Antioxidant effects of Maillard reaction products (MRPs) derived from glucose-casein model systems

By

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"Those who complete the course will do so only because they do not, as fatigue sets in, convince themselves that the road ahead is still too long, the inclines too steep, the loneliness impossible to bear and the prize itself of doubtful value."

Thabo Mvuyelwa Mbeki – President of South Africa (1999 – 2008)

DECLARATION

I, **Vusi Vincent Mshayisa**, hereby declare that the work contained in the research report is my own original work and that it has not previously, in its entirely or in part, been submitted to any other university for a degree. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Vusi Vincent Mshayisa	01 October 2016
Signed	Date

ABSTRACT

The Maillard reaction (MR) involves the condensation reaction between amino acids or proteins with reducing sugars, which occurs commonly in food processing and storage. Maillard reaction products (MRPs) were prepared from glucose-casein model system at pH 8, heated at 60, 75 and 90°C for 6, 12 and 24 h, respectively. Browning intensity (BI) of MRPs, as monitored by absorbance at 420 nm increased with an increase in reaction temperature. The reducing power (RP) of MRPs increased ($p < 0.05$) as the reaction time increased at 60 and 75°C, while at 90°C an increase in RP was observed from 6 to 12 h and thereafter a slight decrease was observed up to 24 h. The 2,2-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) radical scavenging activity (ABTS-RS) and Peroxyl radical scavenging (PRS) activity of glucose-casein MRPs produced at 90°C decreased as the reaction time increased. In this study, the ferrous chelation activity of MRPs was higher than that of tert-butylhydroquinone (TBHQ) (0.02%) and Trolox (1 mM), respectively. Moreover, the 1, 1-diphenyl-2-picryl-hydrazil radical scavenging (DPPH-RS) of MRPs increased ($p < 0.05$) as the reaction time increased irrespective of the heating temperature. The primary and secondary lipid oxidation products were measured using the Peroxide value (PV) and Thiobarbituric acid reactive substance (TBARs) assay in sunflower oil-in-water emulsion, respectively. MRPs derived at 90°C for 12 h had the lowest peroxide value, while the TBARs inhibitory by MRPs ranged from 39.05 – 88.66%. Glucose-casein MRPs displayed superior antioxidant activity than TBHQ (0.02%) and Trolox (1 mM), respectively, as measured by the TBARs assay. The differential scanning calorimetry (DSC) and Rancimat techniques set at 110°C were used to evaluate the oxidative stability the lipid-rich media containing MRPs. At the same temperature program, DSC gave significantly lower reduction times than the Rancimat. Furosine (N-ε-Fructosyl-lysine) and Pyrraline (2-amino-6-(2-formyl-5-hydroxymethyl-1-pyrrolyl)-hexanoic acid) were determined using high pressure liquid chromatography to evaluate the extent of the MR. Furosine concentration of glucose-casein MRPs ranged between 0.44 – 1.075 $mg.L^{-1}$ in MRPs derived at 60°C, while at 75°C an increase as function of time was observed. MRPs derived at 60 and 75°C exhibited a varied concentration of pyrraline as the reaction time increased with higher temperatures resulted in higher concentrations (0.39 $mg.L^{-1}$).

The results of this study clearly indicated that MRPs possess antioxidant activity and can be used as natural antioxidants in the food industry.

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DEDICATION

Dedicated to the memory of my cousin brothers
Jabulani (†21.12.2009) and Sizwe († 04.01.2016)

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GLOSSARY

Acronyms/Abbreviations	Definition
ABAP	2'-azo-bis (2-amidinopropane) dihydrochloride
ABTS	2'2- azinobis (3 ethylbenzothioazoline -6 sulfonate)
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxyl toluene
DHA	Docosahexaenoic acid
DPPH	2, 2-diphenyl-picrylhydrazyl
EDTA	Ethylenediaminetetraacetic acid
EPA	Eicosapentaenoic acid
ET	Electron transfer
FCR	Folin-Ciocalteu reagent
FID	Flame ionisation detector
FRAP	Ferric ion reducing antioxidant power
GC	Gas Chromatography
HAT	Hydrogen atom transfer
HDL	High density lipoprotein
HPLC	High-performance liquid chromatography
IP	Induction period
LDL	Low density lipoproteins
MRPs	Maillard reaction products
MS	Mass spectrometry
PG	Propyl gallate
PUFAs	Polyunsaturated fatty acids
ROS	Reactive oxygen species
TBHQ	Tertiary butyl hydroquinone
TEAC	Trolox equivalence antioxidant capacity

CHAPTER 1

MOTIVATION AND DESIGN OF THE STUDY

1.1 Introduction

The Maillard reaction (MR) is a non-enzymatic reaction between carbonyl groups of reducing sugars and amino acids, peptides, or proteins. Maillard reaction products (MRPs), which are naturally produced during thermal processing and storage by reducing sugars that interact with available amino acids, modify important food properties such as colour, flavour and texture, which are key factors responsible for the consumer acceptance of many food products (Osada & Shibamoto, 2006; Maillard *et al.,* 2007). The MR occurs during thermal processing such as baking, boiling and roasting of different types of food products and during drying and storage. Non-volatile compounds in MRPs were considered as the major contributors to the antioxidant activity of MRPs. However, there have been reports of volatile MRPs which are responsible for flavours in food, possessing antioxidant activities (Yu *et al*., 2012), such as volatile MRPs formed during roasting of almonds which were found to have an antioxidative effect on lipid oxidation (Severini *et al*., 2000). Antioxidant character of MRPs has been observed *via* radical chain-breaking activity, scavenging of reactive oxygen species (ROS), decomposition of hydrogen peroxide and metal chelating activity, thus potentially providing more than one mechanism of action in combatting lipid oxidation (Gu *et al.,* 2010; Yu *et al*., 2012; Vhangani & Van Wyk, 2013).

Lipid oxidation is one of the prime causes of quality deterioration in natural and processed foods. Oxidative deterioration is a large economic concern in the food industry since it affects many quality characteristics such as flavour, colour, texture and the nutritive value of foods. In addition it produces potentially toxic compounds, thus making the lipid- or lipid-containing food products unsuitable for consumption (Chaiyasit *et al*., 2007).

To avoid or delay this autoxidation process, antioxidants have been utilised with the practice being carried out successfully for over fifty years. The most widely used

synthetic antioxidants in food such as butylated hydroxyanisole (BHA), butylated hydroxy toluene (BHT), propyl gallate (PG) and tert-Butylhydroquinone (TBHQ) have been suspected to cause or promote negative health effects and are perceived negatively by consumers despite being relatively cheap and effective (Osada & Shibamoto, 2006; Dong *et al,* 2011). In addition, these are restricted by legislative rules. Due to these reasons, there is an increasing interest in natural antioxidants due to the safety and toxicity problems associated with synthetic antioxidants in lipid-containing food products. In addition, MRPs could be used as antioxidants as well as colour and flavour additives (Gu *et al.,* 2010; Yu *et al*., 2013).

1.2 Statement of the research problem

Despite numerous studies conducted to understand the MR, many of its chemical pathways are still speculative. In general, the limited knowledge of mechanisms is partially due to the complexity of the reaction, but also due to the analytical techniques that are available. Apart from their contributions to sensorial features of thermally treated foods, MRPs are known to exhibit antioxidant properties. Since research considering the antioxidant activity of MRPs has been performed mostly with sugar-amino acid model systems, relatively little is known about the antioxidant potential of sugar-protein models. Therefore, there is a need to further investigate the antioxidant properties of MRPs at different steps in the MR for sugar-protein systems in order to optimise product formulations and processing parameters.

1.3 Objectives of the research

1.3.1. Broad objectives

The aim of this study was to determine selected antioxidant indices of the antioxidant effect of glucose-casein MRPs as a function of reaction temperature and time; and to determine the lipid oxidation indices as a function of MRP antioxidant type in a lipid-rich model system with a view to identify and characterise natural alternatives to synthetic antioxidants in lipid-rich food products, as well as to quantify the early and advanced Maillard reaction products in glucose-casein model systems.

The dependent variables are five antioxidant indices namely; [1, 1-diphenyl-2-picryl-hydrazil radical scavenging (DPPH-RS), peroxyl radical scavenging (PRS),

reducing power (RP), 2,2-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) radical scavenging activity (ABTS-RS) and Iron chelation and three lipid oxidation indices (Thiobarbituric acid reactive substances (TBARs), Peroxide Value (PV), oxidative stability by Differential scanning calorimeter (DSC), and Rancimat].

The independent variables are reaction temperature, time and antioxidant types.

1.3.2. Specific objectives

The first specific objective was to measure and compare the DPPH-RS, PRS, RP, ABTS-RS and Iron chelation of glucose-casein MRPs as a function of heating temperature (60, 75 and 90°C).

The second specific objective was to measure and compare the DPPH-RS, PRS, RP, ABTS-RS and Iron chelation of glucose-casein MRPs as a function of heating time (6, 12 and 24 h).

The third specific objective was the ranking best glucose-casein MRP model systems which exhibits the highest total antioxidant activity as compared to the industry standard synthetic antioxidant (TBHQ).

The fourth specific objective was to measure the PV, TBARs, and oil stability index (Rancimat and DSC) of MRPs as a function of antioxidant type in a lipid-rich model system.

The fifth specific objective was the selection of the MRPs with the optimum antioxidant activity as compared to the industry standard synthetic antioxidant (TBHQ) towards the elucidation of the Maillard reaction pathway/intermediates.

The sixth specific objective was to identify and quantify early and advanced Maillard reaction products in glucose-casein model systems.

The seventh specific objective was to collate, analyse and interpret all data with the view to identifying and characterising alternatives to a synthetic antioxidant in lipid-rich food products.

1.4 Research Hypotheses

Based on previous research, it was hypothesized that glucose-casein MRPs processed at 90°C for 24 h will exhibit higher antioxidant capacity than those produced at lower temperatures. The browning intensity of the MRPs generated at 90°C for 24 h was

expected to be higher than any other combination. It was also anticipated that the pH of MRPs will gradually decrease as the time and temperature increases (Sun & Zhuang, 2011). The DPPH-RS, ABTS-RS, peroxyl radical scavenging activity and reducing power are expected to increase with an increase in reaction time (Morales & Jiménez-Pérez, 2001).

1.5 Research design and methodology

A full factorial design was used, with the design factors of three reaction temperatures and reaction times, i.e. a 3^2 design. The data was analysed using Analysis of Variance (ANOVA) and significant differences among the means of replicates (n = 2) was determined by Duncan's multiple range tests using the statistical analysis software SPSS 21.0 for Windows®, SPSS.

In this study five antioxidant indices namely; [1, 1-diphenyl-2-picryl-hydrazil radical scavenging (DPPH-RS), peroxyl radical scavenging (PRS), reducing power (RP), 2,2-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) radical scavenging activity (ABTS-RS) and Iron chelation and four lipid oxidation indices (Thiobarbituric acid reactive substances (TBARs), Differential scanning calorimeter (DSC), Rancimat and Peroxide Value (PV)] were determined.

HPLC analyses were carried out to identify and quantify early and advanced Maillard reaction products.

1.6 Delineation of the research

A single sugar-protein (glucose-casein), three reaction temperatures (60, 75 and 90 °C), and three reaction times (6, 12 and 24 hours) were used to synthesise MRPs at a constant pH (pH 8). A freeze-dried model system was used to measure the antioxidant indices of the glucose-casein MRPs. Sunflower seeds were subjected to a screw oil press to extract the oil. Pasteurised unsalted egg yolk was used for emulsion preparation and the lipid oxidation indices were established in the emulsion.

1.7 Significance of the research

Lipid oxidation of monounsaturated and polyunsaturated foods during processing and storage is a major concern to the food industry since it leads to millions of Rands in terms

of product losses. In addition, the oxidative instability of polyunsaturated fatty acids often limits their use in nutritionally beneficial lipids in functional foods (Chaiyasit *et al*., 2007).

The oxidation of unsaturated fatty acids results in the formation of peroxides, which are susceptible to further oxidation by-products such as short chain aldehydes and ketones. The presence of these molecules reacting with oxygenated compounds in foods, will adversely affect flavour, taste, nutritional value and overall quality of the food product. In addition, the oxidation of fats in foods can lead to the accumulation of toxic compounds such as peroxides and aldehydes. These are potentially detrimental to human health when consumed in high concentrations and have been implicated in the development of several diseases including cancer and atherosclerosis (Laguerre *et al*., 2007; Gu *et al*., 2009).

To inhibit or prevent oxidation, antioxidants such as BHT and TBHQ are commonly used as food additives at levels or limit specified by legislation. However, use of such compounds has been related to health risks resulting in strict regulations over their use in foods. Alternative sources derived from plant and animals have received considerable attention. Natural preservatives such as spices and other plant-based additives may negatively impart odour and flavour to food product. Previous studies have demonstrated the effectiveness of natural antioxidants in inhibiting lipid oxidation (Kulisic *et al*., 2004; Wang *et al*., 2011).

The antioxidant activities of MRPs have been extensively studied. In some studies, MRPs with antioxidative activity were identified, such as amino reductones, heterocyclic compounds or high molecular weight melanoidins, but most of the active antioxidants in Maillard mixtures or foods rich in MRPs are still unknown (Hwang *et al*., 2011).

MRPs are presumed to be safe and are obtained from food which has been used for centuries; moreover, the question of safety of synthetic compounds can be avoided (Maillard *et al.,* 2007). The benefits of using natural MRPs include GRAS (generally recognized as safe) status, allowing the use of higher concentrations and worldwide acceptance. MRPs are also advantageous in that they may not impart odours and flavours undesirable to consumers and are not destroyed during heating. Therefore, incorporation of the preformed MRPs directly into food systems or application of food processing practices to form MRPs within food can result in improved oxidative stability of foods, and thus can be considered as substances that are naturally present in foods which can solve

the problem of potentially hazardous synthetic antioxidants (Kim & Lee, 2009; Yu *et al.*, 2013). Detailed understanding of the mechanism of MR may provide the technical knowledge required to control different pathways leading to the formation of desirable and undesirable products. The food industry today is facing the challenge of understanding the parameters that control the MR so successfully manipulate the conditions for the selective generation of aromas, flavours, antioxidants, While at the same time minimising the formation of toxic and off-flavour components. The knowledge of the reaction conditions and pathways that generates MRPs with specific antioxidant activity could therefore be a valuable tool for use in food processing strategies.

1.8 Expected outcomes and contributions of the research

Novel knowledge of the reaction conditions and antioxidant activity of MRPs from glucose-casein systems will be generated and identification and characterisation of MRPs with enhanced antioxidant activity in the MR will be established and information generated regarding the mechanism, pathway/intermediates.

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CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Maillard reaction products (MRPs) derived during food processing, packaging and storage have been found to exhibit strong antioxidant activity. As a result of their antioxidant character, MRPs are considered a value-added component. Lipids are a major component of food and essential structural and functional constituents of cells in biological systems. However, this diverse group of substances is prone to oxidation through various pathways. Their oxidative stability depends on a number of factors, including the unsaturation of their fatty acids, presence of minor components, environmental conditions and use of antioxidants. Lipid oxidation has detrimental effects on both food quality and human health, and efforts must be made to minimize oxidation, thus improving oxidative stability of lipid-containing food products. This chapter focuses on lipid oxidation, its mechanism, factors affecting oxidation as well as strategies for improving oxidative stability of lipids and it further explores the potential use of Maillard reaction products (MRPs) from sugar-protein combinations as antioxidants

2.2 Lipid oxidation

Lipid oxidation is a general term that is used to describe a sequence of chemical changes that result from the interaction of lipids with reactive oxygen species (ROS). Lipids may undergo autoxidation, photo-oxidation, thermal oxidation and enzymatic oxidation under different conditions (Pokorny *et al.,* 2008; Shahidi & Zhong, 2010). Lipid oxidation is one of the major causes of quality deterioration in natural and processed foods. In some foods a limited amount of lipid oxidation is desirable since it leads to the generation of molecular species that have a desired characteristic taste or smell, for example some cheeses, fried foods and virgin olive oils (Chaiyasit *et al.,* 2007; Pokorny *et al.,* 2008). On the other hand, oxidative deterioration is a serious economic concern in the food industry since it affects many quality characteristics such as flavour (rancidity), colour, viscosity, density and nutrition value of foods (loss of essential fatty acids, fat-soluble vitamins and other bioactives) (Morales & Jiménez-Péréz, 2001; Sun *et al.,* 2011). In addition, it produces

potentially toxic compounds, which possess a real danger to the consumers. Moreover, the *in vivo* involvement of lipid oxidation products in the etiology of atherosclerosis is clearly established, along with their role in many other human pathologies such Alzheimer's disease, cancers, inflammation and aging (Matmaroh *et al.,* 2006; Laguerre *et al*., 2007).

Polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) have been reported to prevent coronary heart disease and have a positive effect on the brain and nervous system as well as on stimulating the immune system (Niki *et al.,* 2005; Rahman, 2007). The high susceptibility of polyunsaturated lipids to oxidation has restricted their incorporation in many food products which is unfortunate, since greater consumption of polyunsaturated lipids is beneficial to health and is recommended in dietary guidelines (Leo & Toldra, 2009). PUFAs do not only lose their physiological activity, but may also turn into anti-nutritive agents when oxidised (Laguerre *et al*., 2007). Another consequence is that lipid oxidation products can react with other food compounds such as amino acids or proteins resulting in changes in texture and colour. Therefore, lipid oxidation is very important in terms of the palatability, toxicity and nutritional value of foods and, hence, its prevention or retardation is vital (Jing & Kitts, 2002; Rahman, 2007; Pokorny *et al*., 2008).

Moreover, retarding lipid oxidation not only extends product shelf-life but also reduces raw material waste, nutritional loss and widens the range of lipids that can be used in specific products. Thus, control of lipid oxidation could allow food processors to use more available, less costly and more nutritionally favourable fats or oils for product formulation (Chaiyasit *et al*., 2007; Jayathilakan *et al.,* 2007).

The mechanism of lipid oxidation in a particular food depends on the nature of the reactive species present and their environment, hence a thorough understanding of the lipid oxidation mechanism is important in developing practical methods for controlling lipid oxidation in foods (Ross & Smith, 2006). In this chapter, the mechanism of lipid oxidation and its control in food systems, traditional methods to determine primary and secondary lipid oxidation will be discussed in detail. Apart from that antioxidant capacity methods which have been commonly used in the last decade are revised in order to provide a complete oversight of possible options.

2.3 Lipid oxidation mechanism (autoxidation)

Lipids are susceptible to oxidation in the presence of catalytic factors such as light, heat, ezymes, metal, metalloproteins, and microorganisms, leading to complex process of autoxidation, photooxidation, thermal or enzymatic oxidation, most of which involve free radicals. Photooxidation involves excitation of a photosensitizer and energy transfer to lipid molecules or oxygen(Laguerre *et al.*, 2007). Oxidation can also be catalysed by certain enzymes such as lipoxygenases. Autoxidation is the most common process among all and is defined as the spontaneous reaction of lipids with atmospheric oxygen through chain reaction fo free radicals. The process can be accelerated at higher themperatures as in thermal oxidation (Shahidi & Zhong, 2010). The overall mechanism of free radical-mediated chain reaction involves three stages, *viz.* initiation (formation of free radicals), propagation (free-radical chain reactions) and termination (formation of non-radical products). A simple scheme of free radical mechanism is summarised in Figure 2.1 and the stages are briefly described in the sections that follow (Laguerre *et al.*, 2007; Pokorny *et al.*, 2008).

Initiation	$In^{\bullet} + RH$	→	$R^{\bullet} + InH$	a
Propagation	$R^{\bullet} + O_2$	→	$ROO^{\bullet}$	b
	$ROO^{\bullet} + RH$	→	$ROOH + R^{\bullet}$	c
Secondary initiation	$ROOH$	→	$RO^{\bullet} + {}^{\bullet}OH$	d
	$2ROOH$	→	$RO^{\bullet} + ROO^{\bullet} + H_2O$	e
Termination	$ROO^{\bullet} + ROO^{\bullet}$	→	$ROOR + O_2$	f
	$R^{\bullet} + ROO^{\bullet}$	→	$ROOR$	g
	$R^{\bullet} + R^{\bullet}$	→	RR	h

Figure 2.1 Mechanism of lipid autoxidation (Choe & Min, 2006; Pokorny *et al.*, 2008).

2.3.1 Initiation

The first step is initiation in which lipid radicals are formed from lipid molecules. A fatty acid radical known as the alkyl radical ($R^{\bullet}$) is formed by removal of a hydrogen from a fatty acid in the presence of an initiator ($In^{\bullet}$) (Fig. 2.1a). The energy required to remove hydrogen from fatty acids is dependent on the hydrogen position in the molecule. The

hydrogen atom adjacent to the double bond, especially hydrogen attached to the carbon between the two double bonds, is removed easily (Hidalgo & Zamora, 2000; Niki *et al.,* 2005). Hydrogen at C11 of linoleic acid is removed at 50 $kcal.mol^{-1}$, while the energy required to remove hydrogen at C8 and C14 of Linoleic acid is 75 $kcal.mol^{-1}$ (Choe & Min, 2006; Pokorny *et al.,* 2008). The double bond adjacent to the carbon radical in linoleic acid shifts to the more stable next carbon and from the *cis* to the *trans* form. The autoxidation of linoleic and linolenic acids not only produces conjugated products but also 2-aldehydes. The oxidation process remains slow during this phase (Pokorny et al., 2008). However, in oils there is often a trace quantity of hydoperoxides (ROOH) which may be formed by lipoxygenase action in the plant prior to and during extraction of the oil (Laguerre *et al.,* 2007; Pokorny *et al.*, 2008).

2.3.2 Propagation

At the end of the initiation period, oxidation suddenly accelerates, oxygen consumption becomes high and the peroxide content increases substantially. The first step of propagation involves the addition of oxygen to the alkyl radical ($R^{\bullet}$) resulting in the formation of a peroxyl radical ($ROO^{\bullet}$), which has a higher energy than the alkyl radical (Fig 2.1b). Thus the peroxyl radical can abstract hydrogen from another unsaturated fatty acid to produce a lipid hydroperoxide (ROOH) and a new alkyl radical (Fig 2.1c) (Zamora *et al.*, 2005; Pokorny *et al.*, 2008). The formation of lipid peroxyl and hydroperoxide radicals depends only on oxygen availability and temperature. This reaction can be repeated many times; hence it is referred to as a chain reaction.

2.3.3 Decomposition

Primary oxidation products (lipid hydroperoxides) are relatively stable at room temperature and in the absence of metals. However, in the presence of metals or high temperatures they readily decompose to secondary lipid oxidation products (Akor & Min, 2002). The homolytic cleavage of hydroperoxides (ROOH) between the two oxygen molecules is the most likely hydroperoxide decomposition pathway (Akoh & Min, 2002). This reaction yields an alkoxy ($RO^{\bullet}$) and a hydroxyl radical ($OH^{\bullet}$). The alkoxy radical ($RO^{\bullet}$) (Fig 2.1d), which is more energetic than either the alkyl ($R^{\bullet}$) or peroxyl radical ($ROO^{\bullet}$) (Fig 2.1e), can enter into a number of different reaction pathways. Alkoxy radicals can attack

another unsaturated fatty acid, a pentadiene group within the same fatty acid or the covalent bonds adjacent to the alkoxyl radical. This latter reaction is known as *β*-scission reaction and is important to food quality as it can cause fatty acids to decompose into low molecular weight volatile compounds that cause off-flavours or rancidity (Ross & Smith, 2006). After electron rearrangement, the addition of hydroxyl, or hydrogen transfer, the ultimate secondary lipid oxidation products are mostly low molecular-weight aldehydes, ketones, alcohols, furans, hydrocarbons and acids. A more detailed discussion of *β*-scission reaction can be found in Ross & Smith (2006).

2.3.4 Termination

The interaction of two free radicals to form a non-radical will terminate the process (Fig. 2.1f – 2.1h). This step is sometimes not very important in food since initiation and propagation has taken place and the food is already rancid before significant termination reactions take place. An exception is in the low oxygen environment of frying oils where the termination reaction can occur between alkyl radicals to form fatty acid dimers (Chaiyasit *et al.,* 2007).

2.3.5 Induction period

According to McClements & Decker (2000), the understanding of the mechanism by which lipids deteriorate developed rapidly during the twentieth Century and more focus has been paid towards studying autoxidation of lipids. Autoxidation reactions commonly show an induction period (IP), which is a period during which very minute changes occur in the lipids. After the end of the IP, oxidative deterioration of lipids occurs much more rapidly as shown in Figure 2.2. Off-flavours become most noticeable after the end of the IP. One consequence of the sharp rise in the concentration of off-flavour components after the end of this period is that the rate of deterioration of foods is relatively insensitive to the precise fat content of the food. The induction period is very sensitive to small concentrations of components that shorten the IP, e.g. prooxidants, or those that lengthen the IP, namely antioxidants (Chaiyasit *et al.,* 2007; Pokorny *et al.*, 2008; Sun *et al.,* 2011). The length of the induction period is often considered as a measurement of oil stability. Consequently, the shelf-life and the final use of any lipid depend on its resistance to oxidation or oxidative

stability. The factors affecting oxidative stability of lipids are discussed in the sections that follow.

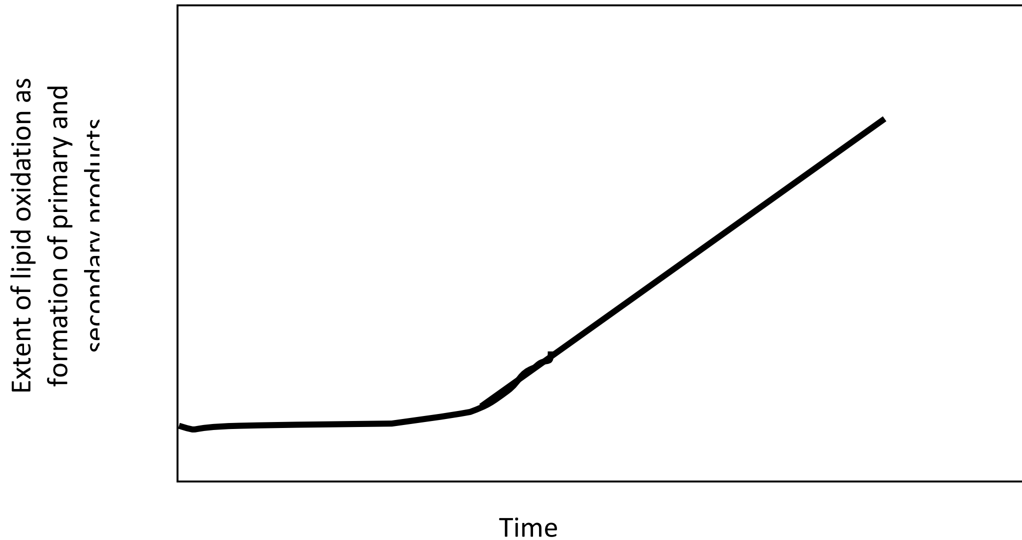


Figure 2.2 A typical induction period in fat deterioration (Pokorny *et al.*, 2008).

2.4 Factors influencing oxidative stability

Lipids are used as main ingredients in the manufacture of various foods such as ready-to-eat foods, soups, butter, among others in the food industry and other products such as lipsticks and creams for the cosmetic and pharmaceutical industries. Therefore, Changes in food formulations of a product and packaging might require changes in processing conditions and these can have an impact on long the term oxidative stability of the product (Kalakowska, 2002).

There are various intrinsic and extrinsic factors that can influence the oxidative stability of foods, some of which are fatty acid composition, oxygen concentration, temperature, surface area and transition metals (Anthony *et al.,* 2002; Shahidi & Zhong, 2005). In addition to the nature of the lipid, environmental factors to which lipids are exposed during processing and storage may also affect its oxidation rate. Although lipids are naturally occurring in various sources, production of commercial fats and oils requires extraction and refining operations, which may introduce modulations in the oxidative stability of the final products through exposure to high temperature, light, atmospheric oxygen and moisture, and possibly the contact with worn metal surfaces. The refining processes may affect minor components (e.g. phospholipids) which affect the oxidative stability of the lipids. Lipids may also undergo oxidative changes during packaging and

upon storage of bulk oils as well as during processing of the lipid-containing materials such as roasting and deep frying (Sun *et al.,* 2011).

2.4.1 Fatty acid composition

The rate of lipid oxidation is affected by the number, position and geometry of the double bonds (Anthony *et al.,* 2002). The fatty acid composition gives important information regarding the stability of oil or fat-containing food products. Unsaturated fatty acids such as oleic (C18:1), linoleic (18:2), linolenic (18:3) and eicosapentaenoic acids (C20:5) are easier targets of oxidation (Figure 2.3). According to Rahman (2007), the oxidation of saturated fatty acids (e.g. stearic acid) occurs mostly at higher temperatures and at a much slower rate as compared to PUFAs. Waraho *et al.* (2011) have found that linoleic acid is 10 – 100 times more susceptible to oxidation than saturated fatty acids during initiation and propagation steps in lipid oxidation (Choe & Min, 2006). In addition, fatty acids in the *cis* form oxidize more readily than their *trans* isomers (Anthony *et al.,* 2002; Pokorny *et al.*, 2008).

2.4.2 Oxygen concentration

Two types of oxygen can react with edible oils. One is known as atmospheric triplet oxygen ($^3O_2$), and the other is singlet oxygen ($^1O_2$). $^3O_2$ reacts with lipid radicals and causes autoxidation, which is a free radical chain reaction. When oxygen is present at low levels the rate of oxidation is approximately proportional to the oxygen concentration. If high levels of oxygen are present, the rate of oxidation is independent of the oxygen concentration (Sun *et al.,* 2011). Since the addition of oxygen to the alkyl radical is a diffusion-limited reaction, the majority of the oxygen must be removed from the system in order to inhibit lipid oxidation. Vacuum conditions are often needed to reduce oxidation since the removal of oxygen can be difficult due to its solubility being higher in oil than in water (Sun *et al.*, 2011). $^1O_2$ is an excited state of oxygen, which can be generated from $^3O_2$ by chemical, photochemical and enzymatic means as well as by decomposition of hydroperoxides. $^1O_2$ is a major reactive oxygen species (ROS) that readily (1500 times faster than $^3O_2$).

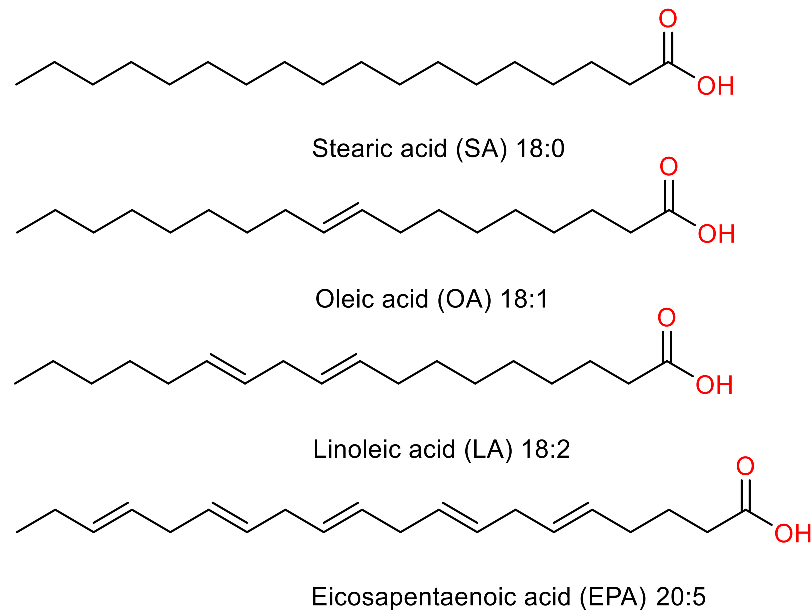


Figure 2.3 Chemical structures of stearic acid, oleic acid, linoleic acid and eicosapentaenoic acid.

$^1O_2$ is a major reactive oxygen species (ROS) that readily (1500 times faster than $^3O_2$) participates in the oxidation process (Shahidi & Zhong, 2010). Light-induced production of $^1O_2$ requires the presence of chlorophyll, pheophytins, riboflavin, myoglobin and heavy metals, which are widely found in nature. These photosensitive compounds absorb energy from light (visible or ultraviolet) and are activated to an excited state. The excited singlet photosensitizers are very unstable and tend to return to ground state either by reacting directly with the lipid substrates or activating $^3O_2$ to $^1O_2$, which readily initiates the oxidation process (Akoh & Min, 2002; Shahidi & Zhong, 2005).

2.4.3 Temperature

The activation energy (E_a) for lipid oxidation is relatively high (16.2 kcal.mol^{-1}), therefore, oxidation increases with temperature. At room temperature, autoxidation of saturated fatty acids is slow. At high temperatures, saturated fatty acids can undergo oxidation at substantial rates (Choe & Min, 2006; Sun *et al.,* 2011). In a storage trial conducted by Tazi *et al.* (2009), the peroxide value increased with an increase in temperatures from 60, 80, 100 to 120°C. However, in bulk oils, increasing temperature can decrease oxygen

solubility which, consequently, has the ability to slow oxidation rates (Anthony *et al.,* 2002). Moreover, according to studies conducted by Soyer *et al.* (2010), chicken meat was found to be prone to lipid oxidation during frozen storage. This was attributed to the high content of unsaturated fatty acid content in chicken. Also during freezing, ice crystals size and distribution in the intra- or extracellular-spaces of frozen meat vary with freezing rate, and this facilitates oxygen diffusion thus accelerating lipid oxidation.

2.4.4 Water activity

In general, the rate of lipid oxidation is low at water activities close to the water monolayer, which falls between 0.2 and 0.3 for most foods, because of a decrease in the catalytic effect of transition metals, quenching of free radicals, and singlet oxygen and/or retardation of hydroperoxide decomposition (Márquez-Ruiz *et al.*, 2013). However, the rate of lipid oxidation increases rapidly when the water activity is either decreased below or increased above the monolayer. Besides, moisture may lead to physical changes in the solid matrix of foods and affect the oil distribution and, consequently, the accessibility of oxygen to the oil.

2.4.5 Transition metals

Trace amounts of heavy metals, such as iron and copper, are commonly present in edible oils and muscle foods. These metals originate from animals, plants, soil, dust, and the metallic equipment used in processing and storage. The concentration at which these trace metals occur in natural lipids is a major factor determining the rate of oxidative deterioration of lipids (Chaiyasit *et al.*, 2007). Even when present at concentrations as low as 0.1 $mg.kg^{-1}$, transition metals can decrease the induction period and increase the rate of oxidation (Tazi *et al.,* 2009).

Dimakou *et al.* (2007) reported that transition metals are the major prooxidants in oil-in-water emulsions. This is in agreement with the well-known fact that in many commercially available oil-in-water emulsions metal chelators such as citric acid, phosphoric acid and ethylenediaminetetracetic acid (EDTA) can decrease the pro-oxidant effect of metal ions by forming a thermodynamically stable complex and reducing their redox potentials (Laguerre *et al.*, 2007; Tazi *et al.,* 2009).

2.5 Prevention and control of lipid oxidation in foods

As has been mentioned previously, food processors apply a variety of methods to increase the oxidative stability of food products. Increased stability can be attained by reducing PUFAs concentration. This is achieved by replacing PUFAs with fats having a high content of saturated fatty acid. However, this practice is contrary to nutritional recommendations that advocate increasing dietary unsaturated fatty acid for the purpose of decreasing the occurrence of coronary heart disease (Chaiyasit *et al.,* 2007; Tazi *et al.,* 2009). Another method to decrease oxidation is to use partial hydrogenation to remove the most highly unsaturated fatty acids that are very susceptible to oxidation e.g. linolenic acid. However, partial hydrogenation leads to the formation of *trans* fatty acids. Several studies have demonstrated that these are more atherogenic than saturated fats since they increase the low density lipoproteins (LDL) and decrease high density lipoprotein (HDL), which is often referred to as the "good cholesterol" (McClements & Decker, 2000).

Lipid oxidation can also be prevented by excluding oxygen from the system, for example packing under vacuum or nitrogen. This technique is used commercially to minimise lipid oxidation in mayonnaise and salad dressings during storage. Nevertheless, once the product is opened and oxygen enters, lipid oxidation will begin, thus reducing the shelf-life of the product (Chaiyasit *et al.,* 2007, Dimakou *et al.,* 2012).

The susceptibility of food products to lipid oxidation could be greatly improved by ensuring that the ingredients used in their manufacture are low in hydroperoxides, transition metals, or other pro-oxidants. This could be achieved by purchasing high-purity ingredients or by using a processing step that purifies the ingredients before use. Once pure ingredients have been obtained, it may be necessary to store them under carefully controlled conditions to avoid formation of hydroperoxides (for example refrigerated temperatures, reduced oxygen and low light) or contamination from pro-oxidants (such as pure ingredients and clean containers). From a practical standpoint, it may not be economically feasible to purchase highly purified ingredients or to use extensive clean-up or purification procedures prior to using them. Therefore, food manufacturers have to find alternative methods of dealing with the fact that most food ingredients contain significant amounts of impurities that can promote lipid oxidation.

One of the most effective means of retarding lipid oxidation in fats and oils is to incorporate antioxidants (Pokorny *et al.,* 2008; Shahidi & Zhong, 2010). Antioxidants are

substances, synthetic or naturally occurring, that can delay the onset or slow the rate of oxidation of autoxidisable materials when present at low concentrations (Chaiyasit *et al.,* 2007; Laguerre *et al.,* 2007). The following subsections briefly discuss the types and mechanism of antioxidants that can be applied in food systems.

2.6 Antioxidants

Halliwell & Gutteridge in 1995 defined antioxidants as "any substance when present in low concentrations compared to that of the oxidizable substrate significantly delays or inhibits the oxidation of that substance". Frankel & Meyer (2000) later defined food antioxidants as "substances that in small quantities are able to prevent or greatly retard the oxidation of easily oxidisable materials such as fats". Antioxidants used in food products should be nontoxic and effective at low concentration, have high stability and capability for surviving processing conditions and should not impart odour, taste or colour. Moreover, antioxidants should be easy to incorporate and have good stability in the product. Food antioxidants have been classified into two classes, namely synthetic or natural antioxidants. Synthetic antioxidants are prepared synthetically in the laboratory and natural antioxidants are extracted from plant and animal sources (Prior *et al.,* 2005; Gülçin, 2012). To be used in food applications, antioxidants must be sufficiently active to be used at low concentrations (0.001 – 0.02%) and must not be toxic. They should be able to withstand processing and cooking conditions (e.g. carry over property) and be stable in the finished product. In the food industry antioxidants can be added as direct additives or indirectly through diffusion from packaging material into the food (Shahidi & Zhong, 2010).

In general, antioxidants do not prevent oxidation, but rather extend or retard the induction period. The selection of antioxidants depends on product compatibility and regulatory guidelines and the activity of antioxidants is strongly influenced by numerous factors. Thus, compounds that are effective antioxidants in one system may be unsuitable in other systems. Some factors that influence antioxidant activity are the nature of the lipid substrate, the hydrophilic-lipophilic balance of the antioxidant, physical and chemical environments and interfacial interactions (Zamora *et al.,* 2005; Chaiyasit *et al.,* 2007).

2.6.1 Synthetic antioxidants

As it has been mentioned, synthetic antioxidants are prepared synthetically in the laboratory and were introduced in the food industry in the 1940's. They are generally phenolic compounds. Therefore, the mechanism of their reaction with radicals is the same as that of phenolic antioxidant compounds, e.g. they act as chain breaking antioxidants and involve transfer of a hydrogen atom or an electron to radicals (Kalakowska, 2002; Apak *et al.,* 2013). The more popular synthetic antioxidants used in pharmacological and food systems include: butylated hydroxyanisole (BHA), butylated hydroxyl toluene (BHT), tertiary butyl hydroquinone (TBHQ) and propyl gallate (PG) (Figure 2.4) (Decker *et al*., 2005; Carocho & Ferreira, 2013). However, these synthetic antioxidants are "label unfriendly" additives owing to the growing concern over their potential carcinogenic effects (Chaiyasit *et al*., 2007).

TBHQ is widely used in a variety of fats and oils and has excellent carry-through properties and is a very effective antioxidant for use in frying oils and vegetable oils. It is available as a beige colour powder that is used alone or in combination with BHA or BHT. Chelating agents such as monoacyglycerols and citrates enhance the activity of TBHQ, mainly in vegetable oils and shortenings (Shahidi & Zhong, 2010; Gülçin, 2012).

BHT is a fat-soluble antioxidant that is available as a white crystalline compound that has been used in foods for many years (Chaiyasit *et al.,* 2007). BHA is less stable than BHT at high temperatures and has lower carry-through properties. BHA and BHT act synergistically, and several commercial antioxidant formulations contain both of these antioxidants. BHT is effectively used in oxidation retardation of animal fats (Frankel & Meyer, 2000). In many developing countries, BHT is still used in food products despite the reported safety concerns. The disadvantages of using gallates such as PG lie in their tendency to form dark-coloured complexes with iron and their heat sensitivity (Decker *et al*., 2005; Gülçin, 2012). In most countries, the use of synthetic antioxidants is regulated and the safety of the compounds involved has been tested based on long-term toxicity studies (Kiokias *et al*., 2009; Shahidi & Zhong, 2010). The four major synthetic antioxidants in use (Fig. 2.3) are subjected to a good manufacturing practice (GMP) limit of 0.02% of fat or oil content of the food.

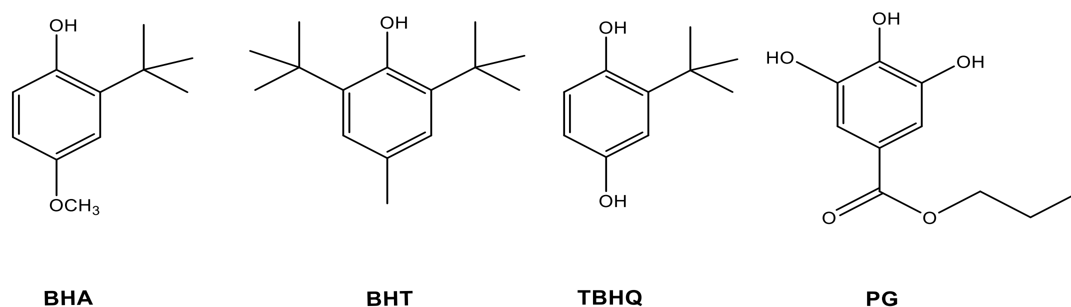


Figure 2.4 Structures of the most recognized and popular synthetic antioxidant.

2.6.2 Natural antioxidants

During the past two decades, intensive research has been carried out on naturally occurring antioxidative compounds from different sources. The main impetus behind this search was to reduce the use of synthetic compounds as food additives due to their potential negative health effects and as a result of consumer demand (Carocho & Ferreira, 2013). Higher plants and their constituents provide a rich source of natural antioxidants. Fruits, vegetables, spices, herbs, cereals, grains, seeds and teas are important source of plant-derived antioxidants, especially phenolic compounds, including tocopherols (Kiokias *et al.,* 2009; Gülçin, 2012).

Natural antioxidants may also act as colourants (flavonoids, condensed tannins or anthocyanins) and they may act as preservation agents (most phenolic antioxidants have bactericidal properties). However, some might impart unfavorable sensory characteristics such as flavour, texture, or color to the stabilized food product (McClements & Decker, 2000). It is useful to consider both the advantages and disadvantages for each particular case and to decide which antioxidant to choose (Table 2.1). Another shortcoming of natural antioxidants is the necessity to use much higher amounts, often at least ten-fold, than synthetic antioxidants.

Ascorbic acid (vitamin C) is considered as one of the most powerful, least toxic natural antioxidants (Gogus *et al.,* 2009). This water soluble vitamin is found in high concentrations in many dietary plants. Typically it reacts with oxidants. Ascorbic acid can terminate radical chain reactions by electron transfer.

Table 2.1 Advantages and disadvantages of synthetic and natural antioxidants commonly used in in food (Márquez-Ruiz *et al*., 2013).

Synthetic antioxidants	**Natural antioxidant**
Widely applied	Usage of in some products restricted
Medium to high antioxidant activity	Wide ranging antioxidant activity
Inexpensive	Expensive
Increasing safety concerns	Clean labelling
Low water solubility	Broad range of solubilities
Usage of some of them banned	Increasing usage and expanding applications
Some of them stored in adipose tissue	Completely metabolised

However, ascorbic acid contributes to browning of foods since it is easily oxidised and decomposed under common storage and processing conditions. For this reason ascorbic acid is responsible for the browning of fruit juices and concentrates (Gogus *et al.,* 2009; Gülçin, 2012).

Considering the safety concerns and consumer demands with regards to synthetic antioxidants, natural antioxidants appear as healthier and safer alternatives to synthetic antioxidants. Hence, there is a growing interest in natural and safer antioxidants for food applications, and a growing trend in consumer preferences towards natural antioxidants, all of which have given impetus to the attempts to explore natural sources of antioxidants, especially Maillard reaction products (MRPs) which are a form of natural antioxidants formed or produced during food processing (Chaiyasit *et al.,* 2007). MRPs allow food processors to produce stable products with ‘clean’ labels and bring less rigorous burden of safety proof than that required by synthetic products. There have also been reports of

volatile MRPs, which are responsible for flavours in food products, possessing antioxidative activities. These studies clearly indicate that some flavour chemicals possess antioxidative activities (Osada & Shibamoto, 2006)

2.7 Classification and mechanism of antioxidants

Antioxidants can inhibit lipid oxidation based on the mechanism of their action, and they are classified into primary antioxidants, secondary antioxidants, and synergistic antioxidants.

2.7.1 Primary antioxidants

A primary antioxidant, also known as a chain-breaking antioxidant is a substance that is capable of accepting free radicals so that it can delay the initiation step or interrupt the propagation step of autoxidation (Huang & Prior, 2005; Prior *et al.*, 2005). Chain-breaking antioxidants react with lipid or peroxyl radicals and convert them to more stable radical, or non-radical products. Chain-breaking antioxidants have increased or higher affinity for peroxyl radicals than lipids and, therefore, tend to scavenge the free radicals produced during the initiation and propagation steps. These differ in their effectiveness in inhibiting lipid oxidation, partly due to their physiological properties, but also due to their physical location within the system. Antioxidants that may be effective in retarding lipid oxidation in bulk oils may not be effective in emulsions. For example, hydrophilic antioxidants are less effective in oil-in-water emulsions than lipophilic antioxidants, whereas lipophilic antioxidants are less effective in bulk oils than hydrophilic antioxidants (Decker *et al.*, 2005; Apak *et al.*, 2013). Synthetic food additives such as BHA, BHT and TBHQ are common chain-breaking antioxidants used in food systems. Naturally occurring phenolics such as flavonoids, eugenol, vanillin, and rosemary antioxidants also possess chain-breaking properties (Apak *et al.*, 2013). MRPs derived from glucose-glycine, fructose-lysine and casein-sugar models have been reported to possess primary or chain-breaking antioxidant activity (Kim & Lee, 2009a). Other researchers have reported that primary antioxidants are effective at very low concentrations but at higher levels they may become prooxidants (Shahidi & Zhong, 2010).

2.7.2 Secondary antioxidants

Secondary or preventative antioxidants can retard lipid oxidation through a variety of mechanisms, including chelation of transition metals, replenishing of hydrogen to primary antioxidants and deactivation of reactive oxygen species (ROS). What distinguishes these from chain-breaking antioxidants is the fact that they do not involve the conversion of free radicals to more stable products (Gülçin, 2012).

As previously mentioned, transition metals are prooxidants capable of accelerating lipid oxidation reactions. Chelators are a group of secondary antioxidants that can bind and thus inactivate or reduce the activity or prooxidant metals. The most common food chelators are citric acid, phosphoric acid and EDTA. The synergistic effect of citric acid is attributed to metal chelation. Other polyvalent acids such as tartaric acid, malic, gluconic, oxalic and succinic acid as well as sodium triphosphate also possess synergistic effects similar to citric acid (Frankel & Meyer, 2000). Moreover, ascorbic acid can act as a synergist with tocopherols by regenerating or restoring their antioxidant properties (Gogus *et al.,* 2009). Oxygen scavengers and reducing agents act by scavenging oxygen and donating hydrogen atoms to peroxyl radicals and primary antioxidants. The hydrogen donation to primary antioxidant radicals regenerates primary antioxidants and this facilitates more effective use of primary antioxidants (Huang & Prior, 2005; Gülçin, 2012). Examples of antioxidants in this group include ascorbic acid and its derivatives as well as sulphides. MRPs derived from glucose-glycine, fructose-lysine and casein-sugar models have been reported to exhibit metal chelation activity (Kim & Lee, 2009b; Vhangani & Van Wyk, 2013).

2.7.3 Antioxidant activity of Maillard reaction products

The Maillard reaction (MR) was first observed by French Chemist Louis-Camille Maillard is classified as a non-enzymatic browning reaction, which is a very complex reaction between carbonyl-containing compounds, such as reducing sugars, aldehydes or ketones, and an amino-containing compound such as amino acids, proteins, or any nitrogenous compound (Liu *et al.*, 2012). The MR occurs during food processing, packaging and storage and produces a wide range of Maillard reaction products (MRPs), which contribute to odour, flavour, and colour of foods (Maillard *et al.*, 2007; Kim & Lee, 2009). The MR is affected by factors such as temperature, time, water activity (a_w),

intrinsic properties of sugar and protein, and the amino group:reducing sugar ratio influence the yields and types of MRPs. Several authors proved that MRPs possess antioxidant activity in food products in which they are formed or are added to (Gogus *et al.,* 2009). Several researchers have reported that the antioxidant activity of MRPs increased with the development of browning intensity and UV absorbance (Chen & Kitts, 2011), while others observed that there was good correlation between fluorescence (374nm/415nm) and good antioxidant capacity in MRPs. Antioxidant character of MRPs has been observed *via* radical chain-breaking activity, scavenging of reactive oxygen species (ROS), decomposition of hydrogen peroxide and metal chelating activity, thus potentially providing more than one mechanism of action in combatting lipid oxidation (Gu *et al.,* 2010; Yu *et al.*, 2012; Vhangani & Van Wyk, 2013). The beneficial antioxidative effects of MRPs are currently gaining much attention. Studies have primarily focused in model systems including ribose-lysine, fructose-lysine, and glucose-casein and in food products such as coffee, bread crust, beer vinegar (Morales & Jiménez-Péréz, 2004; Vhangani & Van Wyk, 2013). These studies and many others have demonstrated that MRPs, independent of the reaction conditions, displayed in vitro antioxidant protective effects on lipids. Despite numerous studies conducted to understand the MR, many of its chemical pathways are still speculative. In general, the limited knowledge of mechanisms is partially due to the complexity of the reaction, but also due to the analytical techniques that are available. Therefore, since antioxidants function at each stage of lipid oxidation *via* different mechanisms, it is crucial to apply the correct knowledge in order to select the correct antioxidant. Laguerre *et al.* (2007) advised the implementation of more than one assay when evaluating the antioxidative capacity of food components, since a single assay cannot determine the effectiveness of all antioxidant types (Laguerre *et al.*, 2007; Pokorny *et al.*, 2008).

2.8 Measurement of antioxidant capacity

The terms “antioxidant activity” and “antioxidant capacity” have different meanings: antioxidant activity deals with the kinetics of a reaction between an antioxidant and the prooxidant or radical it reduces or scavenges, whereas antioxidant capacity measures the thermodynamic conversion efficiency of an oxidant probe upon reaction with an antioxidant. Measuring the antioxidant activity/capacity levels in food and biological fluids

(e.g. serum) is carried out for the meaningful comparison of the antioxidant contents of foodstuffs and for the diagnosis and treatment of oxidative stress-associated diseases in clinical biochemistry (Ross & Smith, 2006; Apak *et al.,* 2013).

In order to accurately evaluate the potential of antioxidants in food systems, the ideal method should be conducted under the chemical, physical, and environmental conditions similar to those expected in biological systems. However, in food products these conditions are not consistent. Therefore, accelerated methods are required (Decker *et al*., 2005). Many simplistic one-dimensional methods that use a broad range of conditions, catalysts of oxidants or prooxidants and those that measure end points of oxidation have been developed to measure the free radical scavenging or “antiradical” ability of antioxidants (Ross & Smith, 2006). Since antioxidant capacity methods are non-specific and one-dimensional, they cannot be used to investigate the multiple protection mechanisms of synthetic and natural antioxidants or synergistic effects between antioxidants. Therefore, having a convenient method for the rapid quantitation of antioxidant effectiveness is appealing to researchers (Huang *et al*., 2005). It was reported by Prior *et al.* (2005) that reliable methods for antioxidant assessment are needed. However, a selection of one ideal method for the determination of antioxidants is not realistic. Nevertheless, the very large number of assays used by different groups constitutes a major difficulty in the search for potent antioxidants since it interferes with the possibility of comparing results of different laboratories. Even when different assays are used to evaluate the same criterion, the result may vary considerably (Frankel & Meyer, 2000).

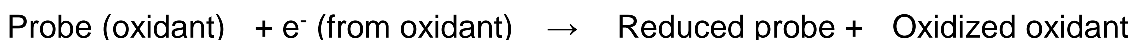
Antioxidant capacity assays can be broadly classified into three types: hydrogen atom transfer (HAT), (single) electron transfer ((S) ET) and a combination of both (Lu *et al*., 2011; Apak *et al*., 2013). Table 2.2 provides a list of some of the most important and widely used HAT and ET assays to determine antioxidant capacity of both synthetic and natural antioxidant. HAT-based methods are more relevant to the radical chain-breaking antioxidant capacity based on their quantification of hydrogen atom donating capacity and involvement of peroxyl radicals versus ET-based methods which measure an antioxidants reducing capacity (Huang *et al*., 2005; Laguerre *et al*., 2007). The following section briefly reviews the indirect and direct antioxidant assays, recent application as well as the advantages and limitations of each method.

Table 2.2 Hydrogen atom transfer (HAT) and Electron Transfer (ET) Assays (Pokorny *et al.*, 2008).

HAT	**ET**
Oxygen Radical Absorbance Capacity (ORAC)	Trolox Equivalent Antioxidant Capacity Assay (TEAC)
Total Radical Trapping Antioxidant Parameter (TRAP)	Ferric Ion Reducing Antioxidant Parameter (FRAP)
Crocin Bleaching Assay	2,2-Diphenyl-1-Picrylhydrazyl (DPPH)
Chemiluminescent assay	Total phenolics assay by Folin-Ciocalteu reagent assay
Total Oxyradical Scavenging Assay (TOSCA)	Total Antioxidant Assays Using Cu^{2+} Complex as an oxidant

2.8.1 ET methods

In most ET-based assays, there are two components involved in the reaction mixture: antioxidants and the oxidant (which is the probe). The probe will abstract an electron from the antioxidant causing a colour change of the probe. The colour change is used to monitor the reaction and works as an indicator of the reaction endpoint. ET-based assays include Trolox equivalence antioxidant capacity (TEAC), 2, 2-diphenyl-picrylhydrazyl (DPPH) (though the first two are considered as mixed HAT/ET-based assays by some researchers), ferric ion reducing antioxidant power (FRAP) and Folin-Ciocalteu reagent (FCR) (Gülçin, 2012; Apak *et al.*, 2013). They are based on the following electron transfer reaction:



Probe (oxidant) + e^- (from oxidant) → Reduced probe + Oxidized oxidant

The probe itself is an oxidant that abstracts an electron from the oxidant, causing colour changes of the probe. The degree of colour change is proportional to the antioxidant concentrations. The reaction endpoint is reached when the colour change stops (Gülçin, 2012; Apak *et al*., 2013).

2.8.1.1 DPPH (2, 2-diphenyl-1-picrylhydrazyl)

One of the earliest synthetic radicals to be used in a substrate-free assay in order to study the effects of structures on the activity of antioxidants is 2, 2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$). This assay was conceptualised by Blois (1958) (as cited by Molyneux, 2004) and has been improved by Brand-Williams *et al*. (1997). DPPH$^{\bullet}$ serves as both the oxidizing radical to be reduced by the antioxidant (RH) and the colour indicator for the reaction as depicted in Figure 2.5, the purple colour changes to yellow (Sharma & Bhat, 2009). The stable DPPH-RS radical scavenging assay is based on electron transfer and has been used widely for determining primary antioxidant activity. This assay determines free radical scavenging activity of all antioxidants, including those in pure form, as well as components of food products and plant and fruit extracts (Kulisic *et al.*, 2004). The effect of an antioxidant on decreasing the absorption of DPPH$^{\bullet}$ in an ethanol or methanol solution at 515 – 517 nm is measured spectrophotometrically until the absorbance reaches a steady state. The usage of methanol is not preferred due to its toxic properties. The assay time could take anywhere from 5 min to 8 hours (Shahidi & Zhong, 2010; Apak *et al*., 2013). The incubation time differs from 1 to 60 min, although 30 minutes incubation was reported in the original paper by Blois (1958) (as cited by Molyneux, 2004). Sanchez-Moreno (2002) classified the kinetic behaviour of antioxidant compounds as follows: < 5 min (rapid), 5 – 30 min (intermediate), and > 30 min (slow). Different types and concentrations of antioxidants can significantly vary the decay slope and the absorbance level reached by the remaining DPPH$^{\bullet}$ radicals. The antioxidant concentration and the time necessary to reach the steady state to 50% of the initial DPPH$^{\bullet}$ concentration are referred to as the antiradical efficiency or IC_{50} (Huang *et al*., 2005; Roginsky & Lissi, 2005).

Compared with other methods, the DPPH$^{\bullet}$ assay has many advantages, such as good stability, credible sensitivity and feasibility (Shahidi & Zhong, 2010; Dong *et al,*

2011). The test requires only a UV-VIS spectrophotometer to perform, which probably explains its widespread use in antioxidant screening.

However, there are limitations to this assay. Firstly, it does not use a substrate, hence providing no information on the protective activity of antioxidants towards food systems. Secondly, $DPPH^{\bullet}$ radicals are artificially generated; therefore the assay is not relevant to real food lipid radicals. Lastly, $DPPH^{\bullet}$ radicals are long-lived nitrogen radicals that are relatively more stable than and bear no direct similarity to peroxyl radicals which are involved in lipid oxidation (Huang *et al.*, 2005; Sharma & Bhat, 2009).

2.8.1.2 FRAP (Ferric reducing antioxidant power)

In the FRAP assay, direct measurement of the ability of antioxidants to reduce a ferric tripyridyltriazine (TPTZ) complex to its ferrous complex at low pH is determined (Roginsky & Lissi, 2005). TEAC is carried out at neutral pH, and the FRAP assay is conducted at acidic pH 3.6 to maintain iron solubility

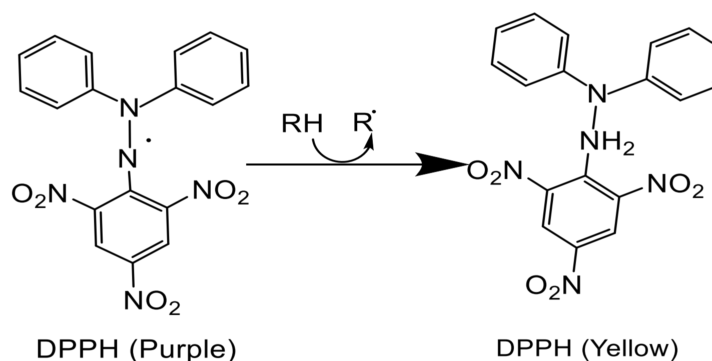


Figure 2.5 DPPH radical scavenging effect by an antioxidant.

Similar to the TEAC assay, except for the decreased pH, this method is based on the redox potential of a ferric complex and the resulting blue colour is measured spectrophotometrically at 593 nm at 0.5 s and then every 15 s for 4 min. The change in absorbance is linearly related to the total reducing power of the electron-donating antioxidants present in the system (Huang *et al.,* 2005; Laguerre *et al.*, 2007). The FRAP assay is simple, speedy and the total antioxidant power can be directly measured (Shahidi

& Zhong, 2010). The FRAP mechanism is totally ET-based rather than mixed ET/HAT-based, so in combination with other methods it can be very useful in distinguishing dominant mechanisms with different antioxidants. However, there are limitations to the assay. The major limitation to this assay is that the measured reducing capacity reflects the total antioxidant concentration, not necessarily specific antioxidant activity. In addition, there is no information provided regarding the protective properties of antioxidants since no oxidizable substrate is included in the assay (Prior *et al.*, 2005; Berker *et al.*, 2007).

2.8.1.3 ABTS [(2'2-azinobis (3 ethylbensothioazoline -6 sulfonate)]

The original 2'2-azinobis (3 ethylbensothioazoline -6 sulfonate) (ABTS) assay was developed by Miller & Rice-Evans in 1993 and subsequently improved (Huang *et al.,* 2005; Prior, 2005). In this assay, ABTS is generated by chemical reaction of $K_2S_2O_8$ (Figure 2.6) is oxidized by oxidants to its radical cation, $ABTS^{\bullet+}$, which is intensely coloured, and antioxidant capacity is measured as the ability of the test compound to decrease the colour reacting directly with ABTS radical (Roginsky & Lissi, 2005). If Trolox is used, the antioxidant capacity is expressed as Trolox equivalent antioxidant capacity (TEAC). $ABTS^{\bullet+}$ is applicable for both lipophilic and hydrophilic compounds. Also, the ABTS radical scavenging assay can be evaluated over a wide pH range, which is useful to study the effect of pH on antioxidant mechanisms for food components. Reaction times ranging from 1 – 30 minutes have been adopted throughout protocols described in the literature. Moreover, this assay has been criticized as the ABTS radical is not representative of biomolecules and not even found in any biological and food systems. Concerning the detection wavelength, the determination at 734 nm is preferred since the interference from other absorbing components and from sample turbidity is minimized (Pinchuk *et al.,* 2012).

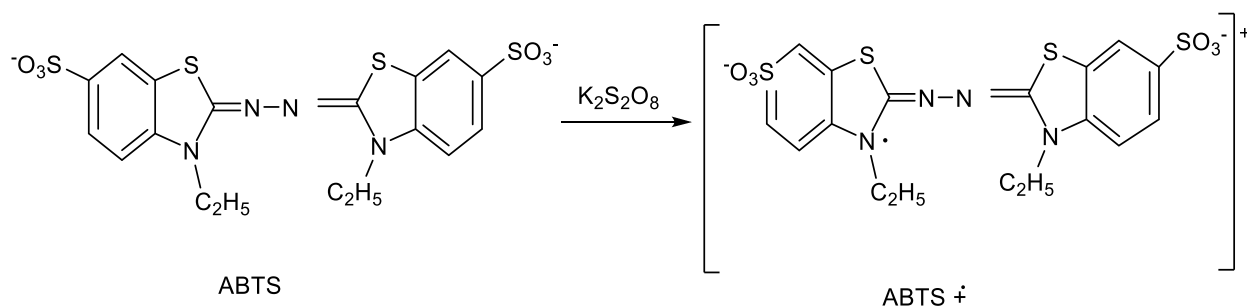
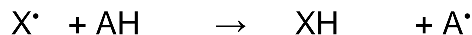


Figure 2.6 Oxidation of ABTS with $K_2S_2O_8$ and generation of $ABTS^{\bullet+}$.

2.8.2 HAT methods

HAT-based assays measure the capability of an antioxidant to quench free radicals by H-atom donation (AH = any H donor). A HAT-based assay, represented by the ORAC assay, involves peroxyl radicals as the oxidant and will provide useful information on radical chain breaking capacity. They are based on the following hydrogen atom transfer reaction:



$$X^{\bullet} + AH \rightarrow XH + A^{\bullet}$$

2.8.2.1 ORAC (oxygen radical absorbance capacity)

The ORAC assay measures the ability of antioxidants to scavenge peroxyl radicals (Roginsky & Lissi, 2005). According to Shahidi & Zhong (2010), the methods which measure antioxidant scavenging activity against peroxyl radical production induced by 2, 2'-azo-bis (2-amidinopropane) dihydrochloride (AAPH or ABAP) at 37°C were first developed by Cao & Alessio in 1993. Various probes can be utilized as the fluorescent probe, including phycoerythrin and fluorescein. The loss of fluorescence of the probe is an indication of the extent of damage from its reaction with the peroxyl radical. The protective effect of an antioxidant is measured by calculating the area under the time-recorded fluorescence decay curve (AUC) and the antioxidant capacity is expressed as µmoles of Trolox equivalents (Huang *et al.,* 2005).

The advantage of ORAC is in its AUC approach. The AUC approach applies equally well for both antioxidants that exhibit a distinct lag phase and those that have no lag phase. It unifies the lag time method and initial rate method and it can be applied to food samples. The ORAC assay has been widely used in academics and the food and supplement industries as the method of choice for quantifying antioxidant capacity (Huang *et al*., 2005). One drawback of the ORAC method is the assumption that the oxidative deterioration of the fluorescent substrate can simulate food substrates (Shahidi & Zhong, 2010). Measuring the effects of an antioxidant by the integrated areas under the decay curves, including total oxidation period, can be misleading because it does not distinguish between the initiation and propagation phases which are significant in relation to oxidative deterioration in foods. ORAC uses Trolox as a reference compound, which is not structurally related to any phenolic compounds found to be sources of antioxidants in foods (Huang *et al.,* 2005). In addition, by using an artificial water soluble azo-compound

as a radical generator, ORAC does not provide a useful estimate of the important protective activities of metal chelators and lipophilic antioxidants in food systems (Shahidi & Zhong, 2010).

2.8.2.2 TRAP (Total Radical Antioxidant Power)

The TRAP assay was developed by Wayner *et al.* (1985). This assay monitors the ability of antioxidant compounds to interfere with the reaction between peroxyl radical generated by AAPH or ABAP and a target probe. Different variations of the method/assay have used oxygen uptake (Huang *et al.*, 2005), fluorescence or R-phycoerythrin (Roginsky & Lissi, 2005) or absorbance of ABTS as the reaction probe. The basic reactions of the assay are similar to those of ORAC (Gülҫin, 2012). Requirements for the assay are that the probe must be reactive with peroxyl radicals at low concentrations, there must be a dramatic spectroscopic change between the native and oxidised probe to maximize sensitivity, and no radical chain breaking beyond probe oxidation should occur. The antioxidant activity is determined as the time required to consume all of the antioxidant, by extension of the lag time for appearance of the oxidized probe when antioxidants are present and by percentage reduction of a reaction. TRAP values are usually expressed as lag time or reaction time of the sample compared to corresponding time of Trolox (Gülҫin, 2012).

It was reported that the main short-coming of this assay is the use of the lag phase for quantifying antioxidant capacity since not all antioxidants possess an obvious lag phase and also the antioxidant capacity profile after the lag phase is ignored. Moreover, TRAP assay involves the initiation of lipid peroxyl radicals and is sensitive to all known chain breaking antioxidants, but it is relatively complex and time-consuming to perform, requiring a high degree of expertise and experience (Huang *et al.*, 2005; Gülҫin, 2012).

2.8.3 Metal chelation

Transition metal ions such as Fe^{2+} and Cu^{2+} can catalyse the generation of ROS such as hydroxyl radical ($OH^{\bullet}$) and superoxide anion ($O_2^{\bullet-}$). In particular, Fe^{2+} generates $OH^{\bullet}$ by the Fenton reaction that accelerates the lipid oxidation chain reaction. Chelators can form complexes with metal ions and inhibit the Fenton reaction. Therefore, chelation of metal ions contributes to antioxidant capacity (Prior *et al.*, 2005; Gülҫin, 2012). MRPs are known metal chelators and their metal-ion binding affinity has been proposed as a possible

mechanism to explain their antioxidant activity (Kitts, 2004). The most common food chelators are citric acid, phosphoric acid and EDTA. Some chelators can be effective when used alone but can greatly enhance the action of phenolic free radical scavengers when used in combination. In addition, some chelators may increase oxidative reaction under certain conditions by increasing metal solubility or altering the redox potential of the metal (Gülçin, 2012). For instance, the antioxidative properties of EDTA is determined by its concentration in relation to prooxidant metals and can act as prooxidants when the ratio of EDTA to iron is less than 1, and antioxidant ratio is equal or greater than 1 (Prior *et al.*, 2005).

2.8.4 Alternative methodologies

In the hydrogen peroxide (H_2O_2) scavenging activity assay, horseradish peroxidases are used to oxidize scopoletin to a non-fluorescent product and antioxidants seem to inhibit this reaction. Its results are ambiguous due to the various pathways that lead to the inhibition (Huang *et al.,* 2005). The antioxidants can inhibit the reaction by (a) reacting directly with H_2O_2, (b) reacting with intermediates formed from enzymes and H_2O_2, or (c) inhibiting the horseradish peroxidase from binding H_2O_2. Therefore, it is difficult to explain the actual chemical meaning of the data (Prior *et al.,* 2005; Carocho & Ferreira, 2013).

The superoxide anion ($O_2^{-\bullet}$) scavenging capacity assay is optimized for enzymatic antioxidants and relies on the competition kinetics of $O_2^{-\bullet}$, reduction of cytochrome C (probe) and $O_2^{-\bullet}$ scavenger (sample) (Huang *et al.,* 2005).

Biologically, the hydroxyl radical ($HO^\bullet$) is widely believed to be generated when hydrogen peroxide reacts with Fe(II) (Fenton reaction). However, the Fe(II)/H_2O_2 mixture has disadvantages in a scavenging assay because many antioxidants are also metal chelators. When the sample is mixed with Fe(II), it may alter the activity of Fe(II) by chelation. As a result, it is impossible to distinguish if the antioxidants are simply good metal chelators or $HO^\bullet$ scavengers. Antioxidants in food (such as vitamin C) may act as pro-oxidants by reducing Fe(III) to Fe(II) and thus catalyses $HO^\bullet$ generation. In fact, ascorbic acid has been used in combination with catalytic Fe(II) and excess H_2O_2 to generate a constant flux of $HO^\bullet$ radicals (Prior *et al.,* 2005).

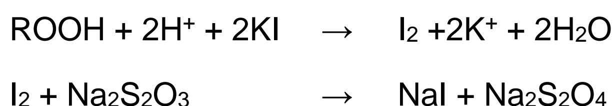
2.9 Measurement of lipid oxidation

Numerous analytical methods are routinely used for measuring lipid oxidation in foods. It is emphasized that evaluating lipid oxidation is a challenging task due to a number of factors such as different compounds which are formed or produced depending on time, the extent of oxidation and the mechanism involved. Therefore, choosing a single assay or parameter to analyse the oxidative stability is rather a difficult and it is frequently convenient to combine different methods. Traditionally, the initial or primary oxidation products are assayed and the secondary products, characterizing sensory changes in lipids, are included in the analysis as well (Laguerre *et al.*, 2007). A summary of lipid oxidation tests is presented in Table 2.3 followed by a discussion.

2.9.1 Determination of primary oxidation products

2.9.1.1 Lipid hydroperoxides

Hydroperoxide is the primary product of unsaturated lipid oxidation. The peroxide value directly measures the concentration of hydroperoxides formed in the initial stage of lipid oxidation. The iodometric titration method is commonly used to monitor the hydroperoxides based on the principle of sodium thiosulphate titrate with iodine produced from a reaction of lipid peroxide with potassium iodine. Thus the content of peroxide can be calculated directly from the equation expressed as:



$$ROOH + 2H^+ + 2KI \rightarrow I_2 + 2K^+ + 2H_2O$$

$$I_2 + Na_2S_2O_3 \rightarrow NaI + Na_2S_2O_4$$

According to this method, PV is considered to represent the quantity of oxygen (in meq) contained in one kilogram of lipid and which could oxidise potassium iodide. This assay, however, shows some drawbacks, mainly derived from the iodine high susceptibility to oxidation in the presence molecular oxygen and accelerated light exposure. Additionally, the presence of water in this assay is very detrimental, thus in case of use in emulsion analysis a previous lipid quantitative and non-altering of lipids is required. The peroxide value does not give a real measure of oxidative degradation; since peroxides are usually further degraded, so simultaneous measurements of secondary products is appropriate.

In addition to the volumetric method (titration), an alternative spectrophotometric assay has been developed. The ferric thiocyanate method is more sensitive than other peroxide methods and requires a smaller sample size (± 5.0 g). This method is based on the oxidation of ferrous to ferric ions by hydroperoxides in an acidic medium in the presence of xylenol orange, which has a UV maximum around 550 – 560 nm. This method is convenient, fast and sensitive, whereas it is required to know the characteristic of the lipid peroxide and to strictly control the test condition which is not the same in a different situation.

Nevertheless, the peroxide value is an empirical measure of oxidation which is useful for samples that are oxidized to relatively low levels under mild conditions so that the hydroperoxides are not appreciably decomposed. During oxidation, the peroxide value reaches a maximum peak and then begins to decrease at more advanced stages of lipid oxidation (Laguerre *et al.,* 2007). The maximum peroxide value can occur at earlier or later stages, depending on the fatty acid composition of the oil and the conditions of oxidation. In oils high in unsaturated fatty acids, such as fish oils, the peroxide value maximum will occur at an earlier stage because their hydroperoxides decompose more rapidly. Hydroperoxides will also rapidly decompose during oxidation conditions involving temperatures over 100°C, exposure to light and the presence of metals (Shahidi & Zhong, 2005). In order to determine individual peroxides, high-performance liquid chromatography (HPLC) methods can be used (Kalakowska, 2002; Rahman, 2007). After the initiation phase, the level of primary oxidation products increases and passes through a maximum. Using PV as a sole determination of oxidation level can therefore be misleading, and it is important to know the history of the oil (Rahman, 2007). However, from the perspectives of food safety and quality, the determination of PV remains the most important quality control measures of edible oils since it is an indicator of primary oxidation status of the oils.

Table 2.3 Summary of Lipid oxidation tests (Shahidi & Zhong, 2005).

Assay	Oxidation level	Oxidation products	Assay type
Peroxide value (PV)	Primary lipid oxidation	Lipid hydroperoxides	Titrimetric (iodometric) Spectrophotometric (ferrous oxidation xylenol orange)
Acid value	Primary lipid oxidation	Free fatty acids	Titrimetric
Oxygen bomb	Primary lipid oxidation	Oxygen depletion	Accelerated (oxygen uptake)
Schaal oven	Primary lipid oxidation	Lipid hydroperoxides	Accelerated (titrimetric or spectrophotometric)
Active oxygen method	Primary lipid oxidation	Lipid hydroperoxides	Accelerated (titrimetric or spectrophotometric)
***P*-Anisidine value (AV)**	Secondary lipid oxidation	Saturated α and β-aldehydes	Spectrophotometric (colorimetric)
Thiobarbituric acid reactive substance (TBARs)	Secondary lipid oxidation	Malonaldehyde	Spectrophotometric (colorimetric)
Volatile profile	Secondary lipid oxidation	Volatile compounds	Chromatographic (HPLC and GC)
Total polar compounds	Secondary lipid oxidation	Polymerised compounds (dimeric and polymeric triglycerides)	Chromatography (HPLC)
Oxidative stability index (Rancimat)	Secondary lipid oxidation	Volatile organic acids	Accelerated (conductivity)
Thermogravimetric analysis (TGA)	Secondary lipid oxidation	Weight gain	Accelerated (oxygen uptake)
Differential scanning calorimeter (DSC)	Secondary lipid oxidation	Thermal transition	Accelerated (exothermic reaction)
Sensory evaluation	Secondary lipid oxidation	Off- flavours and colours	Sensory perception (smell, taste, colour and texture)

2.9.1.2 Oxygen consumption

In the lipid oxidation process, oxygen reacts with lipids. Therefore, the uptake of oxygen can be measured as a parameter that indicates lipid oxidation. The technique to measure dissolved oxygen comprises an oxygen electrode with a Clark-type membrane, where a current that is proportional to the concentration of dissolved oxygen is created (Shahidi & Zhong, 2005). The electrode is connected to an oxygen meter, where the current is converted into oxygen concentration, shown on a display. To measure the oxygen consumption, the sample is located in a closed container. The technique has also been used for emulsion systems (Prior *et al.,* 2005).

Consumption of oxygen during the initial stage of autoxidation results in an increase in the weight of the fat or oil, which theoretically reflects its oxidation level. A classical weight gain method which involves heating an oil and periodically testing it for weight gain. Air samples are weighed and stored in an oven at a set temperature with no air circulation. Samples are then removed from the oven at different time intervals, cooled at ambient temperature and reweighed. The weight gain is then recorded. The induction period is then obtained by plotting weight gain against storage time. This method has several drawbacks such as continuous heating of sample which may give rise to non-reproducible results, requiring long analysis time and intensive human participation. Antolovic *et al,* 2002 suggested an improved technique with more sophisticated continuous monitoring of mass and energy changes as in thermogravimetric analysis (TGA) or Differential scanning calorimetry (DSC).

2.9.1.3 Conjugated diene value

Ultraviolet spectrophotometry is a commonly used method to detect lipid oxidation containing or generating conjugated unsaturation. With this method, conjugated dienes produced during the refining process of oil the course of fatty acid oxidation, can be measured (Roginsky & Lissi, 2005). The samples (oils, fats or emulsions) are diluted in organic solvents. The conjugated dienes have a strong absorption maximum at 234 nm

and are determined directly at this wavelength. Thus, the method is straight forward and rapid but the complication is that it can deliver reliable results only in the early stage of lipid oxidation (Sun *et al.,* 2011). Secondary oxidation products and polymers generated from the decomposition of lipid hydroperoxides at later stages of oxidation also absorb at 234 nm. Therefore, it can become unclear to what extent the measured concentration is due to the presence of conjugated diene or secondary oxidation products. The method has also been described as being unsuitable for samples containing free PUFAs (Sun *et al.,* 2011).

2.9.2 Determination of secondary oxidation products

2.9.2.1 Thiobarbituric Acid Reactive Substance (TBARs) test

The thiobarbituric acid (TBA) method is used to measure the extent of secondary lipid oxidation products. The basis of this test is the absorbance of a pink colour complex at 532 – 535 nm which is formed between thiobarbituric acid (TBA) and oxidation products of polyunsaturated lipids. This colour complex is formed from the condensation of two moles of TBA with one mole of malonaldehyde (MDA) under acidic thermal conditions (Roginsky & Lissi, 2005; Laguerre *et al*., 2007). The determination of the TBA value is expressed as mg of malonaldehyde per kg of sample ($mg.kg^{-1}$). Temperature, time of heating, pH, metal ions and antioxidants are all factors that can affect the production of the pink colour complex. Variations to the TBA test are designed to increase the sensitivity, including heating in acids and adding ferric ions, or reducing production of decomposition materials during the assay by adding antioxidants or metal chelators (Laguerre *et al*., 2007). This assay has been criticised for some reasons lie the fact that TBA is not selective to MDA, since it also reacts with many other compounds such as aldehydes, carbohydrates, amino acids and nucleic acids, interfering with in the TBA assay and resulting in considerable overestimation, as well as variability in results. This is why it is also referred to as thiobarbituric reactive substances (TBARs) assay. Despite the mentioned limitations, the TBARs assay is preferred due to its simplicity and provides an excellent means for evaluating lipid oxidation in foods, especially on a comparative basis.

2.9.2.2 p-Anisidine Value (p-AnV)

When hydroperoxides breakdown they produce volatile aldehydes like hexanal, leaving behind a non-volatile portion of the fatty acid that remains as part of the lipid molecule. The reaction of these non-volatile aldehydes with *p*-anisidine reagent under acidic conditions affords yellowish products that absorb at 350 nm (Antolovic *et al.,* 2001). The *p*-anisidine value (*p*-AnV) is one of the oldest methods use for evaluating lipid oxidation in animal fats and vegetable oils and is defined as 100 times the absorbance solution containing 1 g of fat in 100 mL of solvent. The *p*-AnV is a reliable indicator of oxidative rancidity fats and oils and fatty foods. However, caution must be exercised when performing the assay due to toxicity of the anisidine reagent. PV and *p*-AnV allow calculating the total oxidation, the Totox value is determined as:

$$\text{Totox} = 2 \times \text{PV} + p\text{-AnV}$$

The Totox value combines evidence of about the past history and present state of an oil, and at present is used frequently to estimate the extent of oil oxidation in the food industry (Sun *et al.,* 2011).

2.9.2.3 Volatiles

A great diversity of compounds have been included under this group of secondary lipid oxidation products, presenting very different functional groups such as aldehydes, ketones, alcohols, short carboxylic acids and hydrocarbons. They all share the property of giving moderate to high smells and are related to rancidity in sensorial tests. Measurement of these compounds is of great importance since, their formation closely relates to deterioration of flavour. Volatile compounds formed as a result of lipid oxidation can be analysed using electronic noses/gas-sensor array systems (Sun *et al.,* 2011). Different types of headspace analyses can be used, where the headspace volatiles over the samples are sampled, separated, and identified using different gas sensors. Of these methods, static headspace and solid-phase microextraction (SPME) are the least sensitive. Purge and trap techniques, where the samples are flushed or purged with nitrogen and the volatiles in the gas flow are trapped on a solid absorber, are highly sensitive. After sampling, the volatiles can be thermally desorbed into a gas

chromatograph for separation. The mass spectra of the compounds can also be compared with spectra of pure standard compounds and identified (Kalakowska, 2002, Niki et al., 2005). The advantages of this method are that it is flexible, and the amount of sample and sampling conditions can be varied according to the needs. However, quantification of headspace data, especially from solid matrixes, is complicated, and the results are dependent on the sample material. Small variations in sampling procedures can give large variations in the data; the data handling is also difficult (Kalakowska, 2002).

2.9.2.4 Oxidative stability index

Thermal analysis (TA) has long been one of the analytical techniques available to the fats and oils industry. Historically, the Schaal Oven test and the Active Oxygen Method (AOM) have been the most widely used tests to evaluate oil stability (Rudnick *et al.*, 2001; Arain *et al.*, 2009; Pardauil *et al.*, 2011). Currently, oxidative stability of fats and oils can be determined by two commercially available instruments, the Rancimat from Metrohm Ltd. (Herisau, Switzerland) and the oxidative stability index instrument form Omnion Inc. (Rockland, MA, USA). The Rancimat is an automated instrument that measures the conductivity of low molecular weight fatty acids produced during autoxidation of lipids at 100 and above. The principle of the conductivity determination on the Rancimat is based on measuring the resistance of the solution of the recovered volatile acids. The Rancimat apparatus can determine the induction time of the lipid and antioxidative effect of different oils respectively. Multiple samples can be analysed simultaneously and software controls instrument parameters and data collection. In this regard, the Rancimat test convenient for time saving (Pardauil *et al.*, 2011).

During lipid oxidation, fat or oil materials reveal a number of thermally induced transitions, such as the transfer of oxygen molecules to unsaturated fatty acids. Since this transfer of oxygen molecule requires energy (exothermic process). Therefore, oxidative stability of vegetable oils can also be established by the DSC technique. Recently differential scanning calorimetry (DSC) has been used to determine the oxidative stability (Tan *et al.,* 2002). The calorimeter measures heat flow into (endothermic) or out of (exothermic) a sample undergoing a phase change, as well as heat capacity of a sample. The principle of DSC is to keep, for a given temperature program, sample and reference,

placed in separate micro ovens, at equal temperature. The electrical power needed for the compensation is equivalent to the calorimetric effect. Examples of thermal transitions are melting, crystallization, decomposition, outgassing or change in heat capacity (Tan *et al.,* 2002; Pardauil *et al.*, 2011). Strong correlations between the Rancimat and DSC were reported by Pardauil *et al.,* (2011). Due to its simplicity, quickness and need for small amounts of sample the DSC method could be used in quality control as a routine analysis for vegetable oils.

2.9.2.5 Instrumental methods

In recent times attempts have been made to replace the tradition methods of lipid oxidation analysis with instrumental ones: GC and HPLC. Various detectors and tandems of detectors and chromatographic techniques are applied. Spectroscopy is more and more often applied to analyse lipid oxidation in food products directly, not only as, for example, detectors at the final stage of the analysis. Direct determination of lipid peroxides by LC-MS is one of the most common measurements (Watson *et al.*, 2003). LC-MS or combined techniques such as LC-MS-MS and LC-ESI-MS tandems allow detection of fatty acid hydroperoxides with regioisomers (Watson *et al.,* 2003). Application of GC-MS and GC-ESI-MS renders the analysis to be much more sensitive in determining carbonyl compounds than TBA. However, it requires more complex sample preparation, including derivatization. Mendes *et al.* (2009) compared, using three fish species, the applicability of the traditional TBA and HPLC separation after TBA or DNPH derivatization to determine lipid oxidation on storage. The performance (accuracy and specificity) of the methods used followed the order of HPLC > MDA-DNPH > HPLC > MDA-TBA > traditional spectrophotometric TBA test.

Other instrumental methods which have been developed for the determination of oxidation parameters in oils and foods, including near-infrared spectroscopy (NIR), Fourier-transform near-infrared (FT-NIR), and FT-IR spectroscopy methods (Rustad, 2003). Lipid oxidation products can produce very weak chemiluminescence (CL). It has been demonstrated in literature that sodium hypochlorite-induced decomposition of hydroperoxides gives strong CL. The level of hydroperoxides in fish oil can be determined using a rapid CL method, while electron spin resonance (ESR) spectroscopy can be used

for the assessment of free radicals. In the studies conducted by Carlsen *et al.* (2001) and Velasco *et al.* (2004), strong linear correlations were reported between the ESR and Rancimat analyses. Nissen *et al.* (2002) used the ESR to evaluate antioxidant activity of potato flakes. The ESR spectroscopy is a suitable technique for measuring lipid oxidation and estimating shelf life of food products and biological tissue as it provides high sensitivity and requires little sample preparation.

2.10 Maillard Reaction

Recently, special attention has been given to the use of natural antioxidants due to worldwide trend to avoid or minimise the use of synthetic food additives. The Maillard reaction (MR) is one particular area that has been focused on due to its promising results. The MR is named after the French chemist Louis-Camille Maillard who first described it in 1912 upon heating sugars and amino acids in water, a yellow-brown colour developed (Martins *et al.,* 2001). The MR is often described in food systems but it also occurs in living organisms and in this case it is called glycation. In biological systems the ramifications of the MR have been observed and analysed as this reaction has become vital in both medicine and food science. It is a series of non-enzymatic reactions involving the reaction between the carbonyl groups of reducing sugars with the amino groups of amino acids, polypeptides, proteins, nucleic acids or phospholipids, forming Schiff-bases, and followed by the subsequent rearrangement (Chawla *et al*., 2007; Hwang *et al*., 2011). The MR develops into a complex set of reactions that generate numerous products, including early volatile compounds, intermediate products, and large molecular weight polymers (Jing & Kitts, 2004; Zeng *et al*., 2011). These compounds contribute specifically to aroma and colour characteristics and are collectively referred to as Maillard reaction products (MRPs), similarly in biological systems the end products are referred to as Advanced Glycation End Products (AGEs). MRPs derived during thermal processing and home-cooking *via* amino-carbonyl compound interaction modify important food properties such as colour, flavour and stability during processing, distribution and storage (Maillard *et al.,* 2007; Yu *et al*., 2013b).

Apart from their contributions to sensorial features of thermally processed foods, MRPs have been demonstrated to exhibit antioxidant activity *via* radical chain-breaking

activity, scavenging of reactive oxygen species, decomposition of hydrogen peroxide and metal chelation (Vhangani & Van Wyk, 2013). Since the first reports about the antioxidant effect of MRPs was released in 1954, antioxidant activities of MRP have been studied extensively using different model systems. In some studies, MRPs with antioxidative activity were identified such as aminoreductones, heterocyclic compounds, or high molecular melanoidins, but most of the active antioxidant compounds in MRPs are still unknown.

Although the complete pathways for all the MRPs have not been fully elucidated (Figure 2.7), it is well recognised that three distinct stages of MR development occur as proposed by Hodge in 1953 (Sun & Zhuang, 2011). The mechanism of the reaction is thought to occur at seven steps (A–G) with three main steps as initial, intermediate, and final stage:

I. Initial stage (colourless, no absorption in the near-UV range)

A. Sugar-amine condensation
B. Amadori rearrangement

II. Intermediate stage (colourless, or yellow with strong absorption in the near-UV range)

C. Sugar dehydration
D. Sugar fragmentation
E. Amino acid degradation

III. Final stage (highly coloured)

F. Aldol condensation
G. Aldehyde-amine polymerization; formation of heterocyclic nitrogen compounds (melanoidins)

The initial stage of the Maillard reaction is a condensation reaction between the carbonyl group of an aldose or ketose and the free amino group of an amino acid, followed by the elimination of water to give an N-substituted aldosylamine or ketosylamine. This reaction, which is initiated by an attack of the nucleophilic amino compound on the carbonyl carbon, is reversible and requires an acidic catalyst (Martins *et al.,* 2001).

In the next step, the Amadori rearrangement is essential to the continuation of the Maillard reaction. It is an acid-base catalysed conversion of the N-substituted aldosylamine to an N-substituted 1-amino-1-deoxy-2-ketose. The Amadori reaction is

catalysed by weak acids, where the protonation of the Schiff base and the subsequent proton shift constitute the critical steps; the amino acid serve as their own acid catalysts so the reaction is rapid even in the absence of added acid (Manzocco *et al.,* 2001). If a ketose is allowed to react with the amino compound, a ketosylamine is formed. This leads to a 2-amino-2-deoxyaldose through Heyn's rearrangement, which is similar to the Amadori rearrangement. Both rearrangements result in a conversion of an alpha-hydroxyamino compound to an alpha-aminocarbonyl compound (Van Boekel, 2006; Gogus *et al.,* 2009). The formation of the Amadori rearrangement products (ARPs) proceed slowly at room temperature because a tautomeric shift to the open chain form of the reducing sugar is required for the initial reaction to occur. In the reaction of aldoses with the amino acids blocking the Amadori rearrangement step the formation of brown pigment is stopped completely (Gogus *et al.,* 2009). Once the Amadori rearrangement products are formed they are more stable than the glycosylamines in most acidic environments, although they are heat labile. Upon heating, the Amadori rearrangement products undergo fission and colourless reductones and fluorescent substances are formed. Amadori compounds have been isolated in various heated and stored food products including soy sauce, malt, roasted cocoa and dehydrated vegetables. In the intermediate stage, three degradation pathways exist; sugar dehydration, sugar fragmentation, and Strecker degradation (Manzocco *et al.*, 2001; Van Boekel, 2006; Gogus *et al.,* 2009).

Sugar dehydration is the first reaction in the intermediate stage. There are two types of sugar dehydration both of which depend on pH. In acidic conditions, at pH 7 or below, it undergoes mainly 1, 2-enolization with the formation of furfural (when pentoses are involved) or hydroxy methyl furfural (HMF) (when hexoses are involved). In alkaline medium, at pH higher than 7, the degradation of the Amadori compound is thought to involve mainly 2,3-enolization, where reductones, such as 4-hydroxy-5-methyl-2,3-dihydrofuran-3-one, and a variety of fission products, including acetol, pyruvaldehyde, and diacetyl are formed (Manzocco *et al.,* 2001; Gogus *et al.,* 2009).

The next reaction is sugar fragmentation; the accepted mechanism is dealdolization, the reverse of aldol condensation. The products are aldols, amino-free polymers, and free amino compounds (Gogus *et al.,* 2009).

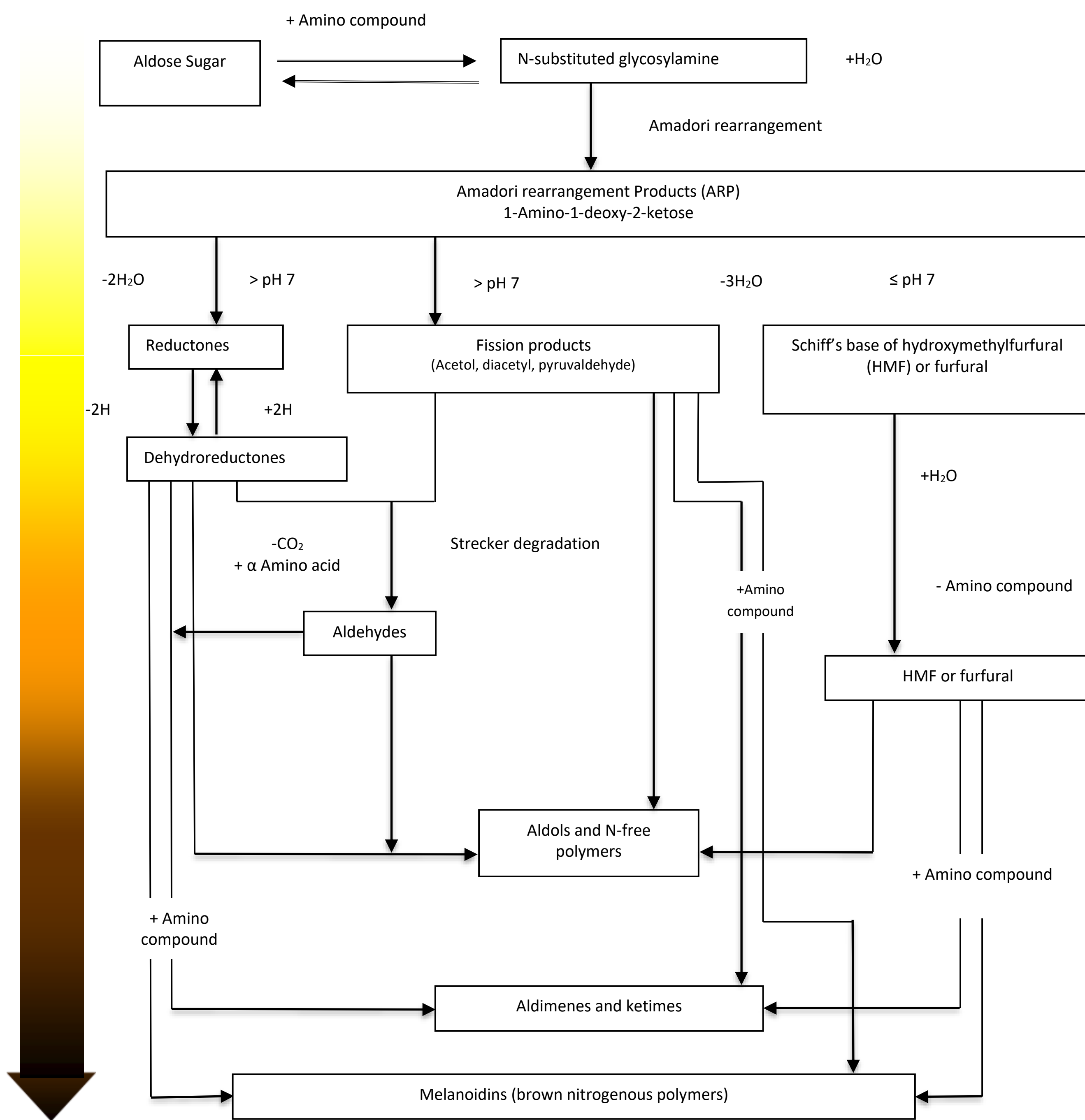


Figure 2.7 General pathway for the Maillard reaction [left arrow indicates that browning intensity increases as the reaction reaches advanced stages (adopted from Martins *et al.*, 2001)].

In the Strecker degradation reaction, all fission products are highly reactive and take part in further reactions. Carbonyl groups can condense with free amino groups, which results in the incorporation of nitrogen into the reaction products. It is actually the reaction of a reductone and an α-amino acid. In this stage CO_2 is liberated to form an aldehyde with one fewer carbon than the original amino acid. The aldehydes formed may be important as auxiliary flavour; these may further condense with themselves, with sugar fragments, with furfurals, and with the other degradation products to form brown pigments (Gogus *et al.,* 2009; Jaeger *et al.,* 2010).

The possible reactions involved in the final stage are aldol condensation, aldehyde-amine polymerization, and formation of heterocyclic nitrogen compounds (pyrroles, imidazoles, pyridines, and pyrazines). The polymerization of products from the second step and co-polymerization with amino compounds yield the coloured products. Finally both water-soluble and water-insoluble pigments, called melanoidins, are produced, and their structure is dependent on the types of amino acid, sugar, and the sugar:amino acid ratio. Melanoidins vary widely in molecular weight and contain several discrete chromophores (Matmoroh *et al*., 2006). Melanoidins may be distinguished from one to another on the basis of their molecular weight and solubility.

Melanoidins with a molecular weight <500 Daltons (Da) represent low molecular weight compounds and are soluble in water or organic solvents. Insoluble melanoidins with molecular weights >12 000 Da can easily be prepared by dialysis of sugar-amino acid reaction mixtures, as the browning reactions proceed the melanoidins eventually become insoluble in water and precipitate (Manzocco *et al*., 2001; Jaeger *et al.,* 2010).

2.10.1 Factors affecting the Maillard reaction

It has been shown that the MR pathways and subsequent products can be modulated by many factors. These can be distinguished as those relating to the reactant concentration e.g. type of amino acid, sugar type, sugar:amino acid ratio, and external factors such as moisture level, temperature and pH (Morales & Jimenez-Perez, 2001, Jing & Kitts, 2004; Matmoroh *et al.,* 2006). In order to reduce the complexity, sugar-amino acid model systems have commonly been used to study the phenomena and mechanism of the MR. The following subsections explain these factors in detail.

2.10.1.1 Amino Acid type

Amino acid type which is used or present in the medium is very important in the MR. Some amino acids have two reactive groups therefore, they rapidly react with the sugars to produce brown pigments (melanoidins). Liu *et al.* (2008) have classified the amino acids according to their reactivity with the sugars at pH 9.0. They classified lysine, glycine, tryptophan, and tyrosine as the most reactive amino acids. They have measured the colour intensity of 60 model systems heated for 3 h at pH 6.5 and 100°C and found that the colour intensity of the MRPs from the lysine was the highest (Gogus *et al.,* 2009).

2.10.1.2 Type of Sugar

Reducing sugars are essential ingredients in these reactions, providing the carbonyl groups for interaction with the free amino groups of amino acids, peptides, and proteins. Low molecular weight compounds tend to be more reactive than the high molecular weight compounds as a result of greater steric hindrance in the latter. Accordingly, aldopentoses are generally more reactive than aldohexoses and monosaccharides are more reactive than di- or oligosaccharides. Aldoses in general appear to be more reactive than ketoses, apparently a consequence of the greater sterical hindrance of the carbonyl group of ketoses. Jing & Kitts (2004) found that xylose, which is a pentose, showed much higher reactivity than glucose and fructose that are hexoses (Guofeng *et al.*, 2012).

2.10.1.3 Effect of pH

Both the initial pH of the product and the buffering capacity of the system, influence the rate and direction of the Maillard reaction. Kim & Lee (2008) systematically investigated the influence of pH on the extent of interaction of glucose and free amino-nitrogen. They made quantitative observations on the interaction of glucose with glycine, and of glucose with various enzymatic digests of protein material. Determinations of pH and Van Slyke amino nitrogen were made with time; in each case the initial pH values were set by phosphate buffers and the solutions contained 1% glycine and 13.2% glucose. An appreciable loss of amino-nitrogen occurred in 48 h and the loss increased with increasing pH. It was shown that the optimum pH for the whole reaction is in the range

pH 6 – 9. With regards to the influence of pH on the MR, Lee *et al.* (2006) observed that the substrate loss increased with increasing pH, up to a pH of about 10, with little, if any, browning occurring below pH 6. The pH-dependence of the MR for the amino acid reagent can, at least qualitatively, be described by the effect of protonation of the amino acid. The amount of unprotonated amino group, which is considered to be the reactive species, increases obviously with increasing pH (Kim & Lee, 2008).

2.10.1.4 Temperature

The most important influence on the kinetics of the MR is temperature. Increasing temperature results in a rapidly increasing rate of browning. Not only the rate of browning but also the character of the reaction is determined by temperature. In model systems, the rate of browning increases 2 – 3 times for each 10°C rise in temperature. In foods containing fructose, the increase may be 5 – 10 times for each 10°C rise (Xiaohong *et al.,* 2010). Temperature also affects the composition of the MRPs formed. If the colour intensity is measured, it may also be increased with increasing temperature because of the changing composition and increasing carbon content of the pigment (Bersuder *et al.,* 2007; Guofeng *et al.*, 2012). Ajandouz *et al.* (2008) worked with equimolecular amounts of D-xylose and glycine in aqueous solution at temperatures of 22°C, 68°C, and 100°C. They reported that an increase in temperature leads to an increase in aromatic character in both high and low molecular weight products. The structure of the melanoidins synthesized at room temperature differs considerably from those synthesized at higher temperatures in that they have different types of aliphatic carbons and fewer unsaturated carbons (Guofeng *et al.*, 2012).

2.10.1.5 Water activity

Non-enzymatic browning reactions may occur as a result of heating, dehydrating, or concentrating food constituents. The role of bound and unbound water in browning reactions has been investigated by several authors. Almost all of the results showed that a maximum browning rate occurs at A_w between 0.4 and 0.6 depending on the type of the food substance. At lower water activities the reaction rate decreases as a result of the increasing diffusion resistance due to high viscosity (Tazi *et al.*, 2009), whereas at higher

water activities the reaction rate again slows down, due to the dilution of the reactants (Yilmaz & Toledo, 2005). Furthermore, at higher water activities, water behaves as a reaction product and blocks the formation of reaction intermediates that are produced together with water (Yilmaz & Toledo, 2005; Tazi *et al*., 2009).

2.11 Desirable and undesirable effects of MR

One of the most significance consequences of the MR is the formation of a probable human carcinogen known as acrylamide which was first discovered in thermally processed foods. It has been shown clearly that the amino acid asparagine is mainly responsible for acrylamide formation in cooked foods after condensation with reducing sugars (Capuano & Fogliano, 2011; Jin *et al.,* 2013). Another possible mutagen formed in the MR is Hydroxymethylflurflural (HMF) and its presence is not desirable in thermally processed foods. HMF is naturally formed as an intermediate in the MR and from dehydration of hexoses under mild acidic conditions during thermal treatment applied to foods (Claus *et al.,* 2008). The desirable and undesirable effects of MR are listed in Table 2.4. The MR is of outstanding importance for the formation of colour, flavour, aroma and texture of heated food products.

Table 2.4 Desirable and undesirable effects of the Maillard reaction in foods (Gogus *et al.,* 2009).

Undesirable	Desirable
Loss in nutritional value of protein	Formation of antioxidants
Formation of undesirable colours and flavours	Formation of desirable food flavours and aromas
Formation of reactive oxygen species	Formation of desirable colours
Formation carcinogenic products	Formation of antimicrobial compounds
Formation of mutagenic products	Formation of antiallergenic compounds

Brown colour development during processing and storage is desirable for many products such as baked foods, coffee, cookies while undesirable in some kinds of food products orange juice, white chocolate, milk and egg powder.

The intermediate and final stages of the MR are the most essential to flavour development, especially the Strecker degradation step, in which amino acids are degraded by dicarbonyls formed previously in the reaction lading to amino acid deamination and decarboxylation. Predicting and controlling food colour and flavour development are particularly important for food companies to satisfy consumer preference. Therefore, this reaction can be used to design or develop food product that present sensory attributes demanded by the consumer. Since the studies of Franzke & Iwainsky in 1954 (as cited by Wang *et al.,* 2013), there is accumulating evidence for the ability of MRPs to decrease the oxidation role of lipids. Several authors attested that these MRPs possess antioxidant activity in food products in which they are formed or are added to (Maillard *et al.,* 2007*;* Chawla *et al.,* 2007). As a result of their antioxidant character, MRPs are considered a value-added component. Moreover, several mechanisms of their antioxidant activity have been recognized, including radical chain-breaking activity metal-chelating ability, decomposition of hydrogen peroxide and scavenging of reactive oxygen species (Morales & Babbel, 2002; Hwang *et al.*, 2011 Vhangani & Van Wyk, 2013).

2.12 Analysis of Maillard reaction products

Several papers indicate that the Maillard reaction can be a good means of producing functional food ingredients since they can be obtained without the use of harmful chemicals and tedious purification procedures. The determination of MRPs along with their beneficial or harmful properties is thus of key importance for the production of safe foods and for the development of novel functional food ingredients (Silván *et al.,* 2006

Analysis of MRPs is also required for a better understanding of their structure and biological activity. The development of sophisticated analytical techniques have made it possible to isolate, characterise and quantify several non-browning reaction compounds *in vitro* and *in vivo*, both at the early and advanced stages of the MR (Silván *et al.,* 2006). Various analytical techniques have been developed for the analysis of lactulsyl-lysine in dairy products due to its usefulness as quality control indicator. Detection of lactulosyl-

lysine has been conducted by spectroscopy techniques such as matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF), electrospray and nuclear magnetic resonance (NMR) analysis. A selective liquid chromatography (LC)-MS procedure allowed the detection and quantification of lactulosyl-lysine after complete enzymatic hydrolysis in milk samples. The first lysine derivative of the advanced MR detected in foods was Nε-(carboxymethyl)-lysine (CML). CML is formed from lysine and glyoxal during the oxidative deterioration of Amadori compounds. This lysine derivative is a commonly used marker compound to evaluate the progress of the Maillard reaction in foods (Kislinger *et al.,* 2003).

The most common isolated compounds include; Amadori compounds (indirectly analysed as furosine), CML and some intermediate derivatives of the reaction, such as hydroxymethylfurfural (HMF) (Figure 2.8). Measurement of fluorescent compounds formed during the reaction is also a reliable tool to evaluate the extension and ratio of nutritional loss due to thermal processing of food (Silván *et al.,* 2006; Wang *et al.,* 2009). However, despite the recent advances in the analytical separation methodologies and increased attention paid to the MR, studying this complex system still presents an interesting challenge to researchers. Fifty-four components were identified in a simple model system such as glucose-glycine and up to seven hundred compounds were reported in roasted coffee (Ames *et al.,* 2001; Delgado-Andrade *et al.,* 2005).

Some of the products formed during this reaction are intermediates and they can further react with other components and increase the diversity of the products. Although numerous attempts have been undertaken to isolate and purify melanoidins in model systems, little is known about the overall structural properties of melanoidins let alone the MRPs that contribute to antioxidant activity (Wang *et al*., 2011). This is caused by the large number of products formed during the MR and the difficulties encountered in their purification, identification and quantification of pure compounds.

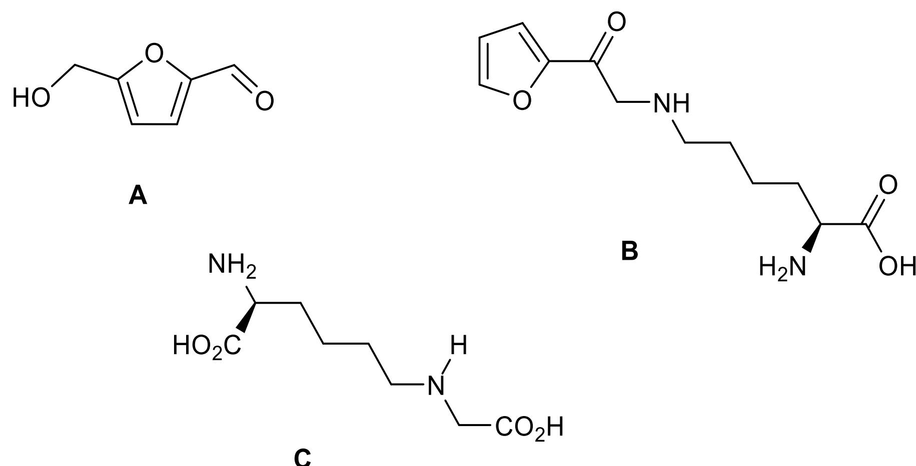


Figure 2.8 Chemical structure of (A) HMF, (B) Furosine and (C) CML.

2.13 Current study

Milk proteins consist of 80% casein and 20% whey protein. Casein is responsible for the white, opaque appearance of milk in which it is combined with calcium and phosphates as clusters of casein "micelles". The casein proteins are four distinct gene products designated α_{s1}-, β-, α_{s2}-, and κ-casein, and are present in a ratio of approximately 40:35:10:12 in cow's milk (Fox & Brodkorb, 2008). The caseins are not globular proteins locked into a secondary conformational structure and are not truly random polymers. They have been described as rheomorphic proteins, indicating that the conformational structure adopted is dictated by, and responsive to, the molecular environment (Akhtar & Dickinson, 2007; Madadlou *et al.*, 2009). The caseins have little α-helical structure, no denaturation temperature and high hydrodynamic volume. The isoelectric point (IEP) of casein micelles is about 4.6. Approaching the IEP by gradually lowering the pH causes the casein micelles to aggregate and form a gel (Liu & Guo, 2008). The major uses of casein until the 1960s were in technical, non-food applications such as adhesives for wood, in paper coating, leather finishing and synthetic fibres as well as for plastic buttons. During the past thirty years however, the principal use of casein has been as an ingredient in food systems to enhance their functional properties such as whipping and foaming, water-binding and thickening, emulsification and texture, and to improve their nutrition (Liu & Guo, 2008; Madadlou *et al.*, 2009).

In order to reduce the complexity, sugar-amino acid or protein model systems are commonly used to study the phenomena and the mechanism of MR. For example; glucose-glycine is a frequently selected model system (Jing & Kitts, 2004). As mentioned previously, incorporation of the preformed MRPs into food systems directly or by the application of food processing practices which result in the formation of MRPs within food, can result in improved oxidative stability of foods, thus they can be considered as substances that are naturally present in foods. Although the antioxidant effect of MRPs has been investigated extensively in sugar-amino acid models, the exact nature of the antioxidants formed still remains undefined. The initial stages of the MR leads to the formation of well-known Amadori and Heyn's products. However, little information is available on the chemical structure of hundreds of brown products formed by a series of consecutive and parallel reactions including oxidations, reductions and aldol condensations amongst others. The knowledge of the reaction conditions that generates MRPs with specific antioxidant activity could therefore be valuable tool for use in food processing strategies.

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CHAPTER 3

ANTIOXIDANT ACTIVITY OF MAILLARD REACTION PRODUCTS (MRPs) AGAINST THE OXIDATIVE DESTABILIZATION OF SUNFLOWER OIL-IN-WATER EMULSIONS

3.1 Abstract

Maillard reaction products were prepared from an aqueous glucose-casein model system at pH 8, heated at 60, 75, and 90°C for 6, 12 and 24 h, respectively. Reducing power (RP), peroxyl (PRS), 2,2'-azinobis (3-ethyl-benzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 1,1 diphenyl-2-picrylhydrazyl (DPPH-RS), ferrous chelating activity were analysed as a measure of their antioxidant activity. The RP of MRPs increased ($p < 0.05$) as the reaction time increased at 60 and 75°C, while the PRS, ABTS and Ferrous chelation decreased ($p < 0.05$) as the reaction time increased at 90°C. The ferrous chelating activity of MRPs was higher than that of TBHQ (0.02%) and EDTA (1 mM), respectively. In addition, DPPH-RS increased ($p < 0.05$) as the reaction time increased regardless of the heating temperature. The lipid oxidation indices (thiobarbituric acid reactive substances (TBARs) and peroxide value (PV)) were determined in a sunflower oil-in-water emulsion. MRPs derived at 90°C for 12 h had the lowest peroxide value (PV), while the TBARs inhibition by MRPs ranged from 39.05 to 88.66%. In the TBARs assay, MRPs displayed higher antioxidant capacity than TBHQ and Trolox, respectively. The oxidative stability index of MRPs was studied using the Rancimat method and DSC techniques set at 110°C. At the same temperature program, DSC gave significantly lower induction times than the Rancimat. However, the results of this study clearly indicated that MRPs possess antioxidant capacity and can be used as natural antioxidants in the food industry.

3.2 Introduction

Free radical mediated autoxidation in food and biological systems has a multitude of diverse effects in these biological systems and thus has an implication in human health as well as in food stability and preservation (Shahidi & Zhong, 2010). In edible fats and

oils such as butter, margarine and salad or cooking oils, oxidative changes observed as the manifestation of “off-flavour” development are the major causes of food spoilage and therefore wastage. Lipid oxidation is also responsible for quality deterioration in muscle foods and dairy products as well as fruit and vegetables (Sun *et al*., 2011). This oxidation reaction is a major concern to the food industry since it leads to product losses amounting to millions of Rands and, therefore, its prevention is vital. In this regard, antioxidants play a major role in preventing or delaying autoxidation and have attracted much attention as stabilizers, dietary supplements and natural health products (Laguerre *et al*., 2007; Pokorny *et al*., 2008). Amongst other methods employed for preventing lipid oxidation, addition of antioxidants is the most effective, convenient and economical strategy for stabilizing food and non-food commodities (Pokorny *et al*., 2008). Antioxidants are substances that, when present at low concentrations compared to that of oxidizable substrate, markedly delay or inhibits its oxidation (Frankel & Meyer, 2000). These substances may be added to food as synthetic antioxidants (e.g. TBHQ), or may be naturally occurring as food components (e.g. tocopherols and ascorbic acid) or induced during processing (e.g. the Maillard reaction) (Sun *et al.,* 2011).

The Maillard reaction (MR) is a very complex non-enzymatic browning reaction between carbonyl-containing compounds (such as reducing sugars) and amino-containing compounds (such as amino acids, peptides and proteins). The MR occurs in three major stages (early, intermediate and final stage), and it is dependent upon factors such as reactant concentration, temperature, time, pH and water activity (A_w) (Guofeng *et al*., 2012). Maillard reaction products (MRPs) are formed during food processing and storage and are widely present in the human diet (Kim & Lee, 2009; Yu *et al*., 2013). MRPs are a complex mixture including volatile compounds (such as aldehyde, reductone, and heterocyclic compounds) and nonvolatile compounds (such as melanoidins) which, respectively, contribute to the flavour and colour of foods (Osada & Shibamoto, 2006; Yu *et al.,* 2012).

Apart from their sensorial role in thermally treated foods, MRPs were also reported to exhibit antioxidant properties, including radical chain breaking activity, metal chelating activity, decomposition of hydrogen peroxide and scavenging of reactive oxygen species (ROS) (Gu *et al*., 2010; Vhangani & Van Wyk, 2013). Although synthetic antioxidants

such as butylated hydroxyanisole (BHA), butylated hydroxyl toluene (BHT), tertiary butyl hydroquinone (TBHQ) and propyl gallate (PG) are most widely used in foods, these have been suspected to cause or promote negative health effects (Kulisic *et al.*, 2004). Considering the toxicity of synthetic antioxidants and the wide presence of MRPs in foods, increasing interest was directed towards the utilization of MRPs as normal food constituents with antioxidant properties (Kim & Lee, 2009; Vhangani & Van Wyk, 2013). In recent years, several researchers have focused on the effect of MRPs as antimicrobial, anti-hypertensive and anti-browning food components (Rufián-Henares & Morales, 2007). In this regard, the antioxidant activity of MRPs produced form sugar-amino acid model systems have been studied by a number of researchers (Morales & Jimenez-Perez, 2001; Chawla *et al.*, 2007; Jayathilakan *et al.,* 2007). Vhangani & Van Wyk (2013) studied the MR in ribose-lysine and fructose-lysine model systems. This approach of using pure solutions of simple sugar-amino acid combinations does not resemble real food systems which comprise more complex solutions (Ames *et al.*, 1998). These simple solutions are also likely to result in the generation of increased browning and flavour formation, both of which may be undesirable in terms of application of MRPs as antioxidants. Therefore, a more complex model system in terms of either or both the sugar and the peptide or protein compound is likely to solve the problem of undesirable colour and flavour development, since more complex systems will be less reactive. With no undesirable colour or flavour contribution, MRPs could be beneficial to the food industry since they can be obtained without the use of harmful chemicals and tedious purification procedures (Silván *et al.,* 2006).

Augustin *et al.* (2006) investigated MRPs from protein-sugar model systems as suitable encapsulants to protect sensitive core material against oxidation, while the glycation of casein with glucose or lactose resulted in enhancement of antioxidant activity when compared to native casein (McGookin & Augustin, 1991). In these studies, heated glucose-casein mixtures decreased lipid oxidation in an emulsified linoleic acid model and increased the shelf-life of full cream milk powder (Augustin *et al.,* 2006).

Antioxidant assays are classified based on the mechanism of action as hydrogen atom transfer (HAT), electron transfer (ET) or a combination of both HAT and ET (Prior *et al.,* 2005). Moreover, in an assay like the ABTS radical scavenging assay, the

generation of the ABTS radical cation forms the basis of one of the spectrophotometric methods that has been applied for the measurement of the total antioxidative activity of solutions of pure substances, aqueous mixtures and beverages. This method for the screening of antioxidative activity is reported as a decolourization assay applicable to both lipophilic and hydrophilic antioxidants (Kim & Lee, 2009). Hence, when determining antioxidant capacity of food components, it is advised to implement more than one assay, since a single assay cannot determine the effectiveness of all antioxidant types (Laguerre *et al*., 2007; Apak *et al.,* 2013).

Therefore the aim of this study was to: (1) measure five indices of the antioxidant activity of MRPs derived from a glucose-casein model system as a function of heating time and temperature and (2) determine the lipid oxidation indices and oxidative stability of a lipid-rich model system in response to treatment with MRPs and synthetic antioxidants with the view of to find a natural alternative to synthetic food antioxidant.

3.3 Materials and Methods

3.3.1 Chemicals

Casein was obtained from Chempure (Pretoria, South Africa). 2,2 diphenyl-1-picrylhydrazyl (DPPH), 2,2' azobis (2-methylpropionamide) dihydrochloride (ABAP), 2,2-Azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), Ferric (III) chloride, ethylenediaminetetraacetic acid (EDTA), tertiary butyl hydroquinone (TBHQ), Pyrogallol red (PGR), Iron(II) chloride tetrahydrate, sodium dodecyl sulphate (SDS) and thiobarbituric acid (TBA) were obtained from Sigma-Aldrich (Kempton Park, South Africa). Glucose, sodium, potassium dihydrogen phosphate, methanol, trichloroacetic acid, potassium ferricyanide, acetic acid, butanol, chloroform, potassium iodide, sodium thiosulphate and starch indicator were obtained from Merck (Modderfontein, South Africa). All the chemicals used in this study were of analytical grade and chemical reagents were prepared according to standard analytical procedures. Prepared reagents were stored under conditions that prevented deterioration or contamination. The water used in the study was ultrapure water purified with a Milli-Q water purification system (Millipore, Microsep, Bellville, South Africa).

3.3.2 Synthesis of MRPs

MRPs were prepared according to the method of Gu *et al.* (2009) with slight modifications. Casein (5.0 g) and glucose (10.0 g) were dissolved in 100 mL of 0.1 M Tris-HCl buffer at pH 8. The samples were transferred into 250 mL Schott bottles and heated at 60, 75 and 90°C in a water bath for 6, 12 and 24 h, respectively. After the heating period had elapsed, the resulting MRPs were immediately cooled in an ice bath. A portion of the MRPs in solution was retained for pH measurements (pH meter: Metrohm, Switzerland). The remainder of the solutions were freeze-dried and stored in air-tight screw-capped glass bottles at -80°C until analysis. Before use, the powder was reconstituted to the required concentration with Milli-Q water and browning intensity was measured with a spectrophotometer (Lambda 25, Perkin Elmer, and Singapore) at 420 nm. The pH and Browning intensity measurements were used as used as non-specific indicators of the Maillard reaction. For all direct antioxidant assays, the same concentration (10 $mg.mL^{-1}$) of MRPs was consistently used as determined in trials as the ideal concentration. Two synthetic antioxidants, TBHQ (0.02%) and Trolox (1 mM) were included in the study in order to benchmark the performance of the MRPs in each antioxidant assay. Milli-Q water was used as a control throughout the study.

3.3.3 Determination of reducing power of MRPs

The reducing power (RP) was determined according to the method of Chawla *et al.* (2007) and Vhangani & Van Wyk (2013) with slight modifications. A one millilitre aliquot of each MRP (10 $mg.mL^{-1}$) sample was mixed with 2.5 mL of 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 mL of 1.0% potassium ferricyanide. The reaction mixtures were incubated in a temperature-controlled water bath at 50°C (Memmert, Germany) for 30 min, followed by the addition of 2.5 mL of 10% trichloro-acetic acid after cooling at room temperature. The mixture was centrifuged at 1 750 *g* for 10 min at 25°C. The supernatant obtained (2.5 mL), was treated with 1 mL of Milli-Q water and 0.5 mL of 0.1% Ferric chloride. The absorbance of the reaction mixture was measured at 700 nm in a spectrophotometer (Lambda 25, Perkin Elmer, Singapore). All measurements were performed in triplicate. The RP was expressed as an increase in absorbance at 700 nm.

3.3.4 Determination of the peroxyl radical scavenging activity of MRPs

Peroxyl radical scavenging activity (PRS) of MRPs was determined according to the method of Vhangani & Van Wyk (2013) with slight modifications. A 5 mL aliquot of 0.1 mM pyrogallol red (PGR) was mixed with 0.5 mL MRP sample in a 1:100 ($w.v^{-1}$) ratio to Milli-Q water and 75 µL of 600 mM 2,2-azobis (2-amidinopropane) dihydrochloride (ABAP), followed by incubation at 37°C for 2 h in a water bath (Memmert, Lasec, Germany). At the end of incubation, the reaction mixture was cooled in an ice bath and the absorbance measured at 540 nm. For control samples, Milli-Q water was used instead of the MRP samples. All measurements were performed in triplicate. The PRS activity was calculated according to the following equation:

$$\% \text{ PRS} = [(A_{\text{control (540 nm)}} - A_{\text{sample (540 nm)}}) / A_{\text{control (540 nm)}}] \times 100$$

Where A_{control} is the absorbance of the control at 540 nm and A_{sample} is the absorbance of the sample at 540 nm.

3.3.5 Determination of ABTS radical scavenging activity of MRPs

The spectrophotometric analysis of $ABTS^{•+}$ radical scavenging activity of MRPs was determined according to a method as described by Yu *et al.* (2012) with slight modifications. The ABTS radical was prepared by reacting 7 $mmol.L^{-1}$ ABTS solution and 2.45 $mmol.L^{-1}$ potassium persulphate solution in equal volume, and the mixture was allowed to stand overnight in the dark at ambient temperature. The ABTS solution was diluted twenty-fold (20-fold) with Milli-Q water to obtain an absorbance of 1.5 – 1.6 at 730 nm. Fresh ABTS was prepared daily. A 4 mL aliquot of diluted ABTS solution was added to 200 µL of aqueous MRPs (10 $mg.mL^{-1}$) solution and the mixture was allowed to stand at room temperature for one hour. The absorbance was then measured at 730 nm using a spectrophotometer (Lambda 25, Perkin Elmer, Singapore). The control was prepared in the same manner with the substitution of distilled water for the sample. All measurements were performed in triplicate. The percentage of ABTS radical scavenging activity was calculated according to the following equation:

$$\% \text{ ABTS-RS} = (A_{\text{control (730nm)}} - A_{\text{sample (730 nm)}} / A_{\text{control (730 nm)}}) \times 100$$

Where $A_{control}$ is the absorbance of the control at 730 nm and A_{sample} is the absorbance of the sample at 730 nm.

3.3.6 Determination of 1,1-diphenyl-2 picryl-hydrazyl (DPPH) radical scavenging activity of MRPs

DPPH radical scavenging (DPPH-RS) of MRPs was determined according to the method of Lertittikul *et al.* (2007) with slight modifications. A 0.12 mM solution of DPPH in ethanol was prepared daily and protected from light. A 4 mL aliquot of DPPH solution was added to 2 mL of MRPs (10 $mg.mL^{-1}$) samples. The mixture was vortexed (Vortex Genie 2, Scientific Industry Inc, USA) and allowed to stand at ambient temperature in the dark for 30 min. The absorbance of the mixtures was measured at 517 nm with a spectrophotometer (Lambda 25, Perkin Elmer, Singapore). A reaction mixture containing 2 mL distilled water and 4 mL ethanolic DPPH solution was used as control. All measurements were performed in triplicate. The percentage of DPPH-RS radical scavenging activity was calculated using the equation:

$$\% \text{ DPPH-RS} = [1- (A_{\text{sample (517nm)}} / A_{\text{control (517nm)}}) \times 100]$$

Where $A_{control}$ is the absorbance of the control at 517 nm and A_{sample} is the absorbance of the sample at 517 nm.

3.3.7 Determination of Iron chelation activity

Chelating activity of MRPs was determined according to the method of Gu *et al.* (2010) with slight modifications. One milliliter MRPs was mixed with 1.85 mL of Milli-Q water and 0.05 mL 2.0 mM $FeCl_2$, and the mixture was allowed to stand at room temperature for 30 s. The reaction mixture thus obtained was added to 0.1 mL of 0.5 mM ferrozine and mixed; the absorbance was measured at 562 nm using a spectrophotometer (Lambda 25, Perkin Elmer, Singapore) after 10 min resting time and 5 min centrifugation at 3 000 *g*. The control was prepared in a similar manner, except that MRPs were replaced with Milli-Q

water. All measurements were performed in triplicate. The percentage of chelating activity was calculated as follows:

$$\% \text{ Chelating activity} = [1 - (A_{\text{sample (562 nm)}} / A_{\text{control (562 nm)}}) \times 100]$$

Where $A_{control}$ is the absorbance of the control at 562 nm and A_{sample} is the absorbance of the sample at 562 nm.

3.3.8 Extraction of oil from sunflower seeds

Oil was extracted from sunflower seeds (Agricol, SA) by weighing off 200 g of hulled and dehulled sunflower seeds in a (60:40) $w.w^{-1}$ ratio. The seeds were introduced into a screw oil press and the resulting oil was centrifuged at 10 000 *g* for 30 min at 4°C (Coulter, Beckman, USA) to separate the hulls and other debris from the clear oil. The acid value of the resulting oil was determined by a titration and to ensure consistent quality, only oil with acid values ≤ 1.1 was used in the study.

3.3.9 Emulsion preparation

Fresh pasteurised unsalted egg yolk (Nulaid, SA) was weighed off (450 g) into bags and frozen for later use. Prior to emulsion preparation, antioxidants were incorporated in the egg yolk. A 0.5% solution of MRPs was dissolved in 49.75 g of thawed egg yolk. A 50:50 ($w.w^{-1}$) ratio oil in water (O/W) emulsion was prepared by weighing off 50 g each of egg yolk and sunflower oil into a 200 mL glass beaker. This was followed by homogenisation using a Polytron (Kinematica, Switzerland) at a speed of 800 rpm for 5 min. A 2 aliquot mL of 0.07 M ABAP solution was added to each emulsion and thoroughly mixed. The resulting emulsions were allowed to oxidise in an oven at 60°C for 12 h, followed by cooling and extracting the oil with hexane (Decker *et al.*, 2005). The hexane was evaporated in a fume cupboard and the residual oil was used as samples in the following tests: Peroxide value and TBARs. For the Rancimat and DSC analyses, the same procedure was followed with the exception of reaction with the ABAP solution was omitted.

3.3.10 Peroxide Value

The peroxide value (PV) was determined according to a protocol adapted from the official American Oil Chemists Society (AOCS) Cd 8-53 Method (Anon, 1999) using an automatic 814 USB sample processor titrator with a Metrohm autosampler and a Pt Titrode electrode. Oil (2.0 g ± 0.5) was accurately weighed into a 200 mL flask and 10 mL of a mixture of acetic acid:1-decanol at 3:2 ($v.v^{-1}$) was added. The solution was stirred gently for 30 s until the oil had dissolved and 0.2 mL of freshly prepared potassium iodide solution was then added. The solution was thoroughly mixed and placed in the dark for 1 min, after which 50 mL of Milli-Q water was added. A titration with a 0.01 N sodium thiosulfate solution was performed with continuous stirring. A control sample where the oil replaced with Milli-Q water was subjected to the same treatment. The volume of added thiosulfate solution was recorded for the oil sample and for the control. All measurements were prepared in triplicate. The PV was calculated using the equation:

$$PV = CVO1 \times (EP1 - CVO2) \times Titre / COO$$

Where CVO1 is 10 for $[Na_2S_2O_3]$ = 0.01 $mol.L^{-1}$, EP1 the volume (mL) of sodium thiosulfate required to titrate the sample, CVO2 is the volume (mL) of sodium thiosulfate required for the control, Titre is the titre for the standardized sodium thiosulfate solution, and COO is the weight of the oil sample (g). The samples were analysed in triplicate and the results were expressed in milli-equivalents of O_2 per kg sample.

3.3.11 Determination of TBARs

The TBARs were determined according to the method of Kulisic *et al.* (2004) with slight modifications. Pre-treated oil samples of 1.5 g were weighed off into screw-cap test tubes. Aliquots of 1.5 mL of 20% acetic acid at pH 3.5 and 1.5 mL of 0.8% ($w.v^{-1}$) TBA in 1.1% ($w.v^{-1}$) SDS solution were added to the test tube. The resultant mixture was vortexed (Vortex Genie 2, Scientific industry Inc, USA) and then heated in a water bath at 95°C for 60 min. After cooling, 5.0 mL of butan-1-ol was added and the mixture vortexed, followed by centrifugation at 1 200 *g* for 10 min. The absorbance of the supernatant was measured at 532 nm using a spectrophotometer. All measurements were performed in triplicate. All

the values were based on the percentage antioxidant index and was calculated according to the following equation:

$$\text{Antioxidant Index \%} = [1 - (A_{\text{sample (532 nm)}} / A_{\text{control (532 nm)}})\ X100]$$

Where A_{sample} is absorbance of the sample at 532 nm and A_{control} is the absorbance of the fully oxidised control at 532 nm.

3.3.12 Determination of the oxidative stability using the Rancimat

The oxidative stability index (OSI) was evaluated by a Metrohm Rancimat Model 743 (Metrohm AG, Herisau, Switzerland) according to a method as described by Velasco *et al.* (2009) with slight modifications. Increasing water conductivities were continually measured while purified air (20 $L.h^{-1}$) was bubbled into the pre-treated oil sample (4.0 ± 0.5 g) heated at 110 °C and the resultant volatile compounds were collected in 60 mL Milli-Q water. At the end of the oxidation induction, the stability of the oil against oxidation was indicated by the induction time in hours. All measurements were performed in duplicate.

3.3.13 Differential scanning calorimetry measurements

The oxidative stability of the oil sample was determined by a Shimadzu DSC – 60 Differential Scanning Calorimeter (Kayto, Japan) according to a method described by Pardauil *et al.* (2011) with slight modifications. The equipment was calibrated with indium (ΔH_f = 28.45 $J.g^{-1}$), and the baseline was obtained with an empty open aluminum pan. The oil sample 5.0 ± 0.5 mg was weighed into an open aluminum pan and placed in the sample chamber. The isothermal temperature was programmed at 110°C, and purified oxygen (99.95%) was passed though the sample enclosure at 20 $mL.min^{-1}$. A preliminary study was conducted to determine the effect of the purge gas using nitrogen (99.99%) and oxygen (99.99%), respectively. When O_2 was used, the induction point (T_o) of the oxidative reaction corresponded closely to the intersection of the extrapolated baseline and the tangent line (leading edge) of the isotherm, while N_2 did not induce any oxidative

reaction (Figure 3.1). Hence O_2 was used throughout the study. All measurements were performed in duplicate.

3.3.14 Statistical analysis

All data were subjected to multivariate analysis and significant differences among means of replicates were determined by Duncan's multiple range tests. All the mean values were converted into Z-scores for each antioxidant index. The sum of Z-scores were ranked in ascending order with the view to obtain a measure of total antioxidant activity for each treatment. SPSS 22.0 for Windows® was used for the statistical analyses and the level of confidence required for significance was selected at $p < 0.05$.

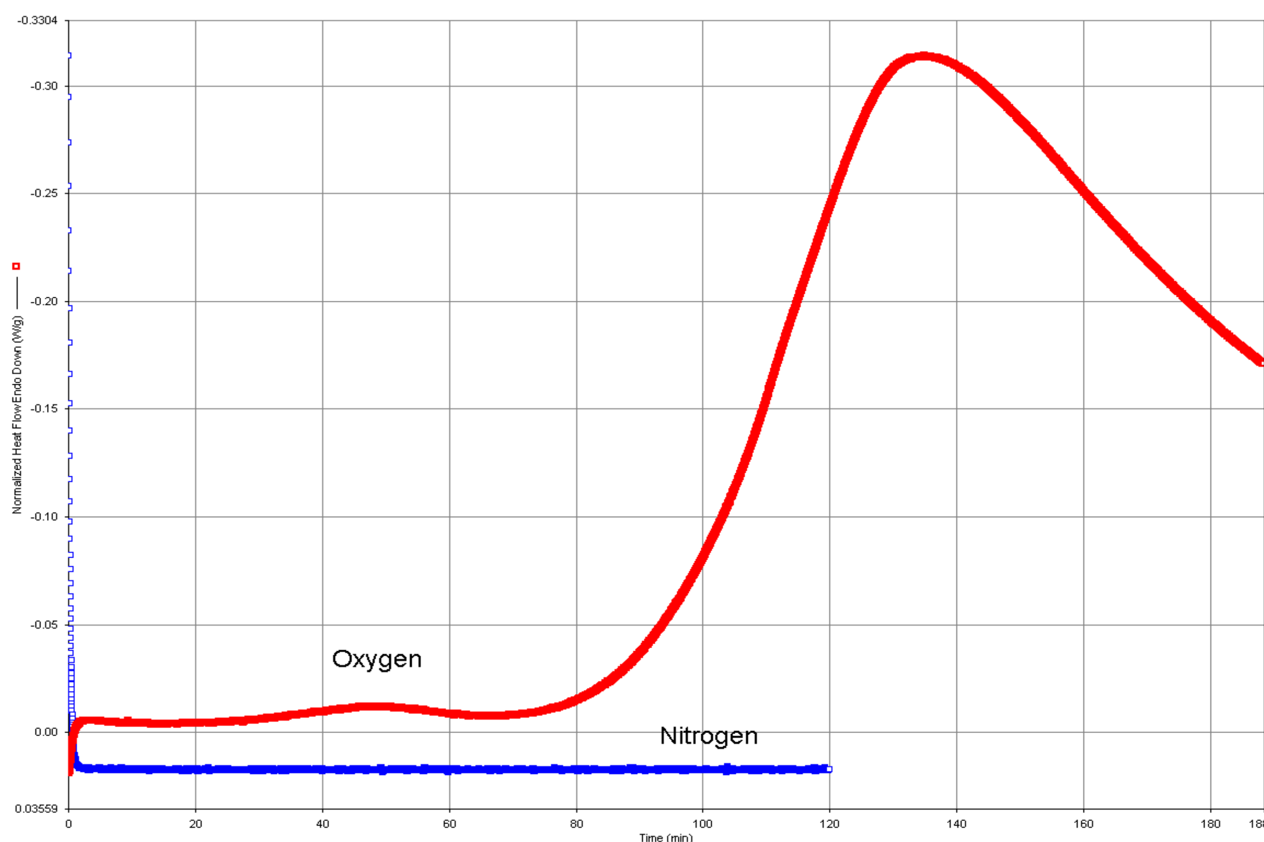

Figure 3.1 DSC oxidation curve of oil sample with MRPs, (O_2) isothermal curve at 100°C with oxygen flowing at 20 mL.min^{-1}, and (N_2) isothermal curve at 100°C with nitrogen flowing at 20 mL.min^{-1}.

3.4 Results and discussion

In the present study, casein was used as a representative food protein along with glucose, the sugar occurring most widely in biological systems (Lima *et al.,* 2010). Glucose-casein MRPs were prepared as reported in Section 3.2.2 and 100-fold dilutions were prepared using Milli-Q water. The results of the ANOVAs and Duncan's multiple range test indicated that the differences observed in the browning intensity were significant ($p < 0.05$). As expected and as can be seen in Figure 3.2 and Table 3.1, the browning intensity of MRPs increased as a function of heating temperature which is characteristic of the Maillard reaction. An exception to this was observed for MRPs produced at 90°C after 24 h, which indicated a decrease in browning index (Figure 3.2).

Figure 3.2 Reconstituted glucose-casein MRPs.

Table 3.1 Browning intensity of reconstituted MRPs[1]

Temp (°C)	Time (h)	Absorbance (420 nm) (n = 3)
60	6	0.265 ± 0.00[b]
	12	0.416 ± 0.00[g]
	24	0.241 ± 0.00[a]
75	6	0.334 ± 0.00[e]
	12	0.320 ± 0.00[d]
	24	0.379 ± 0.00[f]
90	6	0.525 ± 0.00[h]
	12	0.972 ± 0.00[i]
	24	0.286 ± 0.00[c]

[1]Results are reported as Mean ± Standard deviation. Different superscripts in the column indicate significant differences between treatments ($p \leq 0.05$).

3.4.1 Reducing power

During the reducing power (RP) assay, the presence of reductants in the samples lead to reduction of the ferricyanide (Fe^{3+}) complex to the ferrous form (Fe^{2+}). The Fe^{2+} can therefore be monitored by measuring the formation of Pearl's Prussian blue at 700 nm. This assay particularly measures the antioxidant activity of MRPs since the hydroxyl groups of MRPs play a role in reducing activity through their potential to transfer electrons (Gu *et al*., 2010; Hwang *et al*., 2011).

RP of MRPs derived at 60°C was the same regardless of the increased heating time from 6 to 12 h, followed by an increase ($p < 0.05$) at 24 h (Figure 3.3). However, at 75°C a non-significant ($p > 0.05$) increase was observed from 6 to 12 h, followed by a slight increase ($p > 0.05$) at 24 h (Table 3.2). The RP of MRPs at 90°C increased ($p > 0.05$) from 6 h to 12h ($p > 0.05$) and then decreased after 24 h ($p > 0.05$) (Figure 3.3 and Table 3.2). These results are in agreement with the reports of Gu *et al.* (2009) who reported that at a fixed temperature and pH, the reducing power increased at the beginning and decreased slowly with increased heating time.

Table 3.2 Indirect, direct antioxidant activity and oxidative stability of casein-glucose MRPs.

Treatment[1]		Indirect antioxidant assays[2]					Direct antioxidant assays[3]		Oxidative stability Index[4]	
Temp (°C)	Time (h)	RP (Abs at 700 nm)	PRS (%)	ABTS-RS (%)	DPPH-RS (%)	Fe Chelation (%)	PV (meq.kg^{-1})	TBARs (%)	Rancimat (h)	DSC (h)
	6	0.03 ± 0.02^{ab}	37.31 ± 0.00^{c}	56.86 ± 3.60^{c}	0.01 ± 0.85^{a}	35.20 ± 1.34^{de}	2.57 ± 0.03^{bcd}	63.91 ± 0.24^{i}	3.62 ± 0.45^{ab}	1.32 ± 0.09^{b}
60	12	0.03 ± 0.02^{ab}	35.47 ±0.00^{c}	57.26 ± 2.90^{c}	1.52 ± 0.56^{a}	38.64 ± 1.30^{f}	2.82 ± 0.29^{cd}	88.66 ± 0.07^{j}	3.75 ± 0.32^{ab}	1.92 ± 0.01^{e}
	24	0.58 ± 0.02^{abc}	38.02 ± 0.83^{c}	57.34 ± 4.39^{c}	6.12 ± 1.12^{a}	35.66 ± 1.38^{d}	2.46 ± 0.35^{cd}	53.97 ± 0.71^{h}	3.53 ± 0.28^{ab}	1.77 ± 0.04^{d}
	6	0.49 ± 0.03^{abc}	72.88 ± 0.18^{d}	54.30 ± 4.35^{c}	9.35 ± 5.01^{a}	51.04 ± 1.19^{g}	2.66 ± 0.44^{bcd}	63.08 ± 0.02^{i}	2.96 ± 0.82^{a}	1.45 ± 0.05^{c}
75	12	0.78 ± 0.00^{abc}	81.38 ± 0.06^{d}	55.22 ± 3.83^{c}	20.34 ± 0.83^{b}	52.15 ± 1.02^{g}	2.44 ± 0.17^{abcd}	-68.29 ± 1.08^{c}	3.91 ± 0.18^{b}	2.12 ± 0.07^{f}
	24	1.04 ± 0.00^{bc}	94.77 ± 0.12^{e}	54.37 ± 2.65^{c}	42.64 ± 18.51^{c}	33.35 ± 1.41^{d}	2.28 ± 0.03^{abc}	-143.41 ± 2.75^{a}	3.70 ± 0.45^{ab}	1.79 ± 0.02^{d}
	6	1.19 ± 0.04^{bc}	125.16 ± 0.16^{g}	58.37 ± 7.71^{c}	72.05 ± 1.82^{d}	52.59 ± 0.98^{h}	2.55 ± 0.12^{bcd}	-19.09 ± 0.51^{e}	3.75± 0.62^{ab}	2.27 ± 0.01^{g}
90	12	1.37 ± 0.11^{c}	8.26 ± 0.18^{b}	54.95 ± 9.38^{c}	73.28 ± 7.59^{d}	47.66 ± 1.11^{g}	1.89 ± 0.07^{a}	39.05 ± 0.41^{g}	3.45 ± 0.30^{ab}	1.86 ± 0.08^{de}
	24	1.14 ± 0.35^{bc}	1.73 ± 0.71^{a}	39.18 ± 8.65^{b}	81.50 ± 4.30^{d}	35.73 ± 1.37^{d}	2.11 ± 0.25^{ab}	87.34 ± 0.07^{j}	3.79 ± 0.41^{b}	1.44 ± 0.01^{c}
TBHQ (0.02%)		3.09 ± 1.58^{d}	92.76 ± 0.00^{e}	100.01 ± 0.08^{d}	96.53 ± 0.23^{e}	23.21 ± 1.54^{b}	2.16 ± 0.63^{ab}	-34.90 ± 0.83^{d}	5.74 ± 0.00^{d}	1.93 ± 0.05^{e}
TROLOX (1 mM)		0.63 ± 0.01^{abc}	83.29 ± 1.40^{e}	35.87 ± 0.00^{b}	97.66 ± 0.03^{e}	ND	2.51 ± 0.59^{abcd}	-80.41 ±0 .16^{b}	4.68 ± 0.00^{c}	1.78 ± 0.04^{d}
EDTA (1 mM)		ND	ND	ND	ND	26.17 ± 1.60^{c}	ND	ND	ND	ND
Control		0.07 ± 0.01^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	3.00 ± 0.13^{d}	0.00 ± 0.00^{f}	3.24 ± 0.48^{ab}	1.19 ± 0.01^{a}

[1] Set of fixed factors which represented model system. [2] Indirect antioxidant assays (Reducing power, Peroxyl radical, ABTS and DPPH radical scavenging activity, Fe chelation activity as an increase. [3] Direct antioxidant (Thiobarbituric reactive acid substances and peroxide value), [4]Oxidative stability index (Rancimat and DSC method). A one way ANOVA with Duncan's multiple comparison test was performed and [a - j] means with different superscripts in each column are significantly different ($p < 0.05$). ND, Not determined.

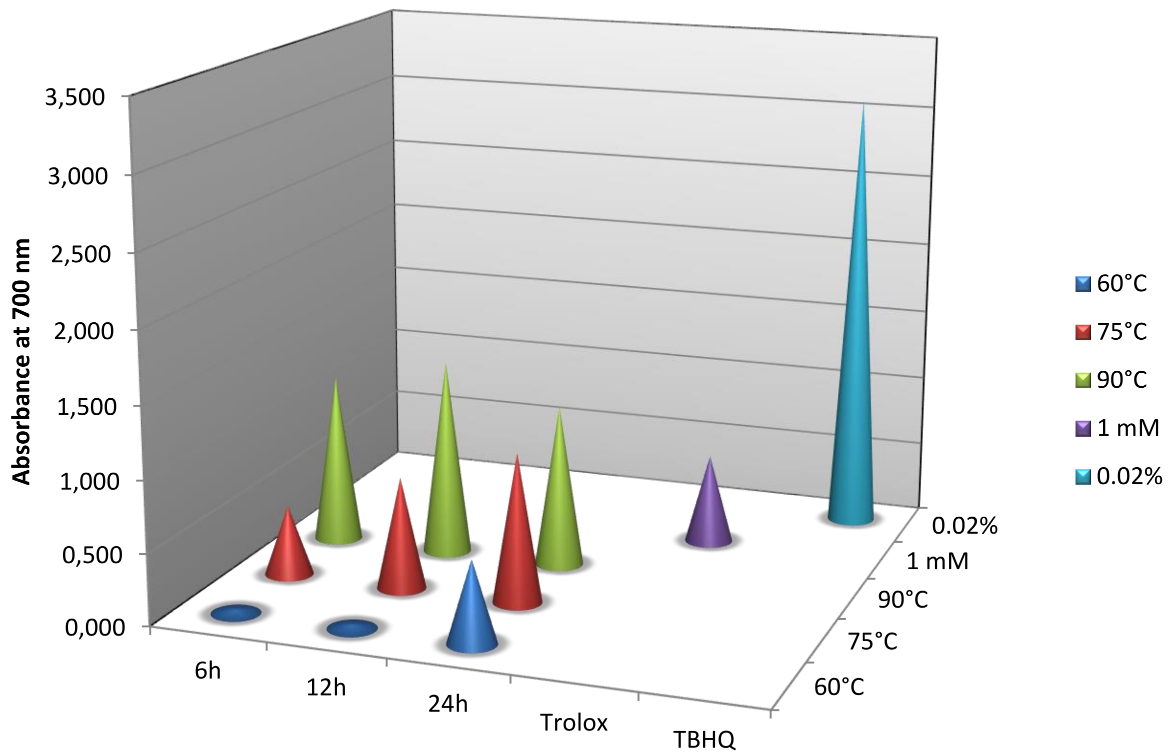

Figure 3.3 Reducing power of the MRPs from glucose-casein.

Although only at 60°C, 12 h v 90°C, 12 h, was a significant increase observed, the increase in reaction temperature resulted in an increase in RP of MRPs with the exception at 90°C for 24 h which indicated a slight decrease ($p > 0.05$) (Table 3.2).

The increase in RP as a function of heating time can be attributed to the high molecular weight compounds formed as the reaction progressed. Delgado-Andrade *et al.* (2005) and Gu *et al.* (2010) reported that high molecular weight MRPs prepared from xylose-lysine and casein-glucose model systems achieved higher reducing power in both cases. The observation was attributed to hydroxyl and pyrrole groups of advanced MRPs were reported to have the capacity to act as reducing agents.

Moreover, all MRPs derived at 90°C had a higher RP compared to Trolox ($p > 0.05$) but lower than TBHQ ($p < 0.05$) (Table 3.2). The antioxidant activity of MRPs is complex

and will vary depending on various factors such as time, temperature, reactant type and concentration and pH (Jaeger *et al.*, 2010), but these results indicate that MRPs could function as electron donors and may have a great potential as effective antioxidants in the food industry.

3.4.2 Peroxyl radical scavenging activity (PRS)

The PRS assay measures the antioxidant activity based on the hydrogen atom transfer mechanism. AAPH, a water soluble radical initiator, spontaneously decomposes at 37°C to form two carbon-centered free radicals which then react with oxygen to produce peroxyl radicals. In the present study, the antioxidative capacity of MPRs was evaluated according to their capacity to scavenge peroxyl radicals (Sachindra & Bhaskar, 2008). The resultant inhibition of oxidation of the substrate by peroxyl radicals was an indication of the scavenging activity of MRPs, thus indicating their chain-breaking activity (Figure 3.4 and Table 3.2).

Figure 3.4 shows the PRS of the glucose-casein model system as function of reaction time. At 60°C increasing reaction times did not have a significant effect ($p > 0.05$) on PRS (Table 3.2). MRPs exhibited a non-significant ($p > 0.05$) increase in PRS from 6 to 12 h at 75°C. Thereafter, increasing reaction time to 24 h at 75°C had a significant effect ($p < 0.05$) and resulted in an increase in PRS activity to 94.77%. (Table 3.2). Increasing the reaction temperature to 90°C and 6 h reaction time, resulted in a further significant increase ($p < 0.05$) (Figure 3.4 and Table 3.2). However, the PRS activity of MRPs derived at 90°C decreased significantly ($p < 0.05$) as the heating time increased beyond 6 h, i.e. at 12 h and 24 h (Table 3.2).

These results are in agreement with Vhangani & Van Wyk (2013) who also reported a decrease in the PRS of ribose-lysine at higher temperature as a function of increasing heating time. The trend in PRS of MRPs derived at 60 and 90°C was not consistent with the results of the RP reported in the previous section, while the trend at 75°C was consistent with the RP results. This phenomenon may be attributed to the multifaceted nature of MRPs and the different mechanisms of antioxidant activity, wherein MRPs are

capable of hydrogen atom transfer and electron transfer reactions, either individually or concurrently.

Furthermore, the potential as lipid antioxidants of the MRPs generated in this study was confirmed by the superior PRS activity displayed by MRPs at 75˚C, 24 h and 90˚C, 6 h compared to Trolox and TBHQ (Figure 3.3).

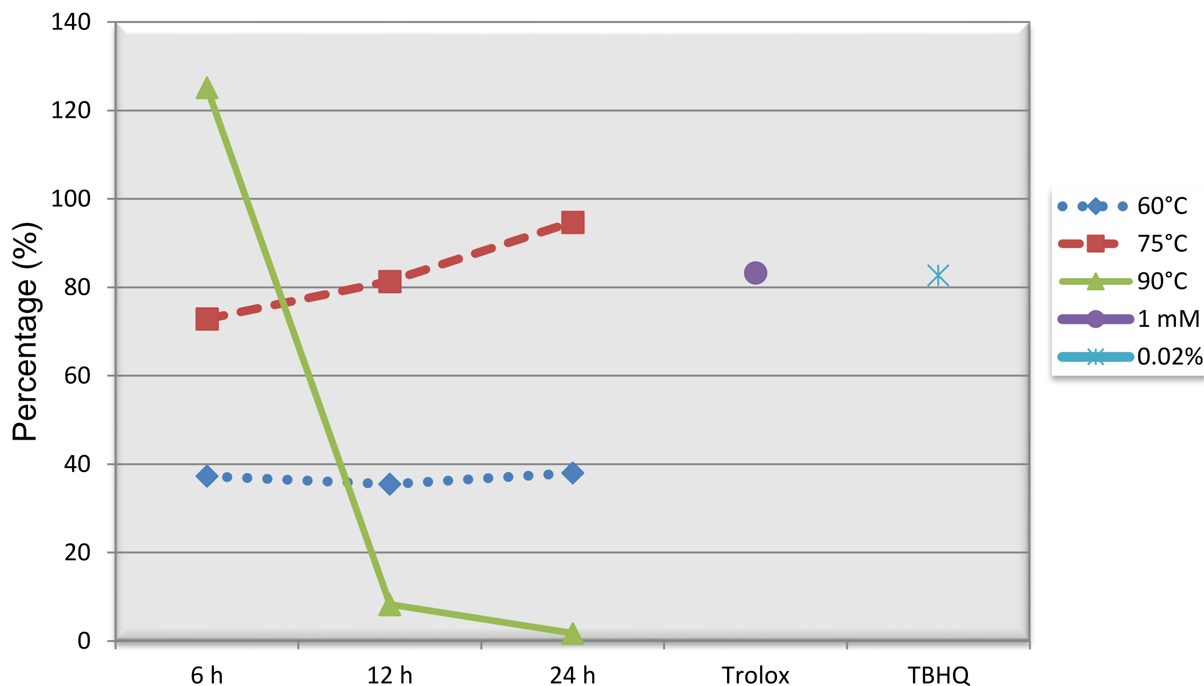

Figure 3.4 Peroxyl radical scavenging activity of glucose-casein MRPs.

3.4.3 ABTS radical scavenging

The changes in the ABTS radical scavenging activity of MRPs derived from the glucose casein model system as a function of heating time are shown in Table 3.2. With one exception, the ABTS-RS of MRPs were not significantly different ($p > 0.05$) in response to an increase either in reaction time (6 – 24 h) or in reaction temperature (60 – 90°C). The exception was a significantly lower ($p < 0.05$) ABTS-RS at 90°C and 24 hours (Table 3.2). A possible explanation for this decrease in ABTS-RS activity is the observations of Knol *et al.* (2010) who found that a reduction in pH slows down the MR, thus lowering the radical scavenging potential of the resulting MRPs. Based on the preliminary study as reported in section 3.2.2, pH was monitored and found to decreases as the reaction time and

temperature increased (results not shown). The results of this study are in contrast to those reported by Kim & Lee (2009) who found an increase in ABTS-RS as the reaction time increased in a glucose-glycine model system. However, this can be explained by the fact that, in the case of proteins, such as the casein used in this study, the reactive amino acid is the ε-amino acid of lysine, since the α-amino groups are tied up in the peptide bond and are not available for Strecker reactions resulting in a different behaviour of individual amino acids compared to proteins (Van Boekel, 2006).

Moreover, the antioxidant activity of MRPs is complex and is influenced by factors such as reactants, reaction time and temperature and pH. ABTS-RS of MRPs was greater than that of Trolox (35.87 ± 0.00%) but lower than that of TBHQ (100.01 ± 0.08%). Hence, since the MRPs displayed superior ABTS-RS compared to Trolox, this indicted the potential of MRPs as hydrophilic antioxidants.

3.4.4 DPPH radical scavenging

DPPH is a chromogen-radical containing compound that can directly react with antioxidants. When the DPPH radical is scavenged by antioxidants through the donation of hydrogen to form a stable DPPH-H molecule, the colour is changed from purple to yellow (Shon *et al.*, 2003; Vhangani & Van Wyk, 2013). Stable DPPH radical has been used widely for the determination of primary antioxidant activity that is the free radical scavenging activities of pure antioxidant compounds, plant and fruit extracts and food materials (Gülçin, 2012). Having been applied to MRPs derived from glucose-casein, the changes in DPPH-RS activity were reported in Table 3.2. This included the antioxidant properties of MRPs in comparison with TBHQ and Trolox (Table 3.2).

The increase in DPPH-RS, with increasing reaction time, of MRPs at 60°C was not significant ($p > 0.05$). Conversely, the DPPH-RS increased significantly ($p < 0.05$) as the reaction temperature increased to 75°C, but only from 12 h, after which an increase in (i.e. to 24 h) also lead to significantly ($p < 0.05$) higher DPPH-RS (Table 3.2).

However, similar to the treatment at 60°C, a non-significant increase in DPPH-RS was observed at 90°C for all reaction times. In contrast, the effect of temperature was significant since MRPs produced at 90°C exhibited significantly higher ($p < 0.05$) radical scavenging activity than the corresponding 60 and 75°C treatments.

The DPPH-RS of MRPs was significantly ($p < 0.05$) lower than that of both TBHQ and Trolox. However, the DPPH-RS of the MRPs produced at 90°C were 72.05 ± 1.82, 73.28 ± 7.59 and 81.50 ± 4.30, respectively, while that of TBHQ was 96.53 ± 0.23 and that of Trolox 97.66 ± 0.03 (Table 3.2). Hence, the DPPH-RS of MRPs produced at 90°C were of the same order of magnitude as that of TBHQ and Trolox.

The DPPH-RS of MRPs displayed a different trend to the RP, PRS, ABTS-RS which may be ascribed to the different mechanism of each assay. However, in all cases MRPs displayed antioxidant activity, often superior or comparable to Trolox and TBHQ (Table 3.2). Hence, based on the reducing power, PRS, ABTS-RS and DPPH-RS activity of MRPs from glucose-casein, MRPs have potential to be used as antioxidants in food products. These results are in agreement with Morales & Jiménez-Pérez (2001) and Vhangani & Van Wyk (2013) who also found that MRPs possess DPPH radical scavenging activity.

3.4.5 Fe^{2+} chelating activity

Table 3.2 shows the Fe^{2+} chelating activity of MRPs at first increased significantly ($p < 0.05$) firstly and then decreased as the reaction times increased at 60°C (Table 3.2). This may indicate the formation of more complex MRPs at 60°C; 24 h resulting in reduced Fe^{2+} chelating activity Zeng *et al*. (2011). A non-significant increase ($p > 0.05$) was observed at 75°C from 6 to 12 h, followed by a significant decrease ($p < 0.05$) at 24 h. At 90°C, The Fe chelation of MRPs decreased significantly ($p < 0.05$) as the reaction time increased from 6 – 24 h (Table 3.2). Increased heating temperatures (60 v 75 v 90°C) resulted in significant ($p < 0.05$) increases at 6 h reaction time, while at 12 h an increase followed by a decrease was observed and at 24 h a decrease at 75°C was followed by an increase at 90°C (Table 3.2).

Therefore, these results indicated that MRPs had different chelating activities according to reaction temperature and time. All MRPs showed high ferrous chelating activity, ranging from 35 – 52%, compared to TBHQ and EDTA (23.21 ± 1.54 and 26.17 ± 1.60%), respectively (Table 3.2). MRPs are known metal chelators and their iron binding affinity has been proposed as a possible mechanism of their antioxidant activity, since transition metals, especially copper and iron, are implicated in the generation of free radicals by the Fenton reaction with the ferrous (Fe^{2+}) ion the most powerful pro-oxidant

among various species of metal ions (Jing & Kitts, 2004; Ruiz-Roca *et al.,* 2008). Metal chelation activity plays and important role in antioxidant activity as it results in reducing the concentration of the transition metal which catalyses lipid oxidation (Delgado-Andrade *et al.,* 2004). The chelating activity can possibly be attributed to hydroxyl groups originating from MRPs (Yoshimura *et al*., 1997).

These results are consistent with the findings of Zeng *et al*. (2011) who reported that fructose-lysine and psicose-lysine MRPs decreased in ferrous chelation activity as a function of heating time at high temperatures. In addition, Ruiz-Roca *et al.* (2008) also thought new compounds with iron-binding properties were produced during thermal treatment while the significant decrease in iron chelation activity of MRPs produced at higher time-temperature combinations could be partially due to the loss of free amino acids. Similar to the PRS assay, the chelating activity of MRPs at 90°C decreased as the reaction time increased. Hence, as demonstrated thus far, MRPs exhibited antioxidant activity through RP, PRS, ABTS-RS, DPPH-RS and Fe chelation and this further confirms the versatile character of MRPs as well as the importance of using more than one assay to evaluate antioxidant activity.

3.4.6 Determination of PV

Lipid oxidation involves the continuous formation of hydroperoxides as primary oxidation products that may break down to a variety of non-volatile and volatile secondary products (Bensmira *et al.,* 2007; Iqbal & Bhanger, 2007). The formation rate of hydroperoxides outweighs their rate of decomposition during the initial stage of oxidation, and this becomes reversed at later stages. Therefore, the PV is an indicator of the initial stages of oxidative change (Roginsky & Lissi, 2005). The PV is conventionally used in the food industry as measure of oxidative deterioration of oil, fat and fatty foods. The requirements according to the Codex Alimentarius Commission (Anon, 2001) are that the PV of refined oils should not exceed 10 meq.kg^{-1} and for cold pressed and virgin oils should not be more than 15 meq.kg^{-1}.

The peroxide values of the lipid-rich emulsion with added MRPs are presented in Table 3.2. The MRPs produced at 90°C, 12 h had the lowest PV (2.11 ± 0.25), followed by those at 90°C, 24 h (Table 3.2). Hence, these MRPs were found to have an inhibition power against the formation of hydroperoxides higher than that of Trolox (2.51 ± 0.59) as

well as TBHQ (2.16 ± 0.63) albeit that the difference was not significant (Table 3.2). All MRPs had PV values lower relative to the control sample (Table 3.2). This data clearly shows that MRPs have an important role in inhibiting free radical formation during the initiation stage of lipid oxidation, interruption of the propagation of the free radical chain reaction by acting as electron donors or free radical scavengers. Moreover, MRPs were able to prevent the formation of lipid hydroperoxides and keep them at a level considerably lower than the Codex specifications.

3.4.7 Determination of TBARs

The thiobarbituric acid (TBA) test is one of the most frequently used methods to assess lipid peroxidation; it is based on the determination of malonaldehyde (MDA) which is accepted to be an important lipid oxidation product in food and biological systems. The reaction of MDA with the TBA reagent produces a pink complex with an absorption maximum at 532 nm (Kulisic *et al.*, 2004). Therefore, the increase in the amount of red/pinkish pigment as oxidative rancidity advances has been applied as a reliable oxidative indicator to a wide variety of foods. In this study the results were expressed as percentage inhibition of formation of TBARs.

As shown in Table 3.2, the antioxidant power of MRPs increased significantly ($p < 0.05$) at 60°C from 6 to 12 h followed by a significant decrease ($p < 0.05$) at 24 h. MRPs derived at 75°C displayed a significant decrease ($p < 0.05$) in antioxidant power as function of increasing heating time (Table 3.2). The data suggest that MRPs produced at this temperature with longer reaction times will be less effective in inhibiting lipid oxidation.

At 90°C the antioxidant power of MRPs increased as a function of reaction time in all cases. All MRPs displayed significantly ($p < 0.05$) higher antioxidant activity compared to both TBHQ and Trolox (Table 3.2). In particular, in comparison with Trolox and TBHQ, MRPs produced at 60°C and 90°C (24 h) exhibited superior antioxidant activity. The most efficient MRPs were 60°C, 12 h > 90°C, 24 h > 60°C, 6 h, reducing the production of TBARs by 88.66 ± 0.07, 87.34 ± 0.07, and 63.91 ± 0.24%, respectively. Hence, the incorporation of MRPs improved the protection of the lipid-rich food model system against lipid oxidation. The result obtained at 90°C; 24 h agree well with those reported for the PV.

3.4.8 Oxidative stability by Rancimat

The Rancimat apparatus determine the induction time of the lipid oxidation in different fats and oils and the inclusion of antioxidants delays the induction time (Tan *et al.,* 2002). As can be seen in Table 3.2, MRPs derived at 60°C exhibited no significant ($p > 0.05$) differences in oxidative stability as a function of heating time. However, MRPs derived at 12 h had the highest antioxidant activity at this reaction temperature (Table 3.2). This result was in agreement with the reported results of the TBARs assay. MRPs at 75°C exhibited a significant ($p < 0.05$) increase in oxidative stability from 6 to 12 h, followed by a slight decrease at 24 h ($p > 0.05$). In this treatment, MRPs produced at 12 h had the highest oxidative stability (3.91 ± 0.18) than any other treatment, although it was not significantly higher that the induction time observed for MRPs derived at 90°C; 24 h (Table 3.2). Moreover, the induction time observed for samples treated with MRPs produced at 90°C decreased and then increased as the heating time increased. The result obtained at 90°C, 24 h was consistent those reported for the TBARs assay. In this study the oxidative stability of the lipid-rich model system treated with MRPs was significantly lower ($p < 0.05$) compared to those treated with either TBHQ or Trolox, but higher than the untreated sample, albeit that the difference was not significant ($p > 0.05$) (Table 3.2). The superior antioxidant capacity of TBHQ was expected, since, as a synthetic food additive, it is one of the most potent fat-soluble antioxidants. However, currently there are growing concerns about the safety of the synthetic antioxidants. Moreover, based on the results of the PV and TBARs and Rancimat analysis, MRPs have potential for application as natural lipid antioxidants in food products.

3.4.9 Oxidative stability by differential scanning calorimetry (DSC)

In general, oxygen reacts with the double bonds present in lipids, following a free radical mechanism, known as autoxidation. Various reactions during lipid oxidation occur simultaneously at different rates. Lipid oxidation is an exothermic reaction and the heat evolved makes it possible to employ differential scanning calorimetry (DSC) for its study (Tan *et al.*, 2002).

The assessment of oxidative stability by DSC was measured by the induction period through exothermic curves of oxidation. T_{onset} vectors were extrapolated from the intersection of the tangent lines of each curve used for analysis.

The results for the oxidative stability of oil with and with MRPs, measured as oxidation induction time (OIT), are shown in Table 3.2. MRPs produced at 90°C, 6 h (2.27 ± 0.01) provided the highest oxidative stability compared to both Trolox (1.78 ± 0.04) and TBHQ (1.93 ± 0.05). The OIT of MRPs derived at 60 and 75°C for 12 h was in in agreement with the result of the Rancimat assay. The values for the induction periods obtained by Rancimat are higher than those obtained by DSC. This difference could relate to the lower quantity of sample used in DSC (5 mg) in comparison to the Rancimat (5 g). According to Tan *et al*. (2002), another important factor to consider is the surface-volume relationship between the oil and oxygen. The small portion samples used for DSC analyses have a higher surface-volume relationship than the samples placed in a test tube for the Rancimat analyses. Generally, oxygen and oil can react more efficiently when a small quantity of oil, or high surface-volume ratio, is used. It should also be highlighted that the DSC measurements used pure oxygen while the Rancimat used air, which contains approximately 21% O_2. In other words, by using DSC, an induction period is reached more rapidly than by Rancimat.

However, the DSC results for MRP-treated samples served as further confirmation that MRPs have potential as food additives, namely as natural alternatives to synthetic lipid antioxidants such as TBHQ.

3.4.10 Z-scores analysis

The analysis of the antioxidant activity was determined using different methods with diverse chemical properties in order to obtain a more complete picture of the antioxidant capacity of MRPs. The z-scores of each assay were ranked with the view to determine the relative contribution to the total antioxidant activity of MRPs, namely the sum of all the z-scores for each MRP. Based on the results for the indirect antioxidant activity assays as shown in Table 3.3, the three top performing MRPs were 90°C, 6 h > 75°C, 12 h; and 90°C, 12 h. In addition, MRPs produced at 90°C, 6h achieved higher z-scores than TBHQ and Trolox, respectively. The predominant antioxidant effect for MRPs produced at 90°C,

6 h was PRS, Fe chelation and ABTS-RS. Hence, the main mechanism of action was observed via metal chelation, ET and HAT (Table 3.3).

With regards to the direct antioxidant activity and oxidative stability assays (Table 3.4), the MRPs derived at 60°C, 12 h, 90°C; 6 h and 75°C, 12 h were the three top performing treatments. The MRPs derived at 60°C for 12 h had the highest z-scores in both PV and TBARs inhibition assays, while the oxidative stability as measured by the DSC was high for MRPs derived at 90°C, 6 h. Considering all the assays applied in this study, the MRPs produced at 90°C, 6h > 75°C, 12 h > 90°C, 12 h achieved the highest z-scores (Table 3.5). Hence, this study has shown the versatile character of MRPs in terms of their mechanism as antioxidants and the importance of applying more than one assay to evaluate the antioxidant activity of MRPs.

Table 3.3 Ranked z-scores for indirect antioxidant assays.

Treatment	RP	PRS	ABTS	DPPH-RS	Fe Chelation	Sum
90°C (6 h)	9	11	10	7	11	48
TBHQ (0.02%)	11	9	11	10	2	43
75°C (12 h)	6	7	6	5	10	34
90°C (12 h)	10	2	5	8	8	33
75°C (24 h)	7	10	4	6	3	30
60°C (24 h)	4	5	9	3	5	26
90° C (24 h)	8	1	2	9	6	26
TROLOX (1 mM)	5	8	1	11	1	26
75°C (6 h)	3	6	3	4	9	25
60°C (12 h)	2	3	8	2	7	22
60°C (6 h)	1	4	7	1	4	17

Table 3.4 Ranked z-scores for direct antioxidant assays.

Treatment	PV	TBARs	Rancimat	DSC	Sum
60°C (12 h)	11	11	7	8	37
90°C (6 h)	8	5	6	11	30
75°C (12 h)	5	3	9	10	27
TBHQ (0.02%)	3	4	11	9	27
TROLOX (1 mM)	7	2	10	5	24
60°C (6 h)	9	9	4	1	23
75°C (6 h)	10	8	1	3	22
90°C (24 h)	2	10	8	2	22
60°C (24 h)	6	7	3	4	20
75°C (24 h)	4	1	5	6	16
90°C (12 h)	1	6	2	7	16

Table 3.5 Sum of ranked z-scored for indirect, indirect antioxidant assays and Oxidative stability index of MRPs.

Treatments	Sum
90°C (6 h)	78
TBHQ (0.02%)	70
75°C (12 h)	61
60°C (12 h)	59
TROLOX (1 mM)	50
90°C (12 h)	49
90°C (24 h)	48
75°C (6 h)	47
60°C (24 h)	46
75°C (24 h)	46
60°C (6 h)	40

3.4.11 Conclusions

Antioxidant activity of glucose-casein MRPs increased with increased reaction time based on the RP and DPPH-RS. The PRS and Fe chelation of MRPs derived at 90°C decreased as the heating time increased while the DPPH-RS increased as the reaction time increased. The antioxidant activity of the MRPs was, in many instances comparable or superior to that of the synthetic antioxidants, namely TBHQ and Trolox. Examples are: RP (MRPs at 90°C > Trolox); PRS (MRPs at 75°C, 24 h and 90°C, 6 h > Trolox and TBHQ) and the ABTS-RS of MRPs was greater than that of Trolox. The latter indicated the potential of MRPs as hydrophilic antioxidants. MRPs also displayed superior Fe^{2+} chelation relative to TBHQ and EDTA. The incorporation of MRPs improved the protection of the of the lipid-rich food model against lipid oxidation, as confirmed by the PV, TBARs, Rancimat and DSC results. Hence, based on the direct, indirect and oxidative stability results, MRPs can be used in food products as natural alternatives to synthetic antioxidants, such as TBHQ and EDTA.

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CHAPTER 4

HPLC determination of intermediate MRPs derived from glucose-casein

4.1 Abstract

Maillard reaction products (MRPs) were prepared from an aqueous glucose-casein model system at pH 8, heated for 1 – 12 h at 60°C and 75°C and 1 – 6 h 90°C, respectively. Furosine (N-ε-Fructosyl-lysine) and Pyrraline (2-amino-6-(2-formyl-5-hydroxymethyl-1-pyrrolyl)-hexanoic acid) were determined using a high pressure liquid chromatography (HPLC) assay to evaluate the extent of the Maillard reaction (MR). Furosine concentration ranged between 0.44 – 1.075 $mg.L^{-1}$ in MRPs derived at 60°C, while at 75°C an increase as a function of heating time was observed. In addition, the levels of furosine of MRPs derived at 90°C were higher than those at 60°C and 75°C irrespective of the heating time. Furthermore, MRPs derived at 60°C and 75°C exhibited a varied concentration of pyrraline as the reaction time increased with the higher temperature resulting in higher concentrations. The result of this study can provide valuable information of the heat effects and the extent of the Maillard reaction in food products containing casein and glucose.

4.2 Introduction

Technological processes applied to food products can give rise to modifications to their composition. One of the most important modifications induced in food by heating is the Maillard reaction (MR), which involves amino acids and reducing sugars. During roasting, baking and frying, different chemical reactions between food components take place, giving rise to new substances or compounds (Kim & Lee, 2009; Delgado-Andrade *et al.,* 2010).

As mentioned, the MR is considered to make a contribution to this pool of newly formed compounds and it is a type of non-enzymatic browning of prime importance to the food industry as it affects the quality of processed food products, in particular the organoleptic attributes (Martins *et al.,* 2001; Shimamura *et al.*, 2011). The MR results from

an initial interaction of a reducing sugar with an amino compound, followed by a cascade of consecutive and parallel reactions to a variety of colourless and coloured products with a range of flavour volatiles (low molecular weight) to melanoidins (low and high molecular weight, brown and nitrogenous chromophores). It can result in either deterioration or enhancement of food quality. Previously, many scientific works focused on the negative biological effects of the MR. The formation of anti-nutritional and toxic MRPs has been reported, including acrylamide, a potential human carcinogen (Claus *et al.,* 2008; Hedegaard *et al.,* 2008; Jin *et al.,* 2013).

The formation of beneficial compounds during the MR has also been demonstrated and is currently gaining a lot of attention. MRPs containing antiallergenic, antimicrobial and cytotoxic properties, amongst others, are reported (Silván *et al.,* 2006). High antioxidant capacity of MRPs in model systems and foods such as coffee, bakery products and beer has also been studied (Delgado-Andrade *et al.,* 2010). In vitro studies demonstrated that MRPs may offer substantial health-promoting activity as they can act as reducing agents, metal chelators and radical scavengers (Wang *et al*., 2011). Several papers indicated that the MR can be a good means of producing functional food ingredients also since they can be obtained without the use of harmful chemicals and tedious procedures (Silván *et al.,* 2006; Vhangani & Van Wyk, 2013).

However, intermediates and specific pathways of the MR still require elucidation (Martins *et al.,* 2001). In this regard, intermediate MRPs such as furosine and pyrraline play a key role in understating the extent of the MR.

Furosine (N-ε-2-furoylmethyl-L-lysine or N-ε-fructose-lysine) is an amino acid derivative formed during the acid hydrolysis of Amadori products (N-substituted 1-amino-1- deoxy-2-ketoses) such as fructose-lysine, lactulose-lysine, and maltulose-lysine, which are generated in the early stages of the MR during the heat processing of foods (Erbersdobler & Somoza, 2007). For this reason, estimates of the extent of protein damage caused by heating in the first stages of the MR are often based on determinations of the amount of furosine that forms during the acid hydrolysis of foods (Drusch *et al.,* 1999). On the other hand, furosine is also monitored during the evaluation of the extent of the early MR *in vivo* such as to predict retinopathy and nephropathy risk (Claus *et al.,* 2008). Furosine can also be considered as a marker of dietary intake of AGEs (Advanced Glycation End products), apart from carboxymethyllysine (CML), which is involved with

several complications of degenerative diseases *in vivo* (for example diabetic nephropathy, uremia and cataracts). Therefore, the determination of furosine is a useful marker of the early stage of the Maillard reaction.

Pyrraline (2-amino-6-(2-formyl-5-hydroxymethyl-1-pyrrolyl)-hexanoic acid) is an advanced Maillard product that is an acid labile pyrrole compound, resulting from a reaction between the ε-amino group of lysine and 3-deoxyglucusulose, which is a degradation product of reducing sugars and aminoketoses (Rufian-Henares *et al*., 2004).

The difficulty to demonstrate the compounds contributing to the antioxidant activity of MRPs lies in the complex composition of MRPs which is widely affected by reactants, reaction time and temperature, pH and water activity (A_w). Moreover, although efforts have been made for devising better and uniform methodologies for evaluation and measurement of oxidation and efficacy of antioxidants, to date there is little evidence or information concerning the absolute chemical structures of MRPs derived from model systems under specified conditions. Most studies are being confined to aqueous solutions of single sugar-amino acid combinations. Very few studies have dealt with the intermediate MRPs from sugar-protein mixtures, although these simulate food products more accurately. Hence, the purpose of this study was to identify/elucidate on the intermediate compounds (Furosine and Pyrraline) formed in MRPs derived from casein-glucose by using High Performance Liquid Chromatography (HPLC) assays.

4.3 Materials and Methods

4.3.1 Chemicals

Casein, Glucose, methanol HPLC grade, Hydrochloric acid, Formic acid were purchased form Merck (Merck, South Africa). Furosine and Pyrraline were purchased from polypeptide (Strasbourg, France), RP-Luna C18 column was supplied by separations (Pretoria, South Africa). All the chemicals used in this study were of analytical grade and chemical reagents were prepared according to standard analytical procedures. Prepared reagents were stored under conditions that prevented deterioration or contamination. The water used in the study was ultrapure water purified with a Milli-Q water purification system (Millipore, Microsep, Bellville, South Africa).

4.3.2 Synthesis of MRPs

MRPs were prepared according to the method of Gu *et al.* (2009) with slight modifications. Casein (5.0 g) and glucose (10.0 g) were dissolved in 100 mL of 0.1 M Tris-HCl buffer at pH 8. The samples were transferred into 250 mL Schott bottles and heated for 1 – 12 h (60 and 75°C) and 1 – 6 h (90°C) in a water bath. After the heating period had elapsed, the resulting MRPs were immediately cooled in an ice bath. The prepared MRPs were stored in a refrigerator at 4°C.

4.3.3 Determination of Furosine and Pyrraline

Furosine (N-ε-Fructosyl-lysine) and Pyrraline (2-amino-6-(2-formyl-5-hydroxymethyl-1-pyrrolyl)-hexanoic acid) determination was performed using the method described by Delgado-Andrade *et al.* (2010) and Serpen *et al* (2012) with minor modifications. A 5 mL sample of MRPs in a screw-cap glass tube was subjected to hydrolysis by adding 10 mL of 8.0 M HCl. High-purity nitrogen (N_2) gas was bubbled through the sample for 2 min. The test tube was capped and heated at 110°C for 23 h. Figure 4.1 depicts the formation of furosine by means of acid hydrolysis of the sample under controlled conditions as depicted. The hydrolysed sample was cooled at ambient temperature and diluted 10-fold with Milli-Q water. This preparation was syringe-filtered (Acrodisc PSF, 0.45 μm, PALL Life Science AP-4426) to remove interfering compounds before HPLC analysis. Direct determination of furosine and pyrraline was performed on Agilent model 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) consisting of quaternary pump, a diode array detector (DAD) and a temperature controlled column oven. The chromatographic separation was performed on an RP-Luna C18 column (250 x 4.6 mm, i.d., 5 μm) by using (A) 1% Formic acid (80%) and (B) methanol (20%) solutions as the mobile phase at an isocratic flow rate of 1.0 mL.min^{-1} at 40°C. The injection volume was 10 μL and detection was performed at 280 nm. Furosine and pyrraline were quantified by the standard external method. The calibration curve was construed form a stock solution (5.0 mg.mL^{-1}) in the range of 0.001 – 0.10 mg.mL^{-1}. Each sample was hydrolyzed and analyzed in duplicate and the mean of two measurements was reported.

4.3.4 Statistical analysis

All data were subjected to multivariate analysis and significant differences among means of replicates were determined by Duncan's multiple range tests using SPSS 22.0 for Windows®. The level of confidence required for significance was selected at $p < 0.05$.

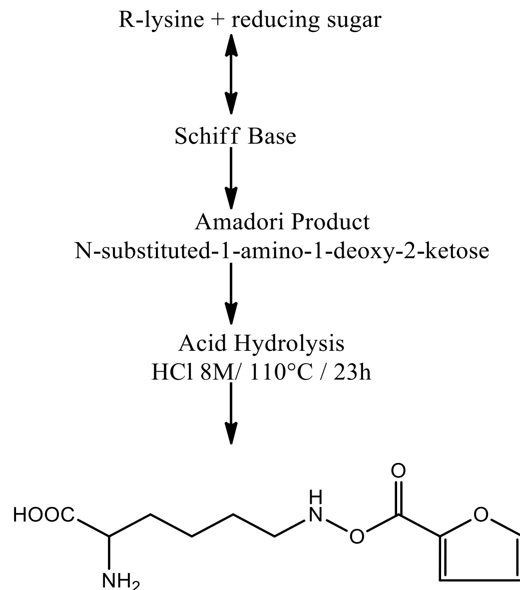

Figure 4.1 Schematic representation of formation of furosine after acid hydrolysis.

4.4 Results and discussion

4.4.1 Furosine content of glucose-casein MRPs

The HPLC chromatogram obtained for furosine and pyrraline shows retention times of 2.12 and 5.14 minutes, respectively (Fig. 4.2). The furosine content of glucose-casein MRPs as a function of heating time is shown in Figure 4.2. The furosine content of MRPs derived at 60°C increased from 0.44 – 1.075 g.L^{-1}, while at 75°C a more pronounced increase was observed as a function of increase in heating time, indicating the formation of this Amadori compound during the heating process. This is in line with the general idea that increasing temperatures promote the MR. However, at 90°C an increase in the furosine content was observed from 1 – 3 hours followed by a decline between 4 and 5 hours, followed by an increase at 6 h. A possible explanation for the observed decrease in furosine content at 4 and 5 hours of heating, could be that, since the MR is a multi-step

reaction, the Amadori products formed firstly continue reacting with other compounds to reach the intermediate steps, potentially leading to a decrease in the furosine levels. However, the changes in furosine levels in these samples clearly indicate that this is a compound of the intermediate phase of the MR. As expected, the formation of furosine was higher at 90°C compared to 75 and 60°C, respectively, since, it is well known that the progression of the MR depends on the reaction temperature.

4.4.2 Pyrraline content of glucose-casein MRPs

The pyrraline content of MRPs derived from glucose-casein MRPs is shown in Figure 4.4. MRPs derived at 60°C and 75°C showed a variation in pyrraline concentration as the reaction time increased, with the higher reaction temperature resulting in a higher pyrraline concentration. Pyrraline is an acid labile compound and as the MR progresses the pH decreases, this may be the attributed to the instability of pyrraline content detected in the samples. Interestingly, it was observed that at 90°C the pyrraline content decreased as the reaction time increased beyond 1 h reaction time. This decrease indicates that the MR has advanced beyond AGEs and have progressed to the third stage of the MR, particularly polymerization. The brown colour of the reaction mixture was testimony to that (results not shown). Morales and Van Boekel (1996) studied pyrraline content in model systems with glucose or lactose and caseinate heated at 110 – 150°C for 30 min. They found large amounts of pyrraline at temperatures over 120°C and higher reactivity with glucose than with lactose. The higher levels of pyrraline found by these authors at the higher reaction temperatures agreed with the high levels found at 90˚C (i.e. the highest temperature) in this study. However, the decreases in pyrraline found in this study beyond 1 h heating at 90°C is very likely related to the fact that the MR had progressed to the third stage and that polymerization products had started forming. In contrast, Morales and Van Boekel (1996) did not study pyrraline levels beyond 30 min reaction time.

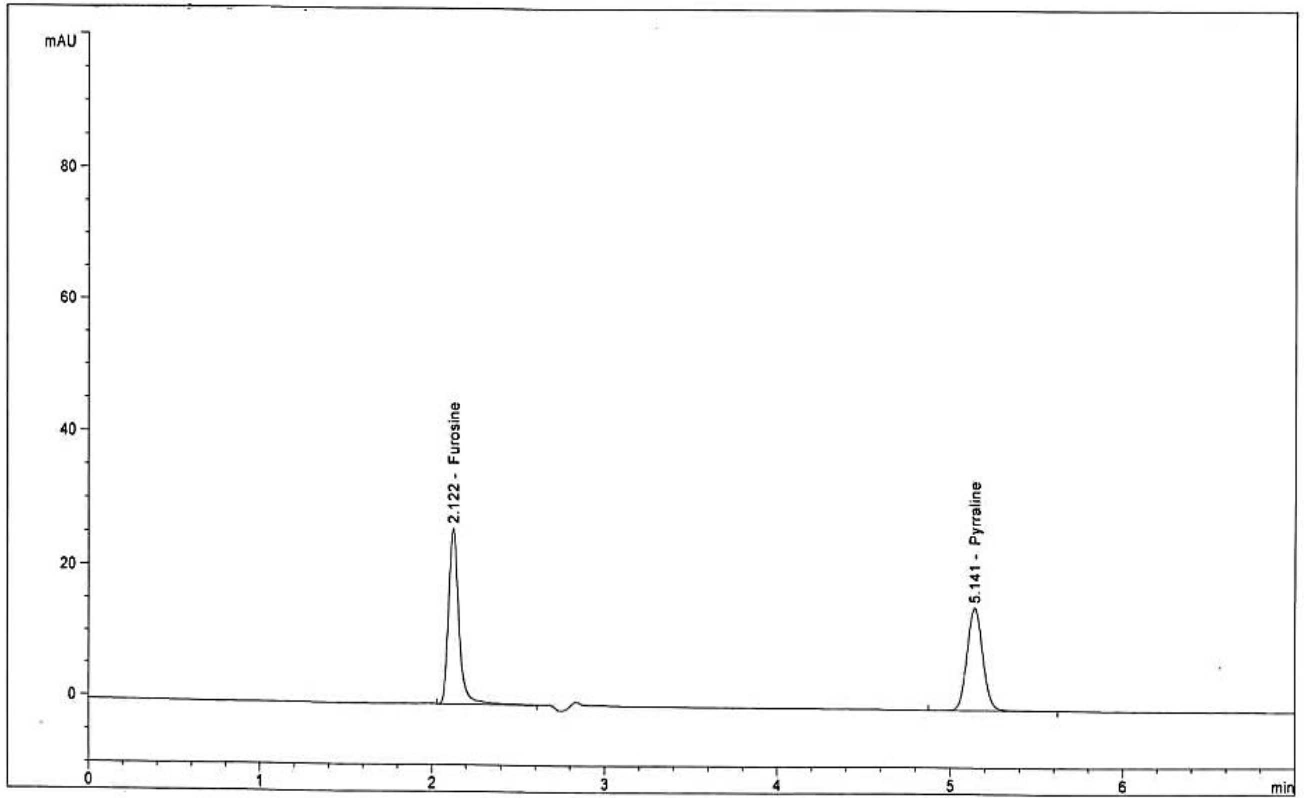

Figure 4.2 HPLC Chromatogram for furosine and pyrraline.

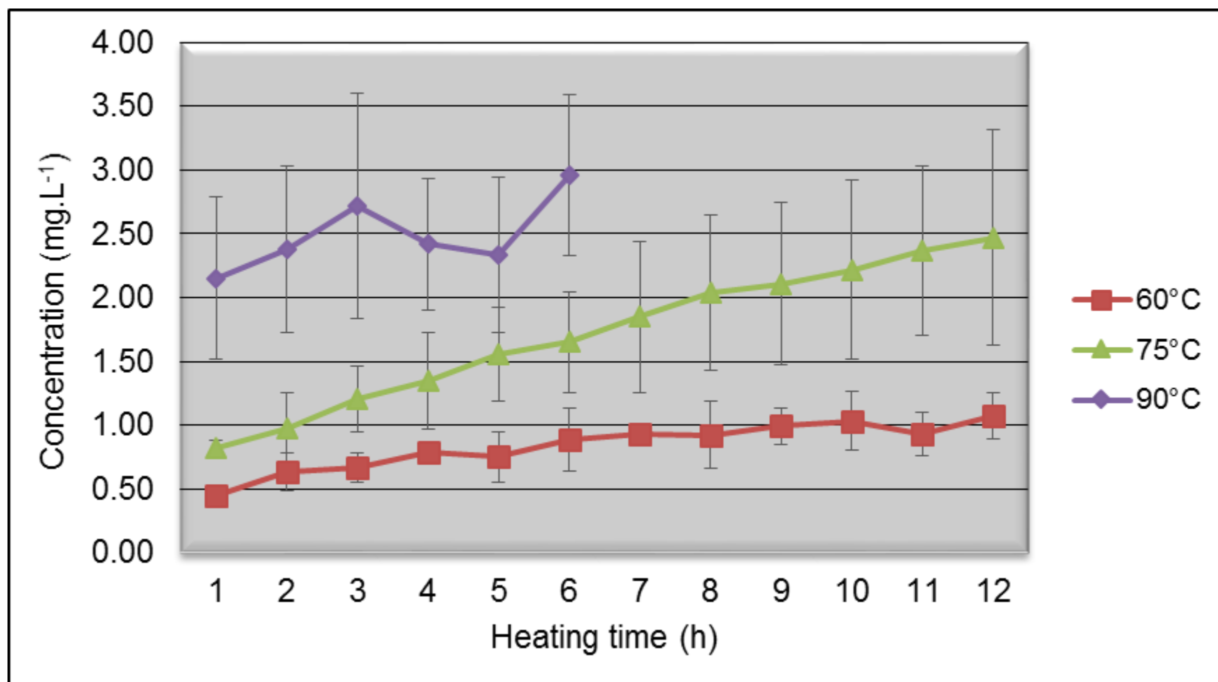

Figure 4.3 Formation of furosine in glucose-casein MRPs.

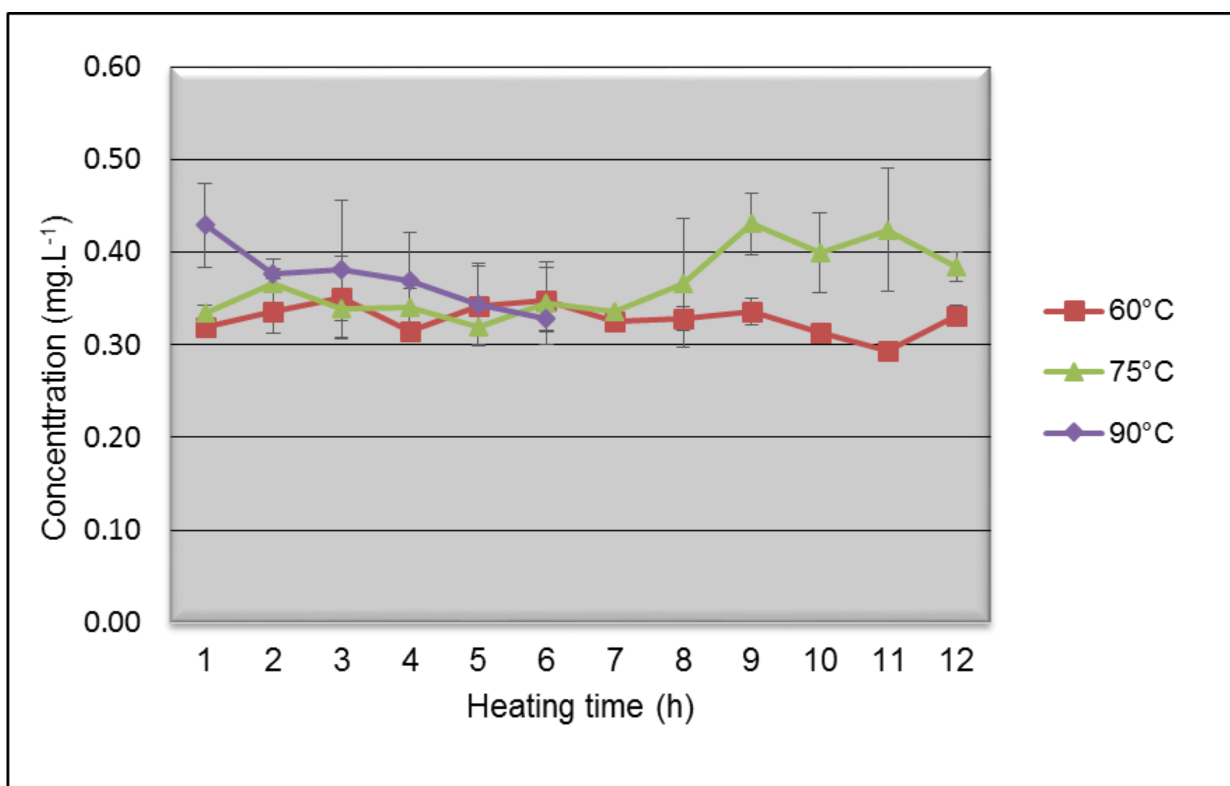

Figure 4.4 Formation of pyrraline in glucose-casein MRPs.

4.5 Conclusion

In the present study, the extent of the Maillard reaction in MRPs was investigated, using furosine as an indicator for the early stage, and pyrraline as an indicator of advanced glycation. The presence of furosine in the MRP samples confirmed that this compound of the intermediate phase of the MR was formed in all samples. Moreover, the increasing of levels of furosine further confirmed that increasing heating temperature and heating time enhances the formation of MRPs such as furosine. The results for pyrraline showed a similar relationship to temperature at 1 h of reaction time, namely that the pyralline level increased with increasing reaction temperature, while the subsequent decrease in pyrraline concentration at the highest temperature strongly indicated that the MR has reached a more advanced phase. In conclusion, as the Maillard reaction is a complex scheme of linked reaction cascades, the results of this study show that the concentration of both furosine and pyrraline in glucose-casein MRPs is influenced by the heating time and temperature and hence serves as some indication of the extent of the MR. However, more compounds, such as N-(carboxyethyl)lysine (CEL), N-(carboxymethyl)lysine (CML), Methylglyoxal and hydroxymethylfurfural (HMF), should be monitored in order to get a more complete picture of the MR intermediates that are formed.

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CHAPTER 5

GENERAL SUMMARY AND CONCLUSSIONS

The aim of this study was to investigate the antioxidant effect of sugar-protein (casein-glucose) MRPs with the view to identifying an alternative to synthetic antioxidants in lipid-rich products. In Chapter 1 a general introduction of the Maillard reaction was given followed by a more complete overview in Chapter 2. The possible routes and type of products that are formed were summarised. Chapter 2 also presented a detailed explanation of the mechanism and factors affecting lipid oxidation in food systems. This is an important step to establish strategies to prevent this reaction where it is not desirable in the food industry. The present thesis focused in particular on preventing lipid oxidation in a sunflower oil-in-water emulsion using sugar-protein MRPs.

In the Maillard reaction, melanoidins (brown nitrogenous polymers and co-polymers) are known as the main products of this reaction. Although the mechanism of their formation is not fully understood and their chemical structure is largely unknown, these melanoidins have a significant effect on consumer perception of food products, since colour is an important attribute in consumer acceptance of food products (Martins *et al.,* 2001; Liu *et al.*, 2012). In Chapter 3, the browning intensity of casein-glucose MRPs increased as a function of heating time and temperature ($p > 0.05$). This further confirms reaction temperature and time as important factors affecting the MR. Moreover, this is significant since MRPs with antioxidant potential produced at lower temperatures and processing time can be applied in food products without imparting colour.

The versatile character of MRPs was demonstrated using different antioxidant assays (DPPH-RS, RP, PRS, ABTS and Fe chelation). This is important since no single antioxidant assay is sufficient to determine the overall antioxidant activity of MRPs. The MR is cascade of consecutive and parallel reaction steps. Such cascade reactions in which several reactants and products take part frequently occurs in food systems.

In Chapter 3, The RP of casein-glucose MRPs increased significantly with an increase in reaction temperature. The hydroxyl and pyrrole groups of advanced MRPs

may act as reducing agents. During the Maillard reaction several pyrroles with hydroxyalkyl side-chains can be formed together with other products. In addition, the potential as lipid antioxidants of MRPs generated in this thesis was confirmed by the superior PRS activity displayed by MRPs particularly at 75°C/ 24 h and 90°C/6 h compared to Trolox and TBHQ, respectively. As illustrated in Chapter 3, in all cases MRPs displayed antioxidant activity, often superior or compared to Trolox and TBHQ. Therefore, based on the RP, ABTS-RS, DPPH-RS and Iron chelation activity of MRPs from glucose-casein, MRPs have the potential to be used as antioxidants in food products. This further confirmed the versatile character of MRPs and well as the essence of using more than one assay to evaluate the antioxidant activity.

The work carried out in this thesis for the first time provides evidence of prevention or retardation of lipid oxidation in a lipid-rich system (mayonnaise) using casein-glucose MRPs. The MRPs derived from glucose-casein model systems were able to inhibit the formation of hydro-peroxides. This is shown by the low PV in the oil in water emulsion. The oxidative stability results by the Rancimat and DSC further confirmed the potential of MRPs as food additives, namely as natural or clean label antioxidants. These results indicate that MRPs have potential as alternatives to synthetic lipid antioxidants such as TBHQ. The hypothesis that DPPH-RS, ABTS-SR, PRS activity and RR are expected to increase with an increase in reaction time is accepted. On the basis of these results MRPs derived from casein-glucose with proven antioxidant properties may be used as antioxidants in food products.

In Chapter 4, the extent of the Maillard reaction in MRPs was investigated, using furosine as an indicator for the early stage, and pyrraline as an indicator of advanced glycation. Furosine concentration in glucose-casein MRPs increased significantly as a function of reaction temperatures. Moreover, the decrease in concentration of pyrraline at higher temperature strongly indicated that the MR has reached a more advanced phase.

The chemistry underlying the Maillard reaction is very complex. It encompasses not one reaction pathway but a whole network of various reactions. A better insight on the antioxidant activity of casein glucose-casein MRPs has been obtained and a major step was taken in identifying and quantifying two important MR indicators (furosine and pyralline). It is further recommended to determine other intermediates such as N-ε-carboxymethyl lysine (CML) and furfurals (2-furaldehyde,5-hydroxymethyl-2-furaldehyde

(HMF), 2-furylmethylketone, 5 methylfuraldehyde) with the view to obtain a complete picture of the MR intermediates that are formed in casein-glucose model systems

Therefore, the conclusion of this study is that casein-glucose MRPs can be used as alternatives to synthetic antioxidants in food product. Future research is required to elucidate the structures of all MRPs formed and the functional groups responsible for the observed antioxidant activity in casein-glucose model systems. In this respect, Matrix-assisted laser desorption ionization-mass spectrometry with time-of-flight detection (MALDI-TOF/MS), ultra-high performance liquid chromatography coupled electrospray ionization tandem mass spectrometry (UHPLC-ESI-MS/MS) and Fourier transform infrared spectroscopy (FT-IR) are promising tools that can be used analyse MRPs.

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