td>
<td>(Seigler, 2003; Tindale &amp; Roux, 1969)</td>
</tr>
<tr>
<td>9</td>
<td>4.679</td>
<td>577.1349</td>
<td>577.1351</td>
<td>C\textsubscript{30}H\textsubscript{26}O\textsubscript{12}</td>
<td>0.43</td>
<td>Procyanidin B2</td>
<td>(Gouveia-Figueira &amp; Castilho, 2015)</td>
</tr>
<tr>
<td>10</td>
<td>5.087</td>
<td>289.0721</td>
<td>289.0718</td>
<td>C\textsubscript{15}H\textsubscript{14}O\textsubscript{6}</td>
<td>1.17</td>
<td>(-)- Epicatechin</td>
<td>(Seigler, 2003; Tindale &amp; Roux, 1969)</td>
</tr>
<tr>
<td>11</td>
<td>6.652</td>
<td>625.1397</td>
<td>625.1410</td>
<td>C\textsubscript{27}H\textsubscript{30}O\textsubscript{17}</td>
<td>2.11</td>
<td>Quercetin diglucoside</td>
<td>(Seigler, 2003)</td>
</tr>
<tr>
<td>12</td>
<td>7.093</td>
<td>441.0819</td>
<td>441.0827</td>
<td>C\textsubscript{22}H\textsubscript{18}O\textsubscript{10}</td>
<td>1.86</td>
<td>Epicatechin gallate</td>
<td>(Seigler, 2003; Tindale &amp; Roux, 1969)</td>
</tr>
<tr>
<td>13</td>
<td>7.360</td>
<td>609.1459</td>
<td>609.1461</td>
<td>C\textsubscript{27}H\textsubscript{36}O\textsubscript{16}</td>
<td>0.34</td>
<td>Rutin</td>
<td>(Seigler, 2003)</td>
</tr>
<tr>
<td>14</td>
<td>7.460</td>
<td>463.0887</td>
<td>463.0882</td>
<td>C\textsubscript{21}H\textsubscript{20}O\textsubscript{12}</td>
<td>1.08</td>
<td>Quercetin-3-galctoside/myricitrin</td>
<td>(Seigler, 2003)</td>
</tr>
<tr>
<td>15</td>
<td>9.175</td>
<td>285.0415</td>
<td>285.0405</td>
<td>C\textsubscript{15}H\textsubscript{10}O\textsubscript{6}</td>
<td>3.63</td>
<td>Kaempferol</td>
<td>(Seigler, 2003)</td>
</tr>
</tbody>
</table>

*Comp=Compound,* *RT=Retention time,* *m/z=mass to charge ratio,* *expt. =experimental,* *calc=calculated,* *MF=molecular formula,* and *δ in ppm = error (ppm),* *ST= standards.*
4.4.4.3 *Eucalyptus rossii* leaves

In table 4.5 and Appendix 4, compound (1) was also assigned as gallic acid, which was also found in *A. implexa* bark. Compound (16) was assigned as (+)-catechin by matching the very closer RT and m/z with those at quality control sample (RT=2.928 and m/z=289.0718). In addition, the assignment was supported by the review of – Vuong et al. (Vuong et al., 2015), in which the compound was reported to be present in the other species of *Eucalyptus*. Similarly, compounds (17-20, 22 and 23) were tentatively assigned as ellagic acid, cypellocarpin B, quercetin-3-glucoside, quercetin-3-rhamnoside (quercetin), 5-hydroxy-7,4´-dimethoxy flavone and sideroxylin on the basis of the report of Vuong et al (Vuong et al., 2015), where the compounds were reported to be found in the other species of *Eucalyptus*. Compound (21) was tentatively assigned as 3-methyl ellagic acid. Because ellagic acid (compound 17) was present in this species of *Eucalyptus* and hence 3-methyl derivative of ellagic acid might also be present. Furthermore, 3-methyl ellagic acid was identified and characterised in root bark in *Anisophyilea dichostyla* (Khallouki et al., 2007). Compound (24) was tentatively assigned as eucalyptin on the basis of the identification and characterisation in one of the species of *Eucalyptus* (Wollenweber & Kohorst, 1981).

In this species, all of the assigned compounds (1 and 16-22) except compounds (23 and 24) were prominent in either EtOAc fraction or crude extract followed by EtOH fraction. Compounds (23 and 24) were prominent in n-hexane or DCM fraction followed by other fractions. All compounds were commonly present in all fractions,
and contributed to similar antioxidant activity to each fraction in the FTC assay (Figure 4.3 E). The presence of very small number of less polar compounds in \( n \)-hexane and DCM fractions contributed to low activity in ABTS assay (Figure 4.2 C). Of the compounds, compound (24) sideroxylin was predominately present in crude extract and all fractions and hence contributed to a general activity to each fraction in both FTC and TBARS assays (Figures 4.3 E and F). Compound 24 was shown in appendix 8 for EIC chromatogram (\( n \)-hexane fraction).
Table 4.5: Tentative assignment of compounds in *Eucalyptus rossii* leaves (crude extract).

<table>
<thead>
<tr>
<th><em>Comp</em></th>
<th><em>RT</em></th>
<th><em>m/z</em> expt</th>
<th><em>m/z</em> calc</th>
<th><em>MF</em></th>
<th>δ in ppm</th>
<th>Tentatively identified compound</th>
<th>Reference</th>
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<td>0.730</td>
<td>169.0146</td>
<td>169.0142</td>
<td>C_7H_6O_5</td>
<td>2.08</td>
<td>Gallic acid</td>
<td><em>ST</em>, (Vuong et al., 2015)</td>
</tr>
<tr>
<td>16</td>
<td>2.961</td>
<td>289.0720</td>
<td>289.0718</td>
<td>C_{15}H_{14}O_6</td>
<td>0.82</td>
<td>(+)- Catechin</td>
<td><em>ST</em> (Vuong et al., 2015)</td>
</tr>
<tr>
<td>17</td>
<td>7.208</td>
<td>300.9994</td>
<td>300.9990</td>
<td>C_{14}H_{16}O_8</td>
<td>1.38</td>
<td>Ellagic acid</td>
<td>(Vuong et al., 2015)</td>
</tr>
<tr>
<td>18</td>
<td>7.250</td>
<td>537.1978</td>
<td>537.1978</td>
<td>C_{26}H_{34}O_{12}</td>
<td>0.09</td>
<td>Cypellocarpin B</td>
<td>(Vuong et al., 2015)</td>
</tr>
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<td>19</td>
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<td>463.0876</td>
<td>463.0882</td>
<td>C_{21}H_{20}O_{12}</td>
<td>0</td>
<td>Quercetin-3-glucoside</td>
<td>(Vuong et al., 2015)</td>
</tr>
<tr>
<td>20</td>
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<td>447.0920</td>
<td>447.0933</td>
<td>C_{21}H_{20}O_{11}</td>
<td>2.87</td>
<td>Quercetin-3-rhamnoside (Quercetrin)</td>
<td>(Vuong et al., 2015)</td>
</tr>
<tr>
<td>21</td>
<td>8.274</td>
<td>315.0150</td>
<td>315.0146</td>
<td>C_{15}H_{16}O_8</td>
<td>1.14</td>
<td>3-methyl ellagic acid</td>
<td>(Khallouki et al., 2007)</td>
</tr>
<tr>
<td>22</td>
<td>12.613</td>
<td>297.0772</td>
<td>297.0768</td>
<td>C_{17}H_{14}O_5</td>
<td>1.18</td>
<td>5-hydroxy-7,4´-dimethoxy flavone</td>
<td>(Vuong et al., 2015)</td>
</tr>
<tr>
<td>23</td>
<td>12.788</td>
<td>311.0927</td>
<td>311.0925</td>
<td>C_{19}H_{16}O_5</td>
<td>0.65</td>
<td>Sideroxylin</td>
<td>(Vuong et al., 2015)</td>
</tr>
<tr>
<td>24</td>
<td>13.612</td>
<td>325.1846</td>
<td>325.1081</td>
<td>C_{19}H_{18}O_5</td>
<td>0.11</td>
<td>Eucalyptin</td>
<td>(Wollenweber &amp; Kohorst, 1981)</td>
</tr>
</tbody>
</table>

*Comp=Compound, * RT=Retention time, *m/z=mass to charge ratio, *calc=calculated,
*MF=molecular formula, and *δ in ppm = error (ppm), *ST= standards.
4.4.4.4 *Exocarpos cupressiformis* leaves

In table 4.6 and Appendix 5, compounds (25, 26, 27 and 28) were tentatively assigned as kaempferol-3-rhamnobiioside, dihydrokaempferol-7-rhamnoside, kaempferol-7-rhamnoside and quercetin-3-rhamnobioside on the basis of the identification and characterisation of these compounds in *E. cupressiformis* studied by Cooke et al. (Cooke & Haynes, 1960). Compound (16) was assigned as (+)-catechin, which was also found in *E. rossii*. Compound 20 was tentatively assigned as quercetin-3-rhamnoside (quercetrin), because quercetin-3-rhamnobioside (compound 28) was present in this species of *Exocarpos* and hence a related compound might also be present. In addition, quercetin-3-rhamnoside (quercetrin) was identified and characterised in *E. rossii* (Vuong et al., 2015).

In this species, all compounds (16 and 25-28) were commonly present in crude and all fractions. These compounds are considered to be powerful antioxidants and hence contributed to substantial antioxidant activity in both FTC assay (Figure 4.3 G) and TBARS assay (Figure 4.3 H). Of the assigned compounds, (20, 26-28) are generally non-polar flavonoids and hence contributed high antioxidant activity in n-hexane and DCM fractions for both (FTC assay) (Figure 4.3 G) and TBARS assay (Figure 4.2 H). The compounds might not effective in scavenging ABSTS radical and hence opposite trend of activity for these fractions was found the ABTS assay (Figure 4.2 D). Compound 26 was shown in Appendix 9 for EIC chromatogram (n-hexane fraction).
Based on the overall results, some of the tentatively indentified compounds are general polyphenolic compounds including flavonoids, which are well-studied antioxidants (Roleira et al., 2015; Shahidi, Janitha, & Wanasundara, 1992) and commonly available in plant kingdom. Of the compounds, rhamnetin, rutin, kaempferol, quercetrin, sideroxylin are flavonoids, which are known antioxidants. These flavonoids are less-polar than other polyphenolic compounds and were found predominately in n-hexane and DCM fractions in some of the tested species. The high antioxidant activity of the non-polar fractions determined by the developed FTC and TBARS assay might be due to the presence of some of the above the plant derived antioxidants.
Table 4.6: Tentative assignment of compounds in *Exocarpos cupressiformis* leaves (crude extract).

<table>
<thead>
<tr>
<th><em>Comp</em></th>
<th><em>RT</em></th>
<th><em>m/z</em> expt</th>
<th><em>m/z</em> calc</th>
<th><em>MF</em></th>
<th><em>δ in ppm</em></th>
<th>Tentatively identified compound</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>2.752</td>
<td>577. 1352</td>
<td>577. 1563</td>
<td>C_{27}H_{36}O_{14}</td>
<td>0.16</td>
<td>Kaempferol-3-rhamnobioside</td>
<td>(Cooke &amp; Haynes, 1960)</td>
</tr>
<tr>
<td>16</td>
<td>2.936</td>
<td>289. 0722</td>
<td>289. 0707</td>
<td>C_{15}H_{12}O_{6}</td>
<td>0.06</td>
<td>(+)- Catechin</td>
<td><em>ST standard</em></td>
</tr>
<tr>
<td>26</td>
<td>7. 432</td>
<td>433.1137</td>
<td>433.1140</td>
<td>C_{21}H_{22}O_{10}</td>
<td>0.74</td>
<td>Dihydrokaempferol-7-rhamnoside</td>
<td>(Cooke &amp; Haynes, 1960)</td>
</tr>
<tr>
<td>20</td>
<td>7. 974</td>
<td>447. 0929</td>
<td>447. 0933</td>
<td>C_{21}H_{26}O_{11}</td>
<td>0.86</td>
<td>Quercetin-3-rhamnoside (Quercetrin)</td>
<td>(Vuong et al., 2015)</td>
</tr>
<tr>
<td>27</td>
<td>8. 523</td>
<td>431.0986</td>
<td>431.0984</td>
<td>C_{21}H_{26}O_{10}</td>
<td>0.53</td>
<td>Kaempferol-7-rhamnoside</td>
<td>(Cooke &amp; Haynes, 1960)</td>
</tr>
<tr>
<td>28</td>
<td>10. 488</td>
<td>593.1292</td>
<td>593.1512</td>
<td>C_{27}H_{36}O_{15}</td>
<td>0.15</td>
<td>Quercetin-3-rhamnobioside</td>
<td>(Cooke &amp; Haynes, 1960)</td>
</tr>
</tbody>
</table>

*Comp=Compound,* RT=Retention time, *m/z=mass to charge ratio, *calc=calculated, *MF=molecular formula, *δ in ppm= error (ppm), and *ST= standards.
4.5 Conclusions

The developed rapid and robust method discussed in Chapter 3 has successfully been applied to screen antioxidant activity in plant materials (bark or leaves) of *A. implexa, E. rossii*, and *E. cupressiformis*. Crude extracts and all fractions showed appreciable antioxidant activity in the FTC assay. Majority of crude extracts and other fractions showed substantial antioxidant activity in the TBARS assay. High antioxidant activity of the non-polar fractions (*n*-hexane and DCM) was observed with the developed method, which showed low activity in ABTS and even in Folin-Ciocalteu test for total phenol contents. High antioxidant activity of these fractions might be due to the presence of identified or tentatively assigned non-polar or less polar, potent antioxidant compounds.

In the course of applying this developed method to screen medicinal plant extracts, it has been found that the tested three species of plants have very potent antioxidant activity. There was variable antioxidant activity in different crude extracts and fractions and even in bark and leaves of same species. Of the species, crude extract of *E. rossii* showed the highest antioxidant activity against both peroxides and TBARS. Among the fractions, *n*-hexane fraction of *A. implexa* bark showed the highest antioxidant activity in FTC. Similarly *n*-hexane fraction of *E. cupressiformis* leaves showed the highest antioxidant activity in TBARS assay. Using UHPLC-qTOF-MS, separation and tentatively identification of compounds in the crude extracts and fractions of the species suggest that there are flavonoids, which are well studied antioxidants. However, more research is needed to obtain comprehensive
photochemical analysis of the three plant species so that novel antioxidants in these species can be found.
CHAPTER 5. CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

5.1 Conclusions

In this thesis, the lipid-based TBARS and FTC (peroxides) antioxidant method has been modified to be rapid and robust in a linoleic acid (LA) oxidation system. Of the two oxidation systems studied, the multi-phase system has been found to show variability in measuring TBARS and hence was excluded in optimising the method. The other version of the system, vis. LA emulsion, shows good intra-and inter-batch precision for FTC and TBARS assays in oxidation and antioxidant activity. Therefore, in this oxidation system, rate of reaction was increased in order to develop a high throughput screening of antioxidant activity. Once the method was optimised to be rapid and robust, the suitability of the method was tested for the screening of antioxidant activity of real samples such as traditional medicinal plant extracts. This thesis presents information on these areas as below:

5.1.1 Issues of variability in thiobarbituric acid reactive substances (TBARS) with multi-phase system

In Chapter 1 (Literature review), variability in measuring antioxidant activity using TBARS assay has been discussed. In Chapter 2, variability in results of TBARS
assay has been investigated in a multi-phase system used by McDonald et al. (McDonald et al., 2001). In this system, lipid substrate LA lay over the surface of the aqueous layer of catalyst (Cu\(^{2+}\)) and antioxidant solution (Trolox) making a multi-phase-air (oxidant), aqueous (catalyst/antioxidant) and lipid (substrate). In this version of assay, variability in substrate and TBARS was originated from the complex system. Oxidation in LA in this system showed low intra-batch precision for TBARS assay, which was due to the variability in mixing of reagents. Catalyst was present in this system at the beginning and exposure of LA to catalyst, combined with differences in amount of air mixing into the system resulted in inconsistent oxidation of LA. Oxidation of LA in this system also showed low inter-batch precision for TBARS assay. The variable and high content of preformed peroxides contributed to the inter-batch variability in LA oxidation. The result of peroxides (Chapter 2) showed that the amount of peroxides after 20 h oxidation was equal to pre-formed peroxides. This indicated that TBARS formation was mostly related to pre-formed peroxides. These pre-formed peroxides might erratically breakdown to variable amount of TBARS or some other secondary oxidation products. In addition, different concentrations of Trolox (250, 1000, 2500 and 5000 µM) also showed variability in TBARS assay.

Low intra-batch precision for TBARS assay due to LA oxidation has been minimised by optimising the order, in which catalyst was mixed last so that the time of exposure of LA to oxygen (air) and/or catalyst became limited. Despite this minimisation, there were difficulties to overcome inter batch variability in TBARS. Even the responsible pre-formed peroxides were removed from LA by Magnesol and
the \textit{de novo} oxidation of cleaned LA still showed the variability in TBARS and peroxides as well. This revealed that this literature method of TBARS assay was not suitable to be used for a reliable antioxidant assay. The investigation also provides information, if pre-formed peroxides are not measured, the results of antioxidant activity may vary from one experiment to the next. Having potential variability in results for the multi-phase system, another version of oxidation system \textit{vis.} LA emulsion was investigated in order to optimise a rapid and robust method (Chapter 3).

\textbf{5.1.2 Oxidation in linoleic acid (LA) emulsion for thiobarbituric acid reactive substances (TBARS) or ferric thiocyanate (FTC) (peroxides) assays}

The results of Chapter 3 indicated that in contrast to the multi-phase system, oxidation of LA in emulsion has resulted in good intra-and inter-batch precision for FTC and TBARS assays in oxidation and antioxidant activity. This is because of the fact that in the emulsion system, the concentration LA has been much diluted compared to multi-phase system. Therefore the pre-formed peroxides were negligible to show batch to batch variability in either peroxides or TBARS. Intra-batch variability for TBARS assay due to LA oxidation was minimised because of thoroughly mixing of LA in emulsion and consistent contact with oxygen. In addition, good intra and inter-batch precision in either peroxides or TBARS due to oxidation of LA in the presence of different concentrations (25, 250 and 400 µM) was found.
Having established that antioxidant activity against both peroxides and TBARS in LA emulsion was consistent, the system was attempted to be made faster. In this context, the oxidation system was accelerated using various conditions although a number of reports (Frankel, 2014; Ragnarsson & Labuza, 1977) highlighted that accelerated conditions can alter the oxidation and antioxidant mechanism compared to that at physiological temperature (37 °C). Therefore in Chapter 3, accelerated conditions were optimised such that the mechanism of oxidation was preserved as close as at 37 °C. This was conducted by comparing the oxidation at accelerated condition with an optimised order of antioxidant activity of several 250 µM antioxidants (Trolox, ascorbic acid, gallic acid and quercetin) for 20 h oxidation at 37 °C.

5.1.3 Challenges in applying accelerated conditions

In Chapter 3, it has been found that most of the accelerated conditions used enhanced breakdown of peroxides. Oxidation at 60°C for 10 h enhanced the breakdown of peroxides and provided no accelerated formation of TBARS compared to that at 37 °C. Although at this temperature for 5 h, oxidation speeded up the peroxides formation, but not TBARS, compared to that at 37 °C. In addition, the order of antioxidant activity was changed for this temperature at both 5 and 10 h and hence 60 °C was excluded in optimising the method. At 50 °C for 10 h, oxidation speeded up the formation of peroxides while TBARS were not increased at these conditions and hence was not chosen. On the other hand, at 50 °C for 5 h, oxidation sped up the
formation of both peroxides and TBARS and the order of antioxidant activity was the same as at 37 °C for 20 h.

Using catalysts Cu$^{2+}$ and Co$^{2+}$, oxidation at 5 and 10 h enhanced the breakdown of peroxides, which led to the increased formation of TBARS. Despite the formation of TBARS, the order of antioxidant activity at 5 and 10 h for both peroxides and TBARS was greatly changed. Therefore uses of both Cu$^{2+}$ and Co$^{2+}$ ions in accelerating oxidation system of the TBARS and FTC (peroxides) assays were not chosen. Similarly, use of oxygen initiator (Fe$^{2+}$ and H$_2$O$_2$) enhanced the breakdown of peroxides and provided no speeding up of the TBARS formation at 5 and 10 h. In addition, the order of antioxidant activity was changed at the oxidation times for both peroxides and TBARS and hence was excluded in optimising the method.

Of the accelerated conditions, only at 50 °C for 5 h, oxidation was consistent in speeding up the formation of both peroxides and TBARS and preserving order of antioxidant activity as found at 37 °C for 20 h. Thus 50 °C for 5 h was finally chosen as the accelerated conditions for the rapid method to measure antioxidant activity. The optimised conditions were tested in another set of antioxidants ((+)-catechin, (-)-epicatechin, caffeic acid and α-tocopherol), which also showed similar effectiveness with those of the initial set of antioxidants. The optimised method with oxidation at 50 °C is comparable to that at 37 °C and 4 times faster than the starting conditions of 20 h.
5.1.4 Applicability of method to traditional medicinal plant as real samples

In Chapter 4, using the developed FTC assay, all crude extracts and fractions showed antioxidant activity for all species (*A. implexa* bark and leaves, *E. rossii* and *E. cupressiformis* leaves). Based on the activity of crude extracts, *E. rossii* was found to give the most active antioxidants compared to other species. Of the fractions in all species, *n*-hexane and DCM fractions showed consistently high antioxidant activity, which were higher or comparable to Trolox. Of the fractions of all species, *n*-hexane fraction showed higher antioxidant activity.

Using the TBARS assay, all crude extracts, and majority of fractions of all species showed antioxidant activity. *Exocarpos cupressiformis* crude extract was found to show highest antioxidant activity compared to other species. All *n*-hexane and DCM fractions of all species showed higher or equal antioxidant activity compared to Trolox. An inverse result of the *n*-hexane and DCM fractions for FTC and TBARS assays were found in ATBS assay and in total phenolic contents (FC).

As all crude extracts and most of the fractions showed antioxidant activity in either FTC or TBARS assay, the developed method has successfully been applied to screen the antioxidant activity of the plant materials.
5.1.5 Tentative identification of active compounds in traditional medicinal plants

The antioxidant activity of the plant species screened by the developed method led to UHPLC-qTOF-MS analyses; identification using standards, and tentative assignment of active compounds present in the plant materials. In Chapter 4, gallic acid, gallochettechin, rhamnetin, 3, 3’-dimethylquercetin and hederagenin were tentatively assigned in *A. implexa* bark. Similarly procyanadin B2, epicatchin, quercetin diglucoside, rutin, myricitrin, and kaempferol were assigned in *A. implexa* leaves. The major compounds tentatively assigned in *E. rossii* included (+)- catechin, ellagic acid, quercetin 3-glucoside, sideroxylin and eucalyptin. In other species, *E. cupressiformis*, kaempferol-3-rhamnobioside, quercetin-3-rhamnoside (quercetin), dihydrokaempferol-7-rhamnoside, kaempferol-7-rhamnoside, and quercetin-3-rhamnobioside were tentatively assigned. The presence of the above compounds in *A. implexa*, *E. rossii*, and *E. cupressiformis* is consistent with the existence of these compounds in same genuses or other plants reported in earlier studies. Of the compounds, gallic acid, (+)- catechin, and (-)- epicatechin were identified and confirmed using standards.

The antioxidant activity screened by the developed method suggests that there is potential medicinal value of the plants tested, and that testing would be warranted by the presence of compounds in the species.
5.1.6 Research Conclusions

This thesis answers the research question, “It is realistic to develop a rapid and robust method to measure antioxidant activity in a lipid system” and by presenting a systematic way of optimisation. Using LA as lipid substrate, thesis has addressed the research objectives (Section 1.15) as follows-

(1) The multi-phase oxidation system is problematic. Hence cannot be used in as antioxidant method development

(2) Of the two types of variability with the above system, intra-batch precision can be minimised. The other intra-batch variability cannot be overcome suggesting that pre-formed peroxides needs to be monitored while measuring antioxidant activity

(3) LA emulsion shows good precision for oxidation and antioxidant activity compared to the multi-phase system and can be used in optimising rapid antioxidant assay

(4) Oxidation of LA emulsion at accelerated at 50 °C for 5 h can preserve the conditions as at that of 37 °C. The optimised 5 h oxidation time is comparatively closer to the method time of DPPH, FC or ORAC (1h)and 4 times faster than the literature 20 h (McDonald et al., 2001).

(5) The rapid and robust method can be applied to screen medicinal plants for antioxidant activity
(6) Finally, method can screen antioxidant activity of non-polar fractions (n-hexane and DCM fractions) more effectively, which is not effectively determined by commonly used ABTS and FC assay.

Thus the developed lipid-based FTC and TBARS assay can perform high throughput screening of antioxidant activity, and also reveals that there may be novel lipophilic antioxidants to be explored in traditional medicinal plants, which may not be found if only the conventional ABTS assay are applied in screening studies.

This finding can be extended to food industry where antioxidant activity of food nutrients is measured using ABTS or other aqueous assay and then add to the real food. In doing so, some of the novel lipophilic antioxidants may be missed if the lipid-based assays are not used. In this case, the developed FTC and TBARS assay can be effectively used.

Furthermore, in food industry, results from FTC and TBARS assays have been used to interpret the potential antioxidant value of compounds added to lipid containing food products - such as for the oil and meat products. In this case, pre-formed peroxides in the system are not routinely measured. The results from Chapter 2 will provide information that pre-formed peroxides may have a large impact on the results of antioxidant studies when TBARS are the measure of oxidation. Hence this would be vital information for food technologist.
5.2 Recommendations for Further Research

There are still some interesting issues to be explored in this area. From this thesis, the following recommendations for future research can be made:

5.2.1 In-depth study of mechanisms of oxidation and antioxidant for thiobarbituric acid reactive substances (TBARS) and peroxides

The implicit mechanism of the oxidation including kinetic studies is needed to be looked into for the overall picture of using the studied LA oxidation in antioxidant studies. In Chapter 2, the erratic breakdown of pre-formed peroxides to TBARS or other secondary oxidation products has not been explored. The mechanism of the breakdown of peroxides and formation of TBARS can be investigated. One of the interesting things, the other secondary oxidation products may not be TBARS and hence provided batch to batch LA oxidation variability in Chapter 2. These products can be identified by using analytical techniques (LC-MS or NMR). If any new compounds are found, it would be important to obtain synthetic standards in order to confirm their identity.
5.2.2 Specific identification of compounds of *Acacia implexa*, *Eucalyptus rossii*, *Exocarpos cupressiformis* as antioxidant.

In Chapter 4, in *A.implexa*, *E.rossii*, *E.cupressiformis*, some polyphenolic compounds have been first tentatively identified in these species, which opened up in exploration of new and novel antioxidant in these plants. In this case, antioxidant activity has been assessed on the basis of fractions conducted by different solvents. The fractions can be sub-fractionated by chromatographic techniques and the specific compound can be separated, identified, and tested for antioxidant activity.

5.2.3 Hyphenation of analytical techniques in thiobarbituric acid reactive substances (TBARS) and ferric thiocyanate (FTC) (peroxides) assays

In this thesis, the oxidation system of the method has been made rapid and robust. The other measurement part (instrumental) of the method for reading peroxides and TBARS can be hyphenated with modern technology so that this stage can be more amenable to use for a large number of samples. In this case, below future research can be made:
5.2.3.1 Flow Injection

Flow injection analysis (FIA) is an automation technique where the sample is inserted in the flow of assay reagents, and after a precisely controlled period of time, products are monitored e.g. by spectrophotometer. Flow injection methods are also widely used for the determination of antioxidant activity based on DPPH (Amatatongchai, Laosing, Chailapakul, & Nacapricha, 2012; Mrazek, Watla-iad, Deachatthai, & Suteerapataranan, 2012), ABTS, and FRAP (Martins et al., 2012) assays. Flow injection analysis can also be adapted to the TBARS/ FTC (peroxides) antioxidant assay. In the flow injection analyser, the carrier solvents (assay solvents) can be pumped through the sample valve, reaction chamber 1, and reaction chamber 2. The plant extract/ antioxidant solution can be added in sample valve. The lipid oxidation reagents used for FTC or TBARS assay can be added to reaction chamber 1 and finally the ferrous solution for FTC or TBA for TBARS can be added to reaction chamber 2. After a precisely controlled period of time, absorbance can be measured at 500 or 532 nm. A disadvantage of flow injection analysis may be that no separation of components occurs.

5.2.3.2 Post Column thiobarbituric reactive substances (TBARS)/ ferric thiocyanate (FTC) (peroxides) assays

Another way of adapting the TBARS/ FTC (peroxides) assay to make it more suitable to high throughput screening of natural antioxidants is to hyphenate the
assay to high performance liquid chromatography-diode array detector (HPLC-DAD) separation of antioxidants and assess antioxidant activity as part of the HPLC analysis. This so-called “post-column” technique is frequently used in assessing antioxidant activity by DPPH, ABTS and FRAPS assays (Kusznierewicz, Piasek, Bartoszek, & Namiesnik, 2011; Z. Q. Ou, Schmierer, Rades, Larsen, & McDowell, 2013). The term post-column refers to the fact that assay reagents are added to the HPLC eluent once the compounds have been separated by the column. The plant extract/ antioxidant sample can be added to the HPLC eluent to be fractionated by the column, the fractionated portions can then be pumped to the reaction chamber 1, where the lipid oxidation reagents for FTC or TBARS assay can be added. After this, the reaction mixture can be reacted with the ferrous solution for FTC or TBA for TBARS in reaction chamber 2. Finally the absorbance can be measured. The advantage of post-column antioxidant assays is that they provide a means to rapidly identify the most potent antioxidant(s) in a complex mixture such as a plant extract. It should be noted that post-column methods are technically more challenging than flow injection analysis because there are back pressure problems are associated with HPLC.
REFERENCES


the fluorescent probe. *Journal of Agricultural and Food Chemistry, 49*(10), 4619-4626.


APPENDIX 1

UHPLC-DAD-qTOF-MS chromatograms of quality control sample (gallic acid (1), (+)-catechin (2), caffeine (3), (-)-epicatechin, (4) and quercetin (5)): (A) total ion chromatogram (TIC); (B) chromatogram detected at 280 nm; and (C) chromatogram detected at 380 nm. Process of identification: (i) prominent peaks in 280 (B) and/or 380 nm (C) chromatograms were selected, (ii) accurate mass (m/z) of a peak at the particular retention time in (i) was obtained from the TIC (A), (iii) the peak was then checked through extracted ion chromatogram (EIC) (D) to ensure that the selected m/z value gave a peak with a retention time consistent with those found in (i) and (ii).
APPENDIX 2

UHPLC-DAD-qTOF-MS chromatograms of crude extract of *Acacia implexa* bark: (A) total ion chromatogram (TIC); (B) chromatogram detected at 280 nm; and (C) chromatogram detected at 380 nm. (Process of identification: same as Appendix 1).
APPENDIX 3

UHPLC-DAD-qTOF-MS chromatograms of crude extract of *Acacia implexa* leaves: (A) total ion chromatogram (TIC); (B) chromatogram detected at 280 nm; and (C) chromatogram detected at 380 nm. (Process of identification: same as Appendix 1).
APPENDIX 4

UHPLC-DAD-qTOF-MS chromatograms of crude extract of *Eucalyptus rossii* leaves: (A) total ion chromatogram (TIC); (B) chromatogram detected at 280 nm; and (C) chromatogram detected at 380 nm. (Process of identification: same as Appendix 1).
APPENDIX 5

UHPLC-DAD-qTOF-MS chromatograms of crude extract of *Exocarpos cupressiformis* leaves: (A) total ion chromatogram (TIC); (B) chromatogram detected at 280 nm; and (C) chromatogram detected at 380 nm. (Process of identification: same as Appendix 1).
APPENDIX 6

UHPLC-DAD-qTOF-MS chromatograms of Dichloromethane (DCM) fraction of *Acacia implexa* bark: (A) total ion chromatogram (TIC); (B) chromatogram detected at 280 nm; (C) chromatogram detected at 380 nm, (D) extracted chromatogram for tentatively identified compound 8. (Process of identification: same as Appendix 1).
APPENDIX 7

UHPLC-DAD-qTOF-MS chromatograms of $n$-hexane (H) fraction of Acacia implexa leaves: (A) total ion chromatogram (TIC); (B) chromatogram detected at 280 nm; (C) chromatogram detected at 380 nm, (D) extracted chromatogram for tentatively identified compound 15.

(Process of identification: same as Appendix 1).
APPENDIX 8

UHPLC-DAD-qTOF-MS chromatograms of n-hexane (H) fraction of *Eucalyptus rossii*: (A) total ion chromatogram (TIC); (B) chromatogram detected at 280 nm; (C) chromatogram detected at 380 nm, (D) extracted chromatogram for tentatively identified compound 24. (Process of identification: same as Appendix 1).
APPENDIX 9

UHPLC-DAD-qTOF-MS chromatograms of n-hexane (H) fraction of *Exocarpos cupressiformis* leaves: (A) total ion chromatogram (TIC); (B) chromatogram detected at 280 nm; (C) chromatogram detected at 380 nm, (D) extracted chromatogram for tentatively identified compound 26. (Process of identification: same as Appendix 1).
APPENDIX 10
Structures of Compounds 1, 16 and 10, identified by standards

**Compound 1: Gallic acid**
MF (molecular formula: M-H): C$_7$H$_6$O$_5$
m/z (mass to charge ratio) experimental: 169.0146
Plant species present: *Acacia implexa* bark, and *Eucalyptus rossii* leaves

**Compound 16: (+)-Catechin**
MF (molecular formula: M-H): C$_{15}$H$_{14}$O$_6$
m/z (mass to charge ratio) experimental: 289.0720
Plant species present: *Eucalyptus rossii*, and *Exocarpos cupressiformis* leaves

**Compound 10: (-)-Epicatechin**
MF (molecular formula: M-H): C$_{15}$H$_{14}$O$_6$
m/z (mass to charge ratio) experimental: 289.0721
Plant species present: *Acacia implexa* leaves