Development of physically stable canola oil-in-water emulsion and evaluation of the effect of endogenous phenolics on the oxidative stability

by

Dayanidhi Singh Huidrom

A Thesis submitted to the Faculty of Graduate Studies of
The University of Manitoba
in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

Department of Human Nutritional Sciences
University of Manitoba
Winnipeg

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Abstract

The present study evaluated the effect of canola (*Brassica juncea*) derived antioxidants on the oxidation in whey protein stabilized canola oil-in-water emulsion at elevated temperature. 10 % canola oil in water emulsion using 1% whey protein as emulsifier was first prepared by passing through homogeniser. Canola antioxidants extracts such as sinapic acid extract (**SAE**), sinapine (**SP**), Canolol (**CAN**) and whole extract, (**WE**) at two concentrations (100 and 350 µM), were added and incubated at 30° C. Sinapic acid (**SA**) and Butylated hydroxyl toluene (**BHT**) standards were also used as references. Primary oxidation marker like peroxides and secondary oxidation volatile products like hexanal, pentanal and 2,4-heptadienal were monitored to assess the anti-oxidative effect. BHT was found to be the most effective AO. WE and SP were also equally good as BHT. Peroxide values were significantly different ($P<0.05$) in case of BHT-100* (*concentration in µM), BHT-350, SP-350 and WE-350 compared to control. Same pattern was also followed in volatiles measurement. WE and SP were also shown to be potent AO as shown by DPPH, chelating and reducing assays. The physical stability of the emulsion was determined by particle size measurement. The particle size (diameter) of oil droplets remains constant throughout experimental period (0.162-0.188 µm). Viscosity was also determined by rheometer and found to be stable over 15 days (7.0 - 8.5 mPa.s). This also demonstrated that WE and SP have the potential to replace other synthetic antioxidants in a concentration dependant manner.

Acknowledgement

It is a great pleasure for me to realise that my master thesis has finally come to see the light. In my opinion, the thesis is the pinnacle of the two years long master program and without the contributions of each and everyone, this could have never happened. I am deeply thankful to all of them no matter how small or big they assist in making it possible.

My advisor, Dr. Usham Thiyam-Hollander, did a remarkable job in pushing me hard to get things done. I should say that due to her persistence and motivation, I could complete my study and research in time. I am very much fortunate to work under her mentorship.

Dr. Martin Scanlon, my co-advisor, played a pivotal role in getting through this thesis. His insight and advice not only shaped the thesis but also help to make be a better student researcher as well. I am deeply thankful.

My committee members, Dr. James Friel and Dr. Curtis Rempel were always kind to me. Their inputs made the thesis strong and better.

I am thankful to Dr. Ann-Dorit for her technical guidance. Her style of detailed planning of works and delegation are something I always looked upon. Dr. Narsi Reddy helped me in a big way. Without him, it would not have been possible to prepare phenolic extract and HPLC run, which are critical in my study. I am very much indebted.

Insight of Dr. Ayappan is something I always looked forward. He gave many critical inputs and fresh approach and strengthen the thesis draft. Thanks Appu (his nickname in our lab).

Our technical team, Arshala Dona in particular, not only helped me technically but also a morale booster. Her confidence transmitted to me as well. Thanks Arshala!

My colleague in Department of Food science, Miss Ginna helped tremendously beginning from preparation of emulsion till analysis of physical characteristics of emulsion. I am indeed fortunate. I am also grateful to Mr. John Thoroski and Mr. Michael Stringer for their kind assistance throughout the experiments.

What should I say more about my family? My parents and my brothers, sisters and relatives are always supportive in any academic endeavour. In every up and down of life, they are always with me. That's what we call 'family'. I pay my deepest regard to all of them.

I would also like to thank Canola Council of Canada, Syngenta Crop Protection of Canada and NSERC for funding the project. I would like to acknowledge Canola Council of Canada for funding my travel to the prestigious CAOCS and AOCS meetings. I would like to acknowledge project funds as stipend from Dr Scanlon.

Lastly, I am thankful to 'Almighty' for what I am today. His divine intervention always gave me the conscience to do good things.

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1. Introduction

According to the well-known lipid chemist, Frankel (1985) **"**the problem of oxidative deterioration is of greatest economic importance in the production of lipid-containing foods". Oxidation not only reduces sensory acceptability of lipid but also produces toxic compounds. With this view in mind, various attempts have been made to retard lipid oxidation, both by physical as well as chemical means. One of the most effective, convenient and economical means is the use of antioxidants (Wanasundara and Shahidi, 2005). The most active and successfully used antioxidants in industry are synthetic (eg. Butylated hydroxytoluene (BHT), Butylated hydroxyanisole (BHA) and tertiary- butylated hydroxyquinone (TBHQ)). However, consumers have concerns about the effect of these synthetic compounds on health. Therefore, natural antioxidants that effectively control oxidation are now being sought. Many plants are rich source of phenolic compounds with potent antioxidative properties (Cartea et al, 2011; Kasprzyk et al., 2012). In recent years, they have been the focus of research, particularly in the area of functional application with respect to disease prevention. Lipids containing polyunsaturated fatty acids (PUFA) are very prone to oxidation and many studies have shown that plant based anti-oxidants were successful in retarding oxidation (Carrasco-Pancorbo et al., 2005; Robards et al., 1999). These results intensified studies to search for plant based antioxidants which are not only effective but also economical. One of the challenges about use of natural antioxidants in food is associated with their high cost of production.

Canola meal is rich in phenolic antioxidants like sinapic acid (SA) and its derivatives. A few studies have also shown that SA and its derivatives are effective in controlling oxidation in bulk oil (Thiyam et al., 2006). Lipid is an ingredient of many foods which is used in complex food systems. Many complex food systems include oil-in-water (o/w) and water-in-oil (w/o) emulsions. Food and non-food emulsions are widely used and familiar to everyone. Given the widespread application of lipid in emulsion(s), it makes sense to study, the impact of lipid oxidation in quality deterioration particularly in a simple o/w emulsion(s). Emulsions are thermodynamically unstable due to high surface tension between the two phases that are used to form the emulsion (Rousseau, 2000). Kinetics and behaviour of lipid oxidation are governed by several factors such as the model system that is used for study. An example of a model system could be bulk oil or a simple oil-in-water emulsion.

As per literature, only a few studies have been documented on the effect of antioxidants extracted from canola in retarding lipid oxidation in emulsion systems (Thiyam et al., 2006). Considering the importance of emulsions, more so as many foods are based on model emulsions, it is imperative to study the impact of natural and economical antioxidants on oil-in-water emulsion stability. The present study aims at evaluating the effect of canola seed (*Brassica juncea*) derived antioxidants to inhibit oxidation of canola oil-in-water emulsion stabilized by whey protein emulsifier at 30 °C.

2. Literature review

2.1. Lipid oxidation

2.1.1. Lipids

Lipids are classically known to be compounds insoluble in water and soluble in organic solvents. More precise definition was given by Fahy et al. (2009). They defined lipids as **"**hydrophobic or amphipathic small molecules that originate entirely or in part by carbanion-based condensations of thioesters and/or by carbocation-based condensations of isoprene units". So, it encompasses a number of molecules such as fatty acids, waxes, eicosanoids, monoglycerides, diglycerides, triglycerides, phospholipids, sphingolipids, sterols, terpenes, prenols, fat-soluble vitamins (such as vitamins A, D, E and K) and others (Vance, 2008; Dennis, 2009; Brown, 2009). Lipids play diverse roles in human nutrition. They not only facilitate absorption of fat soluble vitamins, but also provide essential components in the body. It is reported that 15 – 25 g of fat is required in a day to enable absorption of vitamins (Gottschlich, 1992). Lipid can produce energy twice as much as that from same amount of carbohydrates. Cell signalling molecules called 'eicosanoids' are made from ω-3 and ω-6 fatty acids. Lipids are the integral part of cell membranes and maintain their integrity. Triglycerides (TAGs) are the most common and simple class of lipids commonly found in animals and plants and are of great nutritional and functional importance. In TAGs, fatty acids are esterified with three hydroxyl groups of the glycerol skeleton (Fig.1). Fatty acids are the principal components in determining the characteristics of lipids. Fatty acids are different from one another in their chain lengths (number of carbon atoms), degree of saturation and the position of double bonds.

Saturated fatty acids are relatively stable but unsaturated fatty acids (fatty acids containing more than one double bond) are prone to oxidation. Rate of oxidation is directly proportional to the degree of unsaturation (Frankel, 1998). PUFAs, though good for health and proved to be effective in reducing the risks of cardiovascular diseases, can also be atherogenic when they are not protected from oxidation (Jialal et al., 1996). Positive effects of PUFAs are thus negated when not properly protected. Due to the high susceptibility of unsaturated lipids to oxidation, it is a challenge to protect lipids from oxidation (Chaiyasita, 2007). Oxidised lipids not only produce "offensive" odours and flavours but reduce the nutritional value. Oxidative instability of unsaturated fatty acids thus limits its use as sensory acceptability and reduced its health benefitting potential.

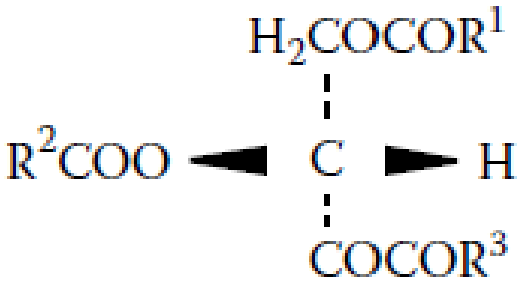


Figure 1. Triglycerides are formed by condensation of glycerol and three fatty acids. Fatty acids may be different or alike. (*Source:* Scrimgeour et al., 2007)

2.1.2. Mechanism of lipid oxidation

Lipid oxidation is a complex process. Lipid oxidation can follow different mechanisms depending upon environments and other factors. Numerous factors like "fatty acid (FA) composition, content and activity of pro- and antioxidants, irradiation, temperature, oxygen pressure, surface area in contact with oxygen, and water activity (a_w)" affect oxidations (Nawar, 1996).

Lipid can either react with triplet oxygen or singlet oxygen. Triplet oxygen has non-radical character and cannot directly react with a lipid but it reacts with a lipid radical. However, singlet oxygen does not need radical formation of lipid and so it can directly react with non-radical lipid (Frankel, 1980). Singlet oxygen is generated in variety of ways; the most important being photosensitizer assisted generation in the presence of light. Oxidation through singlet oxygen, non-radical photoxidation, is believed to be minor compared to triplet oxygen induced free radical chain reaction. It produces hydroperoxides which break down to produce free radicals and initiates autoxidation. It follows three main steps (Languerre, 2007) namely initiation, propagation and termination.

2.1.2.1. Initiation

Initiators like light, heat and metal ions initiate formation of lipid radicals by releasing hydrogen. Allylic hydrogen can be removed easily due to low bond dissociation energy. Once the lipid radical is formed, triplet oxygen can react with lipid radical to form peroxy radical. This step of formation of peroxy radical is known as the initiation step.

$$LH \xrightarrow{\textit{initiators}} L\cdot + H\cdot$$

2.1.2.2. Propagation

Singlet oxygen ($^1O_2$) can react directly with lipid. However, triplet oxygen ($^3O_2$) reacts rapidly with lipid radical to form different radical species including peroxy radical and hydroperoxides. $^1O_2$ is an electronically excited state of molecular oxygen. The outer electrons are paired in a single orbital. On the other hand, in $^3O_2$, two unpaired electrons occupy two degenerate orbitals. They are stable and react only with radicals in contrast to highly reactive $^1O_2$. $^1O_2$ are far more reactive than $^3O_2$.

$$LH + {}^1O_2 \longrightarrow LOO\cdot$$

$$L\cdot + {}^3O_2 \longrightarrow LOO\cdot$$

These propagation reactions have low activation energy and very fast and so the concentration of peroxy radicals increases rapidly. Peroxy radicals again abstract hydrogen from lipid molecule to form hydroperoxides and, at the same time, form a lipid radical.

$$LOO\cdot + LH \longrightarrow LOOH + L\cdot$$

This reaction propagates at a high rate is irreversible and the concentration of hydroperoxides increases. Hydroperoxides are stabilised by formation of conjugated dienes and trienes via double bond rearrangement (Frankel, 1998). Hydroperoxides and conjugated dienes and trienes are the primary oxidation products. Secondary oxidation products (volatiles and non-volatiles) are formed by complex decomposition reactions of hydroperoxides. The volatiles are mostly aldehydes and aldehyde esters and are the main agents for olfactory stimulus.

2.1.2.3. Termination

Termination of oxidation marks with the scission of Hydroperoxides (Frankel, 1998). Hydroperoxides undergo scission of double bond and form secondary non-radical oxidation compounds like hydrocarbons, aldehydes, alcohols and volatile compounds and non-volatile compounds like oxidised triacylglycerols and their polymers (Table 1). Polymer formation also leads to termination of radical chain reaction (Fig. 2).

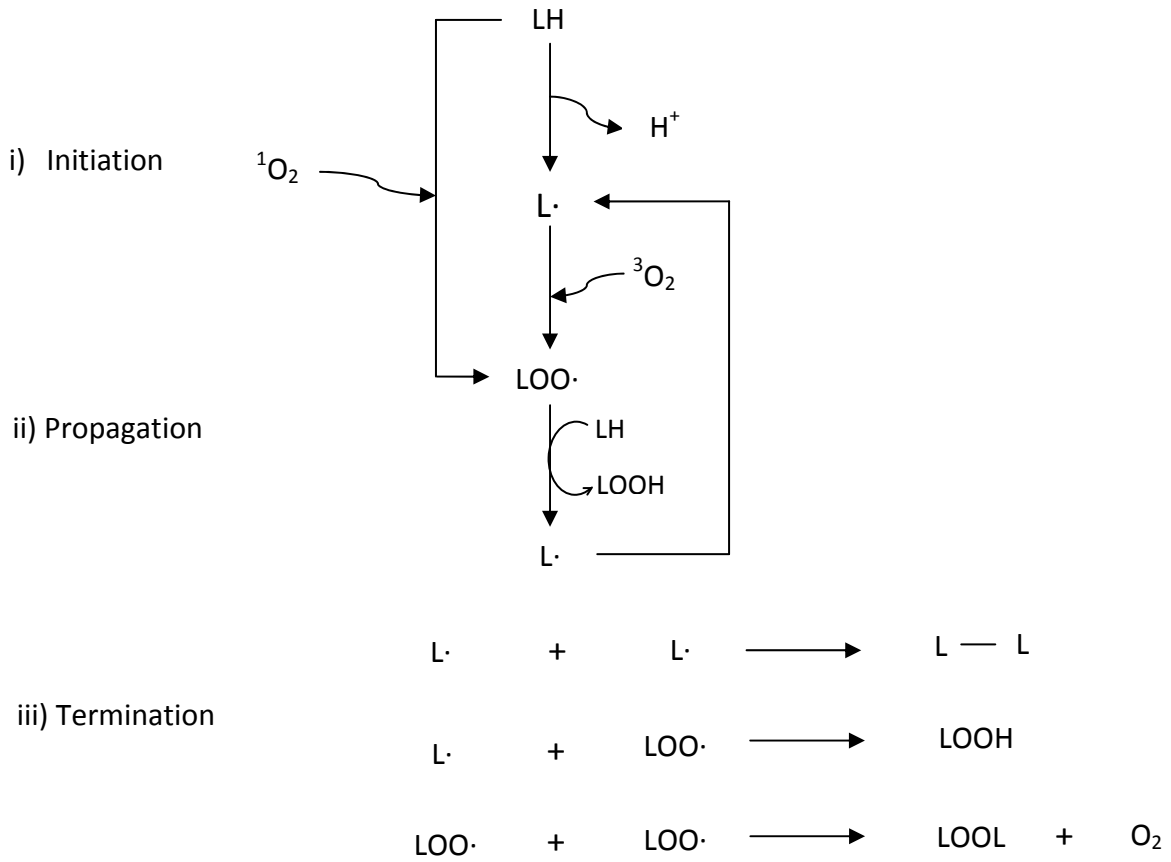


Figure 2. Radical chain reaction is generally divided into three steps i) generation of peroxy radical ii) peroxy radical is further broken into lipid radical and formation of hydroperoxides and iii) reactions are stopped when two radicals are bonded (*Adapted from:* Languerre, 2007).

Table 1. Secondary oxidation products of fatty acid methyl ester by autoxidation (Frankel, 1985)

Class	**Oleic acid**	**Linoleic acid**	**Linolenic acid**
Aldehydes	Octanal Nonanal 2-Decenal Decanal	Pentanal Hexanal 2-Octenal 2-Nonenal 2,4-Decadienal	Propanal Butanal 2-Butenal 2-Pentenal 2-Hexenal 3,6-Nonadienal Decatrienal
Carboxylic acid	Methyl heptanoate Methyl octanoate Methyl 8-oxooctanoate Methyl 9-oxononanoate Methyl 10-oxodecanoate Methyl 10-oxo-8-decenoate Methyl 11-oxo-9-undecenoate	Methyl heptanoate Methyl octanoate Methyl 8-oxooctanoate Methyl 9-oxononanoate Methyl 10-oxodecanoate	Methyl heptanoate Methyl octanoate Methyl nonanoate Methyl 9-oxononanoate Methyl 10-oxodecanoate
Alcohol	1-Heptanol	1-Pentanol 1-Octene-3-ol	
Hydrocarbons	Heptane	Pentane	Ethane

2.2. Factors affecting lipid oxidation

The kinetics of lipid oxidation is governed by many factors including temperature, light, fatty acid composition of lipids, oxygen concentration and types, etc.

2.2.1. Temperature and light

The rate of auto-oxidation is directly co-related with temperature (Shahidi and Spurvey, 1996). Temperature was found to influence significantly the rate of reaction during rapid oxidation of refined oil on exposure to light. Development of hydroperoxides was significantly faster at 40 ˚C after 3 days compared to one stored at 5 ˚C in refined oils (Sang and Jin, 2004). However, no significant difference was observed between the two temperatures in crude oils. This may be due to the presence of endogenous anti-oxidants in crude oil. Light was found to be more important in $^1O_2$ induced oxidation (Rahmani and Csallany, 1998) but it becomes less significant than temperature as oxidation increases (Velasco and Dobarganes, 2002).

2.2.2. Fatty acid composition

The rate of auto-oxidation depends on the rate of formation of fatty acid radical and acylglycerol alkyl radical formation, which in turn depends on the type of fatty acids. Edible oil oxidizes faster as the degree of unsaturation of fatty acid increases. In a study by Tan et al. (2002), soybean, safflower and sunflower oils showed shorter induction time of oxidation compared to coconut and palm oil. Transgenic oilseed containing high oleic and high stearic soybean and cottonseed oils is more auto-oxidative stable than oil from the wild breed containing low oleate and stearate (Evans et al., 1973).

2.2.3. Oxygen

Oxygen is an integral agent in the oxidation of oil. Oxidation takes place when oil, oxygen and catalyst are in contact. Oxygen concentration in oil is directly dependent on the oxygen partial pressure in the headspace (Anderson and Lingnert, 1998). The greater the amount of oxygen in the oil, the greater will be the rate of oxidation. However, oil oxidation rate is independent of O_2 concentration at very high concentration (above 2% of headspace oxygen concentration). At low concentration, rate is dependent on O_2 concentration but independent of lipid concentration (Anderson and Lingnert, 1998). Reaction of lipid and oxygen also depend on the types of oxygen. There are two types of oxygen based on their electronic configuration; singlet oxygen ($^1O_2$) and triplet oxygen ($^3O_2$). Reaction of linoleates and $^1O_2$ was found to be 1450 times faster than $^3O_2$ (Rawls et al., 1970).

2.3. Emulsion

An emulsion is a suspension in which one phase is dispersed into another phase. “An emulsion consists of two immiscible liquids, with one of the liquids being dispersed as small spherical droplets in the other liquid” (Friberg, 1997; McClements, 2005). Oil and water are usually the two immiscible liquids used for emulsion in food industries (Norton and Frith, 2001; Norton et al., 2006).

The diameter of droplets in most emulsion (macro-emulsion) is usually in the range of 0.1 to 100 µm (Dickinson, 1982). Emulsions of lower droplet size are known as micro-emulsion or nano-emulsion. However there is no clear cut line that differentiates one from another. Emulsion can be divided based on the ‘relative spatial distribution’ of oil and aqueous phases

(McClements, 2005). O/W emulsion is a type of emulsion where oil is dispersed in aqueous phase. Milk, creams, dressing, mayonnaise, beverages, soups, etc. are W/O types of emulsion. Whereas, in W/O type of emulsion, water is dispersed into oil. Butter and margarine are W/O of emulsion (McClements, 2005). It was reported that multi emulsions like oil-in-water-in-oil (O/W/O) or water-in-oil-in-water (W/O/W) emulsions can also be prepared (Garti, 1998).

In order to make an emulsion, two immiscible liquids undergo a process called homogenisation wherein one of the phases get dispersed by forming small droplets. Homogenisation involves application of high pressure and shear force using a mechanical device called homogeniser. Homogenisation could produce emulsion but the two phases tend to separate fast by merging the droplets when they collide. Emulsion is a thermodynamically unstable system but it is possible to form kinetically stable system for some period of time by adding substances known as stabilizers. Stabilizers are classified into two types based on the mode of action. Texture modifiers are types of stabilizers that act by retarding the movement of droplets by increasing the viscosity. On the other hand, emulsifiers are surface active stabilisers. Emulsifiers are amphiphilic compounds with a hydrophilic and a hydrophobic head (Fig. 3). Emulsifiers distribute at the interface and hydrophobic and hydrophilic heads are oriented towards oil and water respectively thereby not allowing oil (or water) together (McClements, 2005).

2.3.1. Particle size distribution

Particle size is an important criterion in determining the 'shelf-life, appearance, texture, and flavour' of emulsion (McClements, 2005). Therefore, it is very important to determine the particle size of droplets in emulsion. Droplets in real food systems are mostly in different sizes (polydisperse) and their sizes are generally represented as particle size distribution. Smaller droplets have larger surface area and are more prone to oxidation and thus, stability is also dictated by particle size. When oxygen concentration was limiting, interfacial areas was proportional to oxygen consumption and conjugated dienes (one of the markers of lipid oxidation). However, in later stage of oxidation (after 6-8 h), droplet size was found to have no influence on oxidation (Lethuaut et al. 2002). From the point of our study, uniform size is of particular importance because significantly different particle sizes may alter the reaction kinetics and results may be widely different.

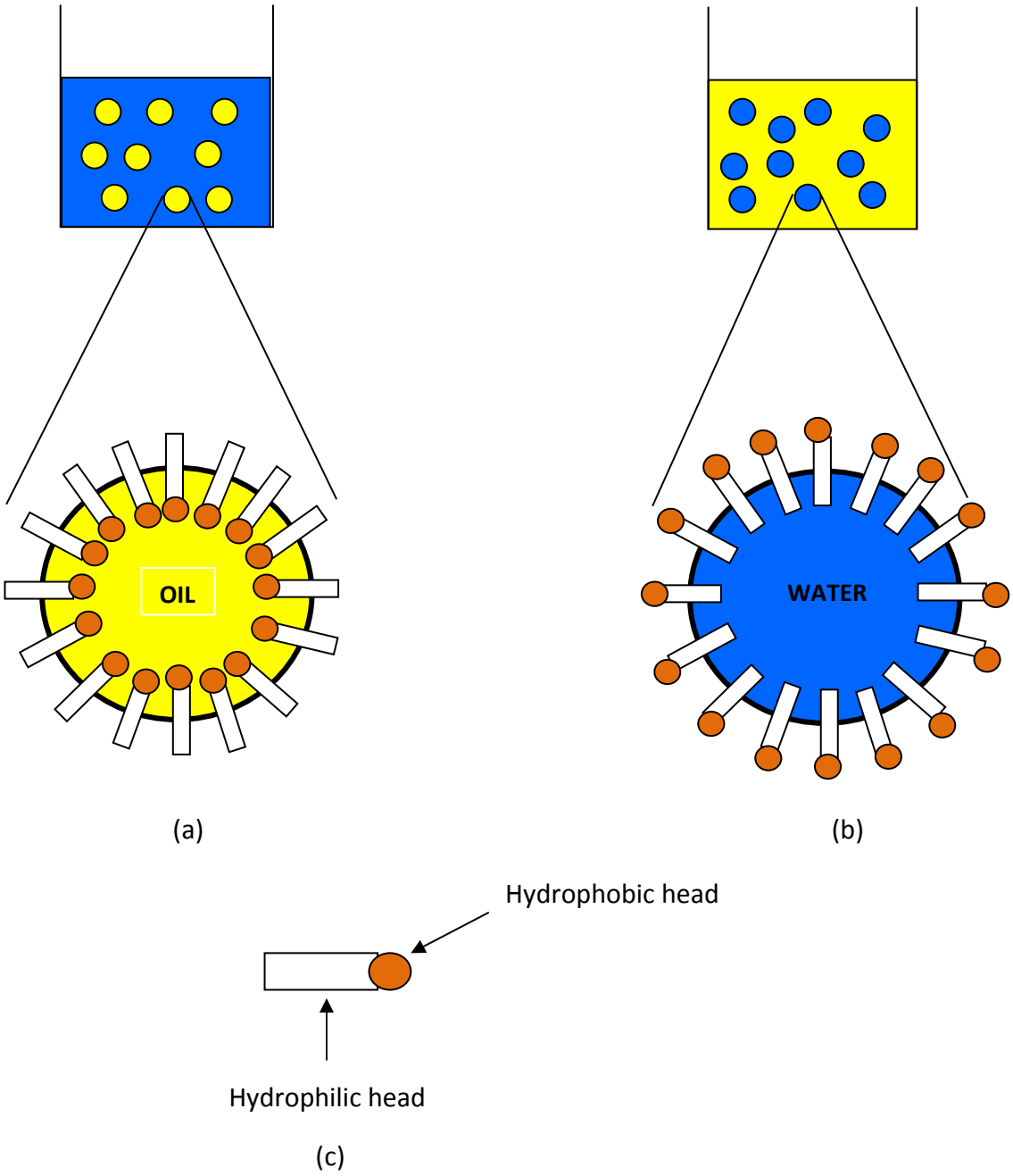


Figure 3. Schematic diagram showing (a) Oil-in-water emulsion (b) water-in-Oil emulsion (c) surface active agent showing hydrophilic and hydrophobic heads.

2.3.2. Rheology

According to McClements (2005), many factors influence rheology in emulsions such as dispersed phase volume fraction, rheology of component phases, droplet sizes, droplet charges, etc. Viscosity increases linearly at a low concentration of dispersed phase. Viscosity rises steeply above critical volume fraction. Colloidal interactions between the droplets are the underlying factor for the role of volume fraction in determining viscosity (McClements, 2005). Though the volume fraction of dispersed phase can adjust the viscosity of an emulsion, it is not a usual practice due to the availability of cost effective options like addition of thickeners. Droplet size can influence the relative Brownian motion and shear stress of droplets when there is no appreciable colloidal interaction (Mewis and Macosko, 1994). Mean droplet diameter and poly dispersity have significant effect on viscosity in concentrated emulsions (Liu and Masliyah, 1996; Pal, 1996). In emulsions containing poly dispersed droplets, packing could be done more efficiently than in emulsion with mono disperse droplets and the emulsions have low viscosity.

2.3.3. Partitioning of ingredients

Food emulsions can be divided into three zones; dispersed oil droplets, continuous water and a narrow phase between the two phases, i.e., the interface. Emulsifiers are oriented in the interface. In terms of thickness, the interfacial layer is a few nanometers. Molecules are distributed among these three phases according to the physical and chemical character of the

molecules. Non-polar molecules and polar molecules are distributed to polar (water) and non polar (oil) phases, respectively, while amphiphilic molecules are located in the interface. Therefore, "molecules in an emulsion partition themselves in the three different regions according to their solubility characteristics and surface activity" (Hiemenz and Rajagopalan, 1997). Porter (1993) propounded a 'polar paradox' theory which stated that 'primary antioxidants that are polar or amphiphiles of high hydrophilic–lipophilic balance (HLB) tend to be more active in low surface to volume (LSV) non-polar lipids (bulk oils), whereas nonpolar or amphiphilic antioxidants with low HLB tend to be more active in high surface to volume (HSV) polar emulsions and polar lipids'. Many studies supported this theory that polar antioxidants like Trolox, gallic acid, etc are effective in bulk oil rather than emulsions (Daniels and Martin, 1967; Sherwin, 1976). On the other hand, lipophilic antioxidants, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) were found to be more effective in emulsions more than in bulk oils (Lea and Ward, 1959). Surface activity and the ability of the antioxidant to accumulate at the interface are also important factors in determining antioxidants effectiveness. *γ*-tocopherol is more surface active than α - tocopherol in corn o/w emulsions stabilized by Tween 20 (Yuji et al, 2007).

2.3.4. Instability

Emulsions can be either deformed by gravitational sedimentation or droplet aggregation (McClements, 2005). In gravitational separation, depending upon the density of the droplets, they can either move upward, which is called creaming, or move downward, which is called sedimentation. If droplets come together and forms aggregates without disturbing their individual integrity, it is known as flocculation. However when they are joined together and form a large droplet, the process is called coalescence. Another process called phase inversion also takes place when o/w emulsion is converted to w/o emulsion or vice versa (McClements, 2005). Alteration in the composition or environmental conditions of emulsion such as dispersed phase volume fraction, emulsifier type and concentration, solvent conditions, temperature or mechanical agitation are also contributing to the stability of emulsions (Shinoda and Frieberg, 1986; Dickinson and Stainsby, 1992).

2.3.5. Lipid oxidation in emulsion

There exists a significant difference between lipid oxidation in bulk form and emulsified form (Coupland and McClements, 1986). Though the basic mechanism of oxidation remains the same, different factors contribute to the kinetics of the reaction. Orientation of the lipid molecules to the interface is also a factor in controlling oxidation in emulsion because this affects the accessibility of water soluble reactive oxygen species like hydroperoxides and other radicals to lipid (Heimanz, 1986). Lipid hydroperoxides can accumulate at the lipid-water interface of emulsion droplets due to their surface-active property (Yuji et al, 2007). The

existence of transition metals (e.g., iron, copper) in the aqueous phase and lipid hydroperoxides at the emulsion droplet surface suggested that lipid oxidation in o/w emulsions primarily occurs at the emulsion droplet interface (McClements, 2000).

Type and concentration of emulsifiers also affects the oxidative stability of o/w emulsion. Fumoso et al. (2002) studied the effect of lecithin, Tween 20, whey protein isolate, mono or diacylglycerols, and sucrose fatty acid ester on oxidation stability of a model o/w emulsion prepared with enzymatically synthesized menhaden oil-caprylic acid structured lipid. It was found that emulsifier type and concentration affected oxidation rate. It was found that 1% emulsifier concentration enhances the oxidative stability than that of 0.25% which indicated that there was minimum level of emulsifier to be incorporated to emulsions.

2.4. Anti-oxidants

Lipid oxidation can be controlled in various ways. PUFAs are highly prone to lipid oxidation. Hydrogenation converts unsaturated fatty acids to saturated fatty acids but from the health point of view this is not recommended due to generation of *trans*-fatty acids which is even more atherogenic than saturated fatty acids (Duxbury, 2005). Another way to control lipid oxidation is to limit the access of oxygen to the food containing unsaturated fatty acids. But it is constrained with practicality during processing and storage. One of the best alternatives is addition of antioxidants. Industries successfully exploited synthetic antioxidants in controlling oxidation. BHT (Butylated hydroxytoluene), TBHQ (tertiary-butylhydroquinone) and BHA (Butyllated hydroxyanisole) are the popular synthetic antioxidants used in food industries. Even polyphenolic antioxidants (rutin, quercetin, morin, myrecitin and kaemferol) have been shown

to be effective in controlling lipid oxidation (Ramanathan and Das, 1992). Natural antioxidants like rosemary extracts are also produced commercially (Herbolax® from Kalsec Inc., USA) for use in bakery products, confectionary, oils, seasonings, fired snacks, etc.

2.4.1. Mechanisms

Antioxidants can act in several ways to slow down the lipid oxidation either singly or in combination; free radical scavenging, metals chelation, quenching of singlet oxygen and photosynthesizers and inactivation of lipoxygenase (Choe and Min, 2009).

2.4.1.1. Radical scavenging

The free radicals in foods are scavenged by donating hydrogen from antioxidants and relatively stable free radicals are formed. Mode of action can be given as:

$$A\text{-}H + LOO\cdot \longrightarrow A\cdot + LOO\text{-}H$$

A-H = Anti-oxidant
LOO· = Peroxy radical
A · = Antioxidant radical
LOO-H = Hydroperoxides

Phenolics like tocopherols, BHT (Butylated hydroxytoluene), propyl gallate, lignans, flavanoids, etc. have the ability to scavenge free radicals (Choe and Min, 2009). Higher stability of free radical anti-oxidant is attributed to resonance stabilisation (Choe and Min, 2006) (Fig. 4). Factors contributing to the effectiveness of antioxidants to scavenge free radicals include bond dissociation energy between oxygen and phenolic hydrogen, pH, reduction potential and

delocalisation of antioxidant radicals (Litwinienko, 2003). Choe and Min (2005) stated that reduction potential of the antioxidant radical can decide their capability to donate hydrogen to food radicals. The lower the reduction potential, the higher the ease to donate hydrogen. Therefore, compounds whose reduction potential is lower than the lipid radicals are good candidates for anti-oxidants. Tocopherol, ascorbic acid, and quercetin radicals have reduction potentials of 500, 330, and 330 mV respectively. On the other hand, hydroxy, alkyl, alkoxy, alkyl peroxy, and superoxide anion radicals have approximately 2300, 600, 1600, 1000, and 940 mV (Steenken and Neta, 1982; Jovanivic et al., 1996). These wide difference and comparatively lower reduction potentials of Tocopherol, ascorbic acid, and quercetin indicated that they can act as anti-oxidants.

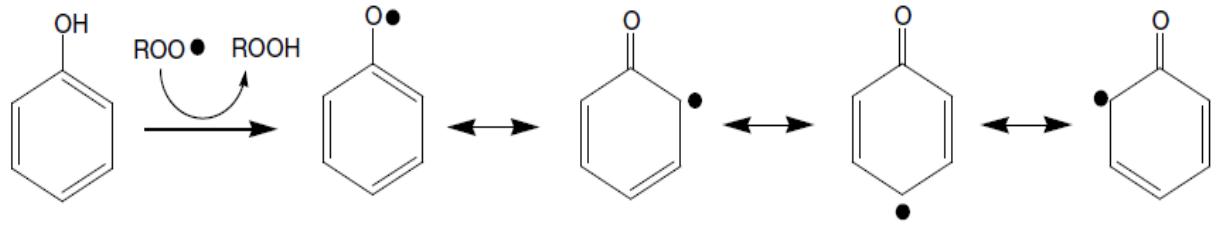
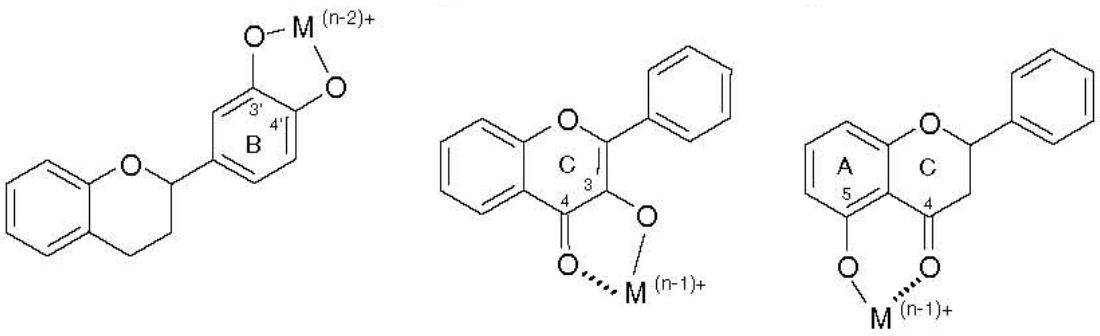


Figure 4. Resonance stabilisation of phenolic antioxidant radical (Choe, 2006).



a **b** **c**

Figure 5. 3'-4'-o-diphenolic in the B ring (a), and ketol structures 4-keto, 3-hydroxy (b) or 4-keto and 5-hydroxy (c) in the C ring of flavonols are the points where transition metals are attached (Rice-Evans, 1997)

2.4.1.2. Metal chelation

Metals decrease the activation energy of the lipid oxidation by forming a transient complex and ease oxidation to take place (Jadhav et al., 1996). Metal chelators can form an insoluble complex with transition metals and decrease oxidation. They also prevent metal redox cycling or provide steric hindrance between metals and food components or their oxidation products (Graf and Eaton, 1990). Examples of compounds having metal chelating capacity are polyphosphates, ethylene diamine tetracetic acid (EDTA), citric acid, phenolic acids and flavonoids (Biaglow and Kachur, 1997; Flora and Pachauri, 2010; Rice-Evans et al, 1997).

2.4.1.3. Singlet Oxygen Quenching

As mentioned above singlet oxygen can directly react with lipid and initiate free radical chain reaction. Free radicals, however, can be quenched by quenchers like carotenoids. Quenching encompasses both physical and chemical mechanisms. In physical quenching, energy or charge transfer takes place whereby singlet oxygen converts to ground state triplet oxygen (Min et al., 1989). Carotenoids are efficient quenchers by energy transfer. Quenching activity is related to the number of conjugated double bonds. The greater the number of conjugated double bonds, more the quenching activity (Buettner , 1993).

Energy from the singlet oxygen is transferred to β-carotene and deactivated to triplet oxygen (Fig. 6). The energy of the excited β-carotene is dissipated as heat through vibrational and rotational interactions with the environment (Min and Lee, 1988).

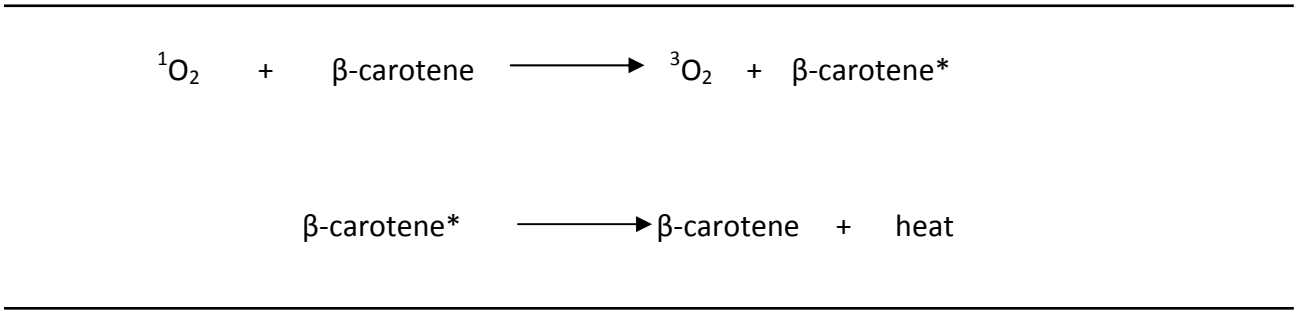


Figure 6. Quenching of singlet oxygen by β-carotene (* represents higher energy state)

2.4.1.4. Inactivation of photosynthesizer and lipoxygenase

Photosynthesizers like chlorophyll and riboflavin are present in food components and help in formation of singlet oxygen by transferring energy to triplet oxygen or superoxide free anion radical by transferring an electron (Min and Lee, 1988). Lipooxygenase is an enzyme that renders lipid oxidation in a non-radical mechanism. Application of heat can, however, deactivate lipooxygenase (Choe and Min, 2009).

2.5. Canola – a source of phenolic anti-oxidants

According to USDA figure (USDA website, 2012), production of rapeseed/ canola occupies second spot next to soyabean (Fig. 7). In 2010/11, 60.55 million metric tonnes of rapeseed/ canola was produced globally. Though it is still low compared to soybean, it is steadily increasing year after year (Table 2). Canada, China, India and European Union (EU) are the major producers of rapeseed/ canola.

Rapessed/ canola after extraction of oil generates meal as a by-product. Meal contains 40% protein and its amino acid profile is reported to be well-balanced and deemed fit for human consumption (Ohlson, 1978). Unfortunately, rapeseed contains high amount of glucosinalates, phytates and phenolics and hulls and their presence made rapeseed meal unsuitable for human consumption. Canola is a relatively new breed of rapeseed developed by canadian breeders. Canola has almost 10 times lower glucosinalates. However, it's still considered too high to be used for humans. The hull constitues 10.5 – 20 % of seed and 20-30% of defatted meal. It is an indigestible material made of 'low molecular weight carbohydrates, polysaccharides, pectins, cellulose, lignins, etc.' (Mitaru et al., 1984).

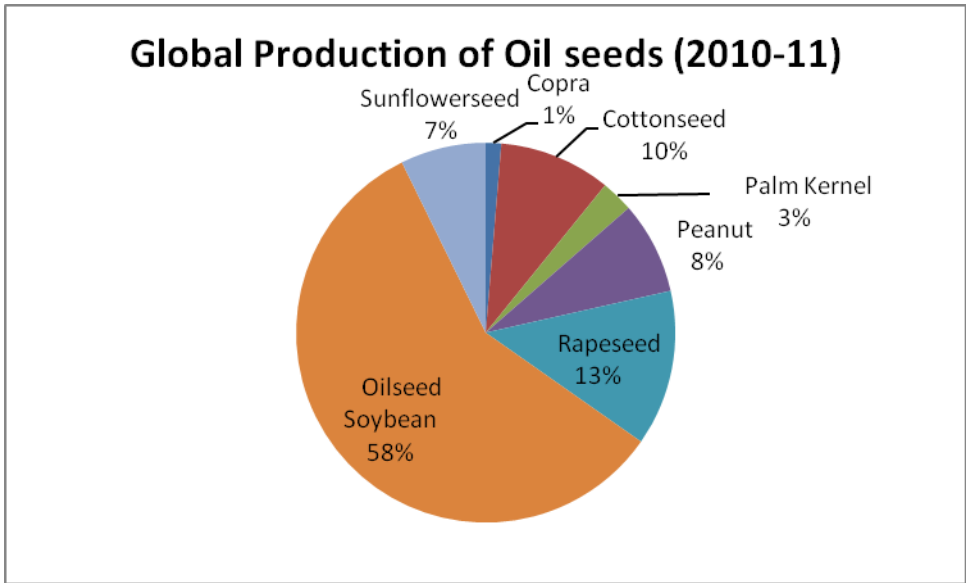


Figure 7. Global production share of different oilseeds (2010-2011) (*Adapted from:* USDA website, 2012; Permission taken from USDA)

Table 2. Global production of different oilseeds since 2007-08 (*Adapted from:* USDA website, 2012; Permission taken from USDA)

Years	*2007/08*	*2008/09*	*2009/10*	*2010/11*
Production				
Oilseed Copra	5.72	5.88	5.88	6.02
Oilseed Cottonseed	45.7	41.08	38.91	43.5
Oilseed Palm Kernel	11.02	11.75	12.22	12.55
Oilseed Peanut	32.81	35.06	33.72	35.88
Oilseed Rapeseed	48.5	57.81	60.96	60.55
Oilseed Soybean	219.56	211.64	261.09	264.69
Oilseed Sunflowerseed	27.44	33.49	32.19	33.29

All values are in million metric tonnes

Rapeseed and Canola has a high content of phenolic compounds. Most of the phenolics are retained in the meal. Phenolic compounds are reponsible for dark colour, astringency, and bitterness (Clandinin and Heard, 1961; Sosulski, 1979). Phenolics are present in different forms in rapeseed/ canola: free, esterified and insouluble bound forms. Free phenolic acid like sinapic acid is esterified with choline to form sinapine. Methods for the extraction of free, esterified and insouluble bound forms of phenolics in rapeseed were developed by Krygier et al. (1982). The total content of phenolics in meal is estimated to be 18. 4 g/kg on dry basis. A kilogram of meal (dry basis) contains more than 2 g of free phenolic acid (Naczk and Shahidi, 1989). Of all the free phenolic acids, sinapic acid contains the highest amount (70 – 85%). Other free phenolics present in rapeseed/ canola include p-hydroxy benzoic, vanillic, gentisic, protocatechuic, coumaric acids, etc (Krygier et al., 1982).

The esterified form of phenolic acids constitutes about 80% of total phenolic acids. Among esterified forms, sinapines, a choline ester form of phenolic acid, are most predominant. Sinapine (Fig. 9b) is formed by esterification of choline with sinapic acid. Phenolic acid glucosides were found in the flour of polish rapeseed varieties (Zadernowski, 1987). Rapeseed/ canola meals also contains insoluble-bound phenolic acids. Naczk and Shahidi (1989) reported the presence of about 1 gm of insoluble-bound phenolics per kilogram of meals. However, in rapeseed flour, it was found to be only 32-50 mg (Kozlowska et al, 1985). Sinapic acid constitutes 30-59 % the total insoluble-bound fraction of phenolic acids (Kozlowska et al, 1985). The major phenolic content (Sinapine, Sinapic acid and Sinapoyl glucose) of defatted canola seeds of canada was estimated by Khattab et al. (2010). Sinapine was in in the range 9.16 – 16-13, Sinapic acid in 6.39 – 12.28 and sinapoyl glucose in the range 0.59 – 1.36 mg/ gm.

Canolol (Fig. 9a) is present in a very small amount in canola seeds (as low as 6 μg/g) (Spielmeyer, 2009). Structurally, canolol constitutes a dimethoxy phenol attached with a vinyl group at the 4^{th} position of the phenol ring. The chemical name is termed as 4-vinyl-2,6-dimethoxyphenol. Sinapic acid, main phenolic compound in canola is regarded as the precursor of canolol which is formed by decarboxylation. Heat treatment like roasting was found to assist in decarboxylation of sinapic acid and as a result, concentration of canolol increases with increased exposure to heat (Spielmeyer, 2009; Wakatamasu et al., 2005). Since its discovery in the early 2000s (Wakatamasu et al., 2005; Tsunehiro et al., 2002; Koski et al., 2003) canolol attracts quite impressively the food scientists, industries and consumers. The remarkable feature about canolol relates to its potent antioxidative property.

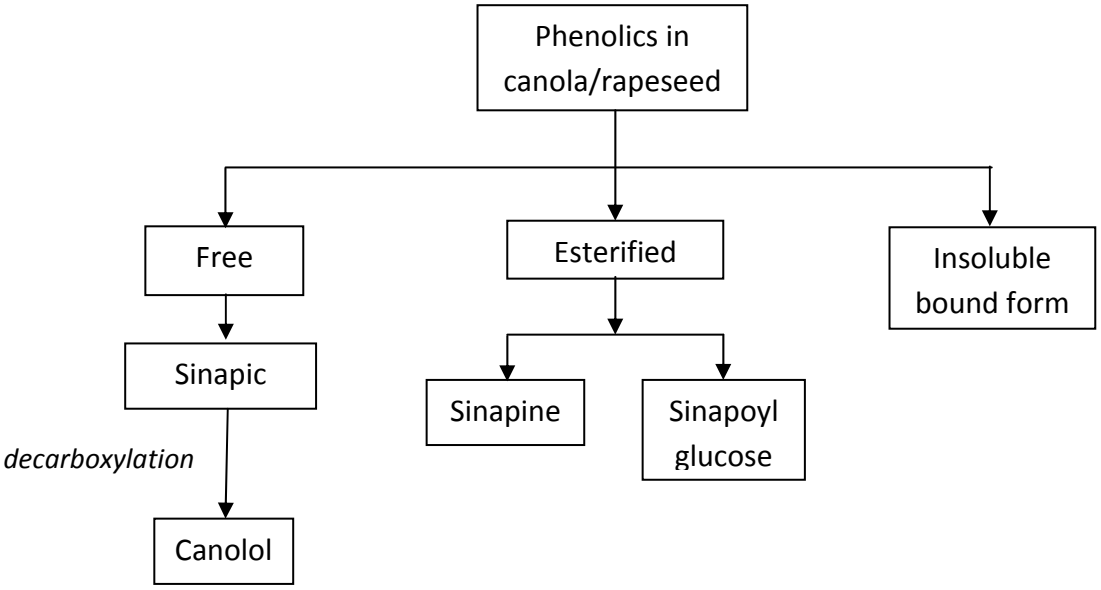


Figure 8. Different forms of phenolics in canola/rapeseed.

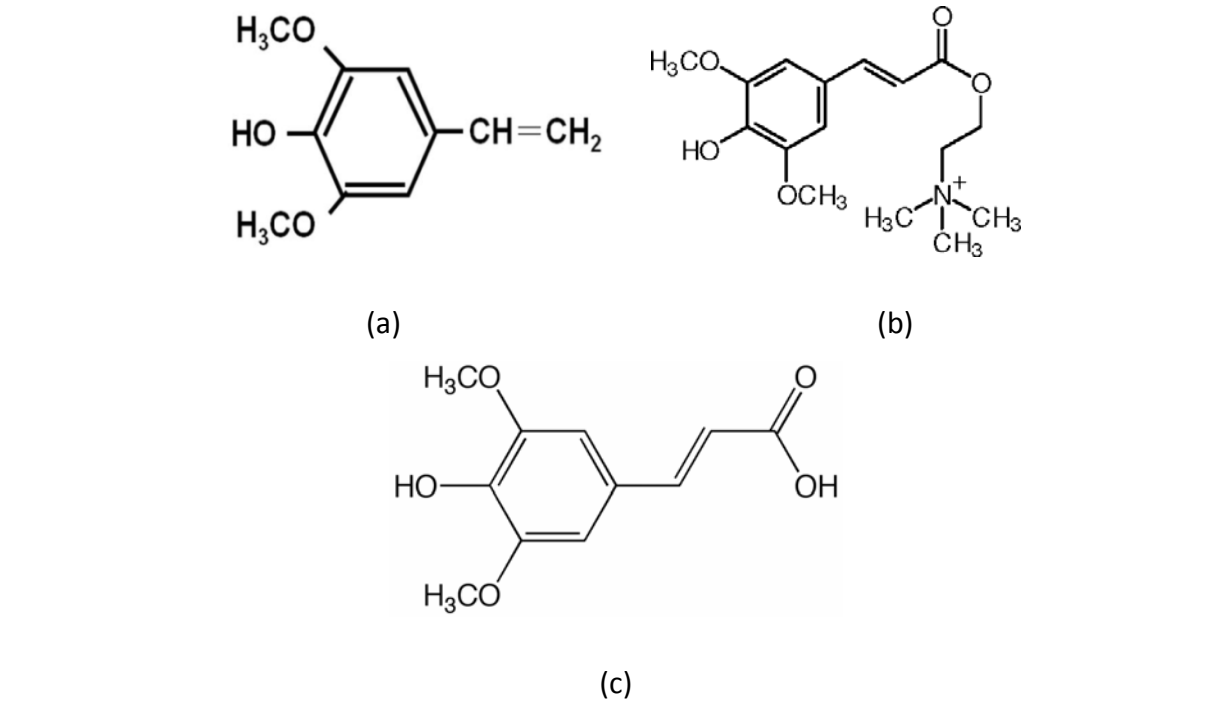


Figure 9. Chemical structures of a) Canolol b) Sinapine c) Sinapic acid

2.5.1. Anti-oxidant activity of canola antoxidants

Anti-oxidant activities of the phenolic compounds extracted from canola/ rapeseed have been extensively studied by many workers. Sinapic acid is known to attenuate kainic acid induced hippocampal cell death (Kim et al., 2010) and thus, helpful in treatment for seizure associated neuronal loss. It was also shown to inhibit peroxynitrite mediated oxidation (Niwa et al., 1999; Zou, 2002). Peroxynitrite ($ONOO^-$) is an unstable structural isomer of nitrate. It can damage molecules in the cells e.g. DNA and proteins. Hypochlorite scavenging activity showed that sinapic acid has higher scavenging activity than chlorogenic acid, ferulic acid and *p*-coumaric acid but lower than caffeic acid (Firuzi, 2003). However, radical scavenging assay as measured by DPPH showed comparatively lower scavenving acitivity. It followed the order: Gallic acid > caffeic acid = ascorbic acid = trolox > sinapinic acid > isoeugenol. Hypochlorites (ClO^-) are the highly reactive oxidants produced by activated phagocytes and it has pathogenic role. Thiyam et al. (2006) analysed DPPH radical scavenging activity of sinapic acid and its derivatives viz. sinapine and sinapoyl glucose. 30.8 mM, 35.8 mM and 47.5 and 21.5 mM of sinapic acid, sinapoyl glucose, sinapine and trolox respectively were required to scavenge 25 x 10^{18} DPPH radicals. Thus, they followed the order: trolox > sinapic acid > sinapoyl glucose > sinapine. Trolox is a water soluble analogue of α-tocopherol and it was used as standard. ABTS (2, 2'-azinobis (3-ethylbenzothiazoline-6-suslfonic acid) assay also showed that sinapic acid was weaker than gallic acid but stronger than many others. Anti-oxidant activity of sinapic acid and its alkyl ester forms were investigated by Gaspar et al. (2010). From their electron donating capacities and lipophilicity, it was found that partition coefficient is affected by ester side chains. Recently, in-depth analysis of anti-oxidant property of sinapic acid was studied from

theoretical perspective by Galano et al. (2011). Density Functional Theory predicted that sinapic acid reacts 32.6 times faster in aqeous solution than in lipid media (Fig. 10). H (hydrogen) atom transfer from the phenolic moiety was the predominant mechanism for reaction of sinapic acid towards OOH· radicals.

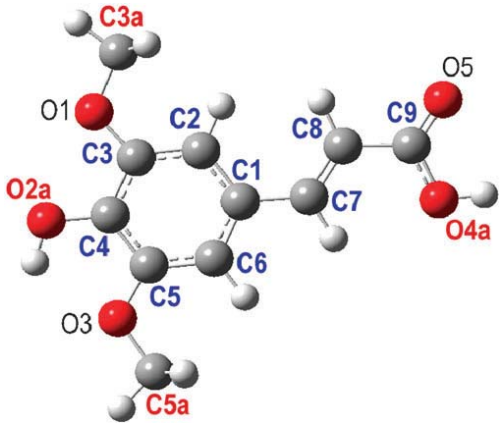


Figure 10. Sinapic acid and its sites of reaction (blue represent RAF sites and red HAT sites). RAF = radical adduct formation, HAT = hydrogen atom transfer (Galano et al, 2011)

The remarkable feature about canolol relates to its potent antioxidative property. It was discovered to possess alkyl-peroxyl radical scavenging capacity which is much higher than established antioxidants such as α-tocopherol, vitamin C, β-carotene, rutin and quercetin *(*Wakatamasu et al., 2005). Since then, it has become an intensely studied subject and new functional properties have been revealed. Canolol was also found to possess anti-carcinogenic (Cao et al., 2008), anti-mutagenic and cyto-protective effects (Kuwahara, 2004).

2.6. Effect of naturally derived anti-oxidants in emulsions

Increasing health concern with respect to the use of synthetic anti-oxidants in food prompted research to look for naturally derived antioxidants. Many foods exist in the form of O/W emulsion. Given the physical difference from that of bulk oil, effectiveness of antioxidants in bulk oil may not be necessarily translated into emulsion systems. The behaviour of the antioxidants may be determined by chemical characteristics of the antioxidants as well as the physical properties of the emulsion systems. Numerous studies have been carried out to determine the antioxidant activity in O/W emulsion system. Salimen et al. (2010) studied the effect of berry phenolics (ellagitannins from raspberry and anthocyanins from black currant) in O/W emulsions incorporated with β-Lactoglobulin as continous phase. Between the concentration of 20 and 50 µM, berry phenolics were able to control lipid oxidation as indicated by inhibition of hydroperoxides and hexanal formation. A similar study by Viljanen et al. (2005a, 2005b) in rapeseed O/W emulsion using whey protein as emulsifier and added with anthocyanins from black currants, raspberry, lingonberry and raspberry and black currant juice showed anthocyanins are protective against lipid and protein oxidation.

Mette (2007) investigated the ability of rapeseed oil and/or different antioxidants (α- and *γ*-tocopherol mixture, ascorbyl palmitate, and EDTA) to protect fish-oil-enriched milk emulsions against oxidation. It was found that rapeseed oil with added tocopherols decreases oxidation in the fish-oil-enriched milk emulsions. *γ*-tocopherol was more efficient than α-tocopherol. Ascorbyl palmitate not only retarded oxidation in the fish-oil-enriched milk emulsions but also prevented the prooxidant effect of tocopherols added to fish oil before emulsification.

3. Objectives and hypothesis

3.4. Objectives

The objectives of this study are:

- To prepare physically stable canola oil-in-water (O/W) emulsion.
- To evaluate the anti-oxidative property of anti-oxidants (sinapic acid, canolol, sinapine and whole extract of canola meal).
- To evaluate the effect of endogenous phenolics in the oxidative stability of the emulsion.
- To examine the physical stability and rheological properties of the emulsion.

3.5. Hypothesis

Earlier studies have shown that phenolic antioxidants from plants are effective in controlling lipid oxidation in oil-in-water emulsion (Ramful et al., 2011; Kiokias and Oreopoulou, 2006; Thiyam, 2006). Moreover, there were also studies which shown that phenolics from canola seeds and meal possessed antioxidative properties (Gaspar et al., 2010; Kim et al., 2010; Galano et al. 2011).

Based on these studies, the following hypothesis was developed:

Addition of endogenous phenolic compounds of canola meal will enhance the oxidative stability of canola oil-in-water emulsions.

4. Materials and methods

4.4. Materials

All chemicals were of analytical grade. Hexane, Methanol, Ferrozine, Ammonium thiocyanate, Barium Chloride, DPPH, BHT and EDTA were from Sigma-Aldrich (USA). Canola oil was a gift from Bunge, Canada while Whey Protein Isolate^{TM} was procured from Bio-X Performance Nutrition Co., North Vancouver.

4.2. Extraction, purification and determination/ identification of phenolics (antioxidants) (Fig. 11)

4.2.1. Extraction of canolol and whole extract

Canola seeds/ meals were subjected to oven treatment at 180 ^{o}C for 15 min (pre-treated) prior to Accelerated Solvent Extraction (ASE) (ASE 300, Dionex). This oven temperature was selected based on high/ optimum content of Canolol as per earlier studies carried out in our lab. The pre-treated seeds/ meal and untreated seeds/ meal were extracted with hexane. The whole extract was obtained from untreated meal. The samples were extracted in ASE using the following conditions (ASE®300 Operators Manual).

50 g of canola meal / seed and 50 g of Ottawa sand were weighed and mixed very well using a spatula. Two filter papers were placed at the bottom of each sample cell prior to the addition of sample. The sample was added and filled up to top level of cell. The cell caps were hand tighten securely for both sides and cells were placed in ASE cell holder. The extraction was carried out using hexane as a solvent and the extract was further purified into different fractions by column chromatography.

4.2.2. Purification and Characterisation

Sinapic acid, sinapine and canolol were obtained from the whole extract by purification and fractionation. 100 ml of hexane extract was further extracted with 100 ml methanol in a separating funnel and the bottom layer was collected. The collected methanol layer was evaporated in a rotary evaporator at 40 ˚C. Briefly, 50 ml methanol was added and mixed again and was filtered through a filter paper followed by syringe filtering (pore size: 0.45 µm). The syringe filtered extract were analysed by RP-HPLC-DAD (Reversed phase - High Performance Liquid chromatography – Diode Array Detector) (Ultimate 3000; Dionex, Sunnyvale, CA, USA) equipped with on-line degasser, binary pump, auto sampler, column heater and diode array detector (Khattab et al., 2010). A gradient elution was performed using water/ methanol (90: 10) with 10% *0*-phosphoric acid as solvent A and methanol 100% as solvent B, using two C18 columns (Gemini 3 µm 110 Å; 150 x 4.6 mm and Synergi 4µ Fusion-RP 80 Å; 150 x 4.0 mm (Phenomenex, Canada)) at 0, 7, 20, 25, 28, 31 and 40 min with 10, 20, 45, 70, 100, 100 and 10% B. The column was maintained at 25 ˚C and solvent flow rate at 1 ml/min. Compounds were detected at 270 nm and 330 nm. Peaks were identified by comparing their relative retention times with peaks of authentic standards establishing the concentration of compounds. This step was followed by fractionation to isolate constituent compounds using a column chromatography procedure.

About 20 g of Sephadex LH-20 was mixed with 80 ml of methanol and packed in a glass column. The crude extract was loaded on the top of the Sephadex LH-20 material and was eluted with methanol. Various fractions were collected in glass tubes and each fraction was analysed by RP-

HPLC-DAD at 270 nm and 330 nm. The fractions which contain maximum concentration of a particular compound were pooled together followed by evaporation of solvent at 40 °C using rotary evaporator.

4.2.3. Quantification

In order to estimate the exact concentration of the isolated compounds, second set of HPLC-DAD analysis was carried out (Khattab et al., 2010). Briefly, 1 ml purified fraction was dissolved in 6 ml methanol (stock). 100 µl was taken from it and dissolved again in 1.9 ml methanol to get a 200 times dilution. The diluted sample was filtered using a 0.45 µm syringe filter and analysed using RP-HPLC-DAD as mentioned in section 4.1.2. Concentration can be calculated directly by fitting into regression equations from the calibration curves of the standards (Fig. 12-14).

Note: For the whole extract, no purification/ fractionation was needed. Extract is directly filtered and total content of phenolic compounds were computed by selecting the major peaks.

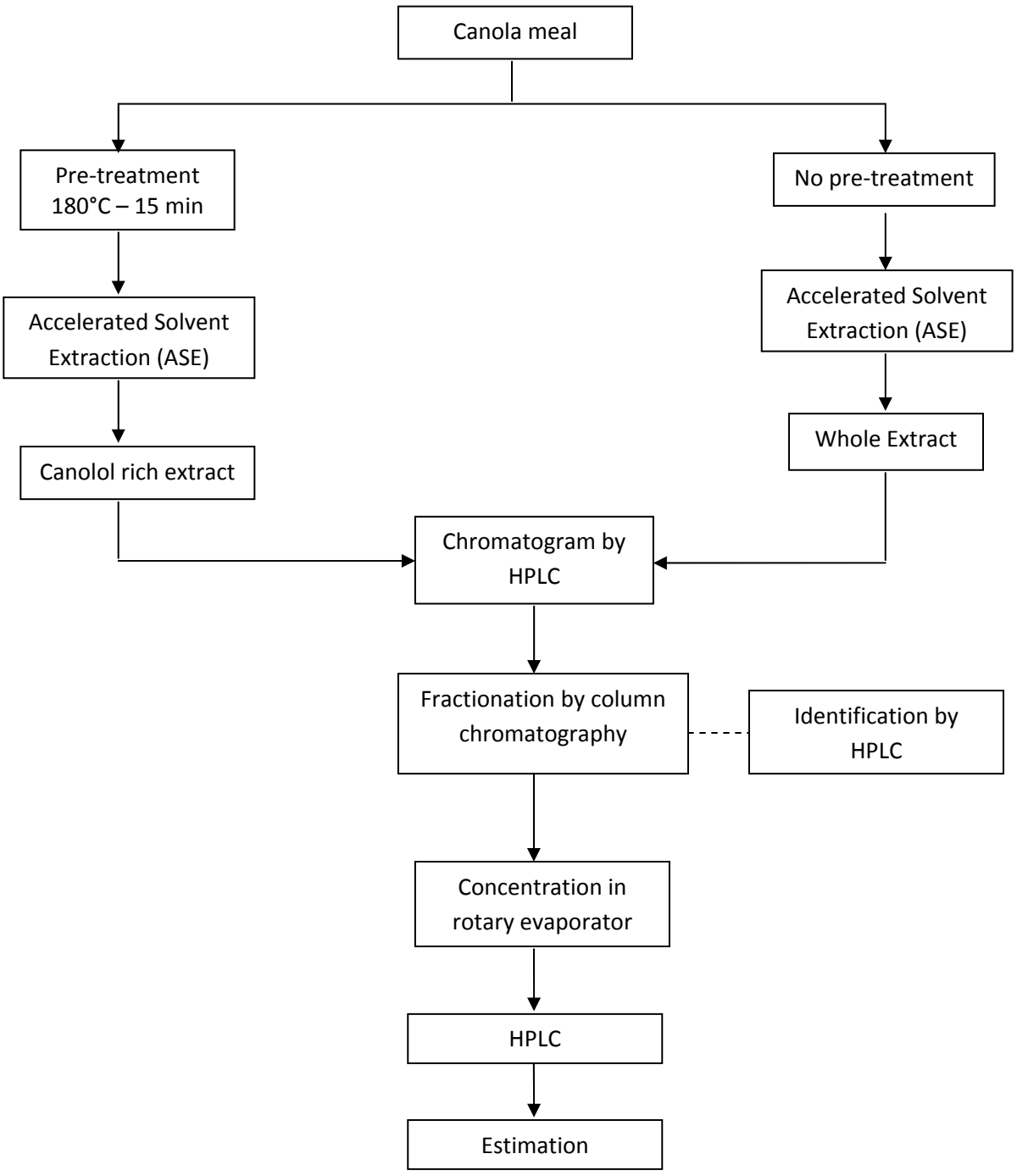


Figure 11. Flow-chart of extraction, purification and estimation of sinapic acid and its derivatives from canola meal.

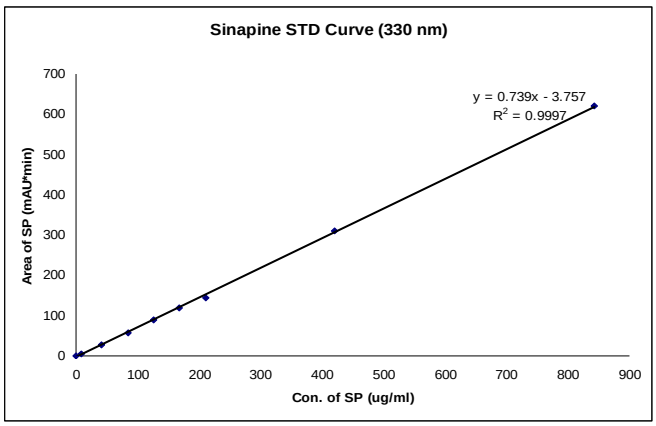


Figure 12. Calibration curve of sinapine standard (SP)

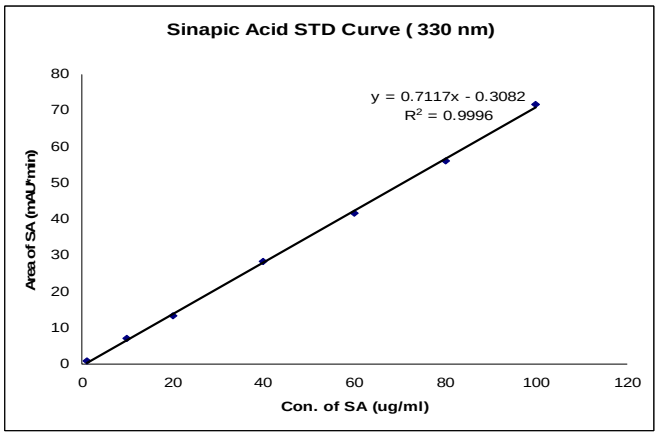


Figure 13. Calibration curve of sinapic acid standard (SA)

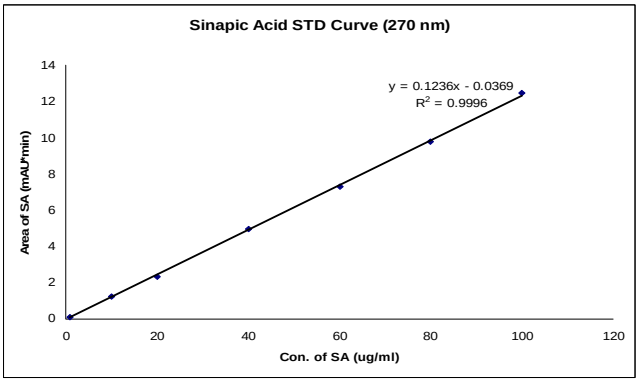


Figure 14. Calibration curve of canolol (CAN) (note: For canolol, sinapic acid maximum absorbance is taken at 270 nm)

4.3. Stripping of canola oil

Edible vegetable oil is mainly composed of triacylglycerol (95% v/v) with the remaining being minor components like phospholipids, tocopherols, free fatty acids, etc. In order to avoid any interference during the experiment from minor components, they are removed by a process called stripping (Yoshida et al., 1992). Column was packed with activated alumina (450 gm) using hexane (about 900 ml). 300 ml canola oil and 300 ml hexane were mixed and the mixture was loaded into the column. After the residual hexane in the column is taken out, stripped oil (with hexane) was started collecting. The collected mixture was rotary evaporated to remove hexane. After hexane is removed nitrogen is flushed for 4 h to remove any residual hexane. The stripped oil is stored at -20 °C to avoid oxidation.

4.3.1. Analysis of tocopherol content in the stripped oil

In order to confirm the absence of tocopherol, stripped oil was subjected to an extraction process followed by determination of tocopherols concentration using RP-HPLC-DAD (Tasioula-Margari and Okogeri, 2001). A mixture of 1 gm of oil was mixed (vortex for 1 min) with 5 ml methanol was centrifuged for 10 min at 5000 rpm to get two distinct layers. The methanolic upper layer was pipetted out and stored in a glass tube. To the lower oil layer, 5ml methanol was added and centrifuged again. Similarly, the upper layer was pipetted out and added to the glass tube. The process was repeated two more times. The collected methanol fraction/ layer was syringe filtered and subjected to HPLC isocratically for any tocopherol content. The procedure followed: methanol/water (99:1, v/v) with 1% glacial acetic acid was isocratically run

as mobile phase at the flow rate of 1 ml/min using C18 column; Synergi 4µ Hydro-RP 80 Å; 150x 4 mm 4 µm (Phenomenex, Canada); temperature maintained at 25 °C; injection volume was 20 µl; data were analysed by *Chromaleon* software (version 6.8). Chromatograms were acquired at 294 nm. Absence of tocopherol was confirmed by comparing retention times with tocopherol standards.

4.4. Anti-oxidant assays

Three *in-vitro* anti-oxidant assays viz. DPPH (2,2-diphenyl-1-picrylhydrazyl), reducing power and iron-chelating assays were carried out to assess the anti-oxidative efficacy of anti-oxidants used in the oxidation studies. Standards were also used for comparison.

4.4.1. 2,2-dipenyl-1-picrylhydrazyl radical (DPPH·) assay

Anti-oxidants of different concentrations were prepared by diluting 500 µM of respective anti-oxidants with diluents i.e. 100% methanol. 0 ml, 0.1 ml, 0.2 ml, 0.4 ml, 0.7 ml and 1 ml of 500 µM of respective antioxidants were mixed with 1.0 ml, 0.9 ml, 0.8 ml, 0.6 ml, 0.3 ml and 0 ml methanol to obtain 0 µM (control), 50 µM , 100 µM , 200 µM , 350 µM and 500 µM. To each diluted anti-oxidants, 3 ml of 0.1 mM DPPH solution was added. They were vortexed and kept in the dark for 30 min. Another set of dilutions in the same concentration range (without control) were also performed without adding DPPH (Sigma Aldrich). Here, samples were made up to 4 ml with methanol. All samples were made in triplicates. Absorbance was measured at

517 nm in a spectrophotometer (DU800 Spectropohtometer, Beckman Coulter) (Bandoniene, 2002). Scavenging capacity of the anti-oxidants was calculated by inhibition percentage.

$$Inhibition\ \ \% = \left[1 - \frac{(As - Ab)}{Ao}\right] \times 100$$

Where,

A_s = absorbance of antioxidant samples with DPPH·

A_b = absorbance of control (without antioxidants) with DPPH·

A_o = absorbance of antioxidant samples without DPPH·

4.4.2. Ferric Reducing Antioxidant Power (FRAP) Assay

The capacity of the anti-oxidants to reduce Fe^{3+} to Fe^{2+} was measured by the procedure by Yang et al. (2008). Thus, it indicated the capacity of the anti-oxidants to donate electrons to free radicals. Antioxidants of different concentration were prepared by diluting stock antioxidants (350 µM) with methanol. 1 ml of each concentration was mixed with 2.5 ml 0.2 M phosphate buffer (pH 6.6) in centrifuge tubes followed by addition of 2.5 ml 30 mM potassium ferricyanide. The mixture incubated for 20 min at 50 ˚C. After incubation, 2.5 ml 0.6 M TCA (trichloroacetic acid) was added and centrifuged at 2000 rpm for 10 min. 2 ml of the upper layer was pipetted out and mixed with 2 ml distilled water in test tubes. Then, 0.4 ml 6 mM $FeCl_3$ was added and kept for 10 min. The absorbance of the sample was spectrophotometrically

measured at 700 nm and was directly reported as reducing power wherein higher absorbance value implied higher reducing power.

4.4.3. Iron Chelating assay

About 0 ml, 0.4 ml, 0.74 ml, 1.5 ml, 2.6 ml and 3.7 ml of 500 µM of respective antioxidants were mixed with 3.7 ml, 3.3 ml, 2.96 ml, 2.2 ml, 1.1 ml and 0 ml distilled water to obtain 0 µM (control), 50 µM , 100 µM , 200 µM , 350 µM and 500 µM. The same concentrations (0 µM (control), 50 µM , 100 µM , 200 µM , 350 µM and 500 µM) of EDTA (Ethylene diamine tetracetic acid, Sigma Aldrich) were also prepared as positive controls. To each diluted anti-oxidant, 0.1 ml 2 mM ferrous chloride was added. After a 3 min gap, 0.2 ml ferrozine was added to all samples. Another set of dilutions in the same concentration range (without control) were also performed. Then, 0.1 ml 2 mM ferrous chloride was added to each diluted samples. Experiments were carried out in triplicates. All tubes were vortexed properly and read the absorbance at 562 nm (Dinis et al., 1994). Iron chelating activity was measured by spectrophotometer (DU800 Beckman Coulter Spectrophotometer).

$$\textbf{Fe}^{2+}\textbf{ chelating activity} = \left[\frac{(A_o - As)}{A_o}\right] \times 100$$

Where,

A_o = Absorbance of antioxidant samples

A_s = Absorbance of control (without antioxidant samples).

4.5. Experimental design

Experimental design of this study consisted of two parts i.e. 1) *In-vitro* antioxidant assay of phenolic compounds extracted from canola meal and 2) study of the effect of extracted anti-oxidants on canola o/w emulsion (Fig. 15).

4.5.1. Preparation of emulsion

1200 g of emulsion was prepared in the following proportion: 10% stripped canola oil (120 g), 89% buffer (Sodium acetate-imadizole buffer solution; 1068 ml; pH 7) and 1% emulsifier (whey protein) (methods moidified from Ries et al. 2010) Canola oil was supplied by Bunge, Canada and whey protein isolate (Power Whey Protein Isolate^{TM}, was purchased from Bio-XPerformance Nutrition Co., North Vancouver).

Steps for the preparation of emulsion:

a. Whey protein (12 g) was mixed with buffer solution (1068 ml) to dissolve the whey protein completely using a magnetic stirrer or spatula.

b. The mixture was thoroughly mixed by a stirrer (Wiggen Hauser D-500, TE Scientific, Malaysia) for a few seconds to completely dissolve the whey protein.

c. Oil was slowly added and stirred for 3 min. Pre-emulsion was obtained in this way.

d. Pre-emulsion was subjected to homogenisation in APV homogeniser (APV Gaulin Laboratory Homogenizer, Model 15MR-8TA, 316 Stainless Steel. 2 Stage valve, 15 gallons per hour, 8000 psi. Driven by a 3 hp, 3/60/208-230/460 volt, 1725 rpm motor) for 4 min at 7000 psi. Then,

emulsion was kept in fridge immediately. Storage glass bottle was also covered by aluminum foil to avoid exposure to light.

e. pH of the emulsion was brought down to pH 7 by addition of 10 M hydrochloric acid (HCl) if necessary. pH was measured by pH meter.

Preparation of sodium-amidazole buffer solution

2.721 g sodium acetate and 1.3616 g Imidazole were added in 2 L volumetric flask and stirred using magnetic stirrer with a little amount of distilled water to dissolve. The mixture was made up to 2 L with distilled water.

4.5.2. Addition of antioxidants

Antioxidants (Table 3) were added in such a way that their final concentration of emulsions are 100 µmol and 350 µmol per litre of emulsion i.e. 100 and 350 µM. In order to achieve this goal, respective anti-oxidants were concentrated by nitrogen flushing and dissolved in 0.5 ml of 100% methanol which was then added to the 40 ml emulsion in 100 ml glass bottle followed by thorough mixing. Duplicates were prepared for each concentration.

Purified anti-oxidants often needed to be diluted to required concentration. The concentrations of the anti-oxidants were determined by fitting into regression equation of calibration curves. Based on the state of the compound (solid or liquid), methods of preparation were also different.

The concentration of the antioxidants in extracts was estimated by HPLC as explained in section 4.2.3 and extracts were diluted to achieve required concentration.

For BHT and sinapic acid standard, required amount is weighed and dissolved in methanol. Dissolved antioxidants were again quantified using HPLC to ascertain the exact concentration.

4.5.3. Incubation of emulsion at 30˚C

The samples were incubated at 30 °C in an oven to facilitate oxidation. Control oil (without added antioxidants) was also kept for oxidation in bottles (duplicates). The samples were taken out in MCT amber tubes periodically and stored at -20°C (Fig. 18). To ensure the chemical stability of the samples, they were flushed with nitrogen.

4.5.4. Analysis of oxidation markers and physical stability

Samples taken out periodically were subjected to the estimation of hydroperoxides content (primary oxidation marker) and hexanal (secondary oxidation product) (Fig. 16). A small quantity of samples of oil without added antioxidants were taken out to measure particle size and viscosity.

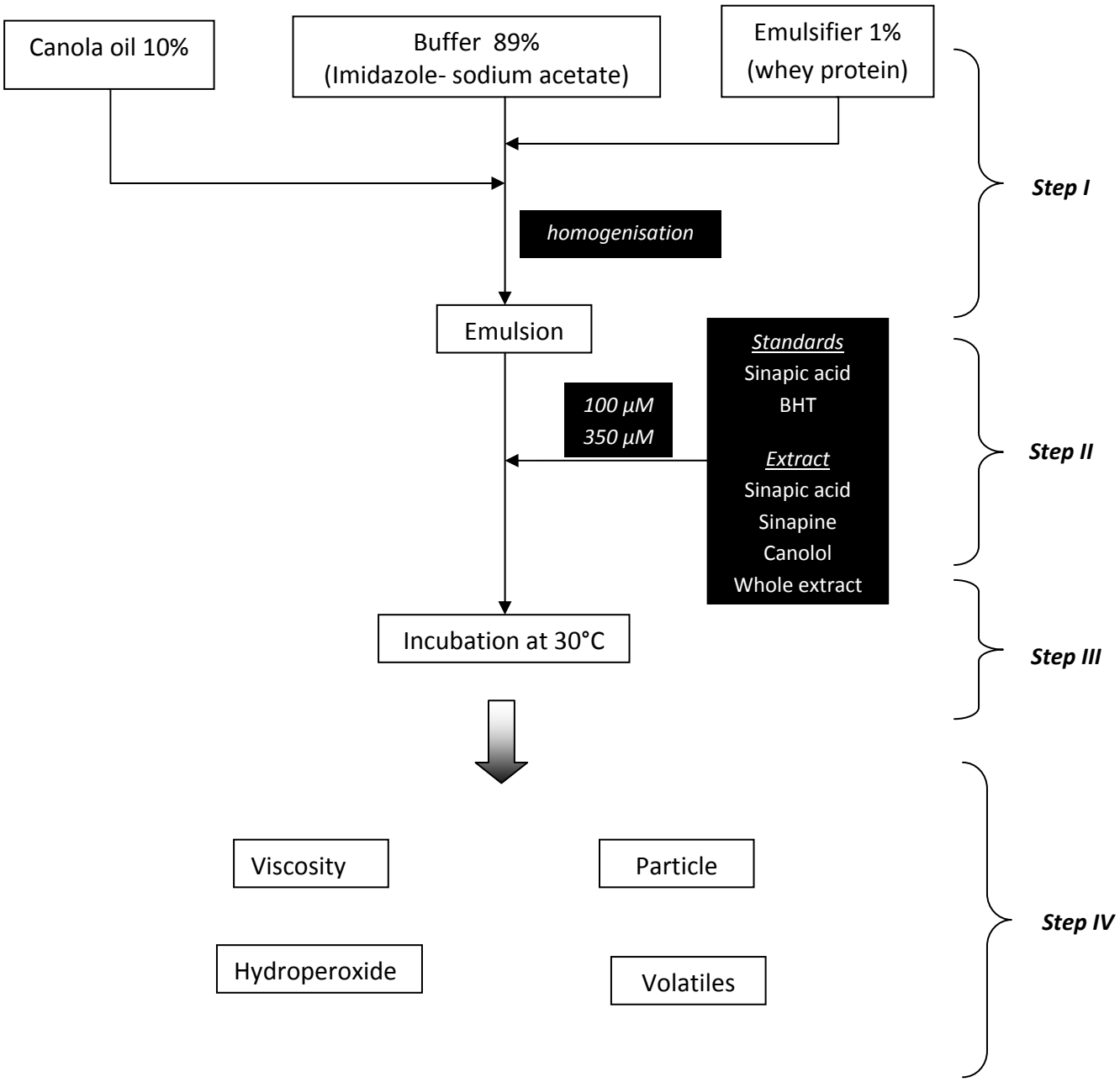


Figure 15. Flow chart showing the steps involved in experimental design.

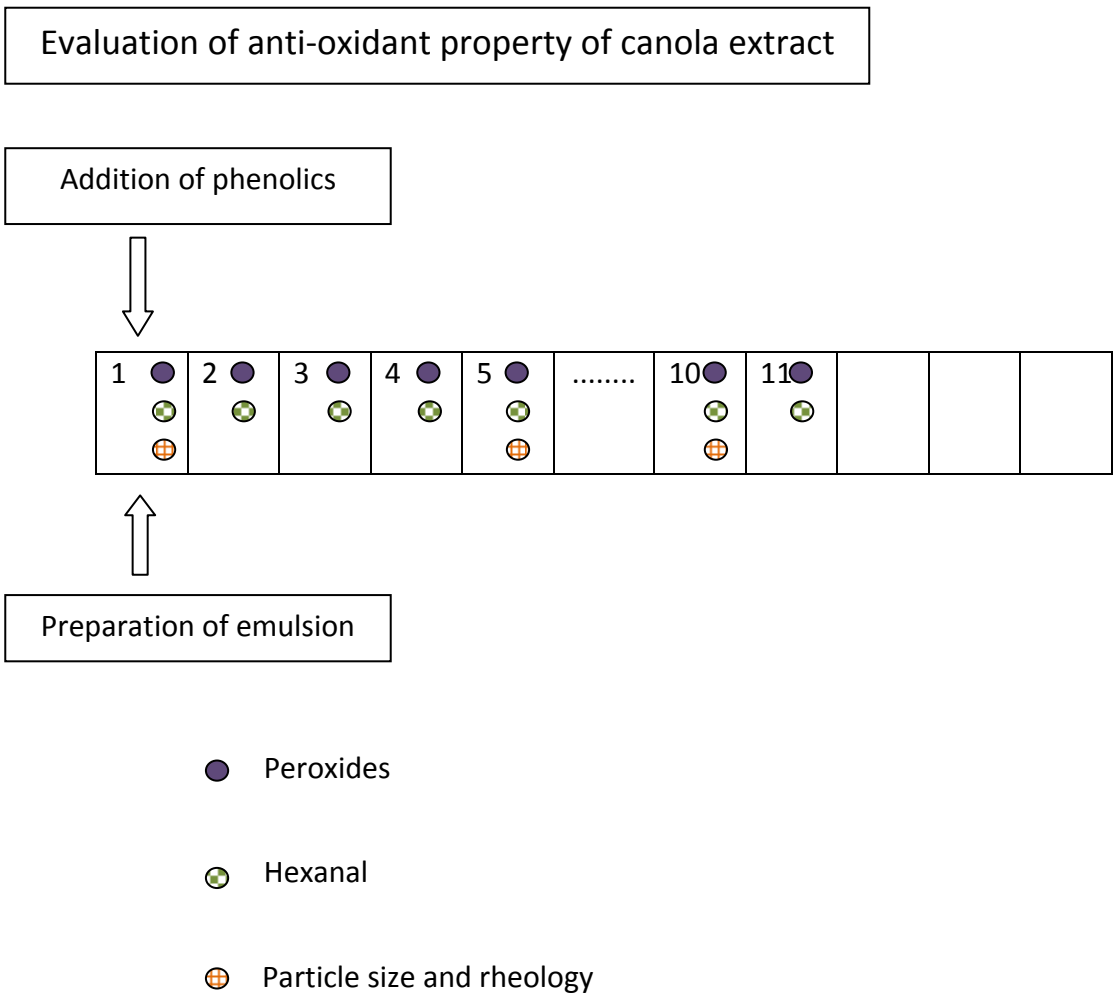


Figure 16. Timeline of addition analysis of oxidation markers and physical stability.

Table 3. Coding system of antioxidant added in the o/w emulsion.

Anti-oxidants		***Abbreviation***	***Concentration (µM)***	***Code***
Extracts	Sinapic acid extract	SA(E)	100	SA(E)100
			350	SA(E)350
	Sinapine	SP	100	SP100
			350	SP350
	canolol	CAN	10	CAN100
			350	CAN350
	Whole extract	WE	10	WE100
			350	WE350
Stds	Sinapic acid	SA	100	SA100
			350	SA350
	Butylated hydroxyl toluene	BHT	100	BHT100
			350	BHT350

4.6. Oxidation studies

4.6.1. Measurement of peroxide value

After bringing the stored samples to room temperature, 300 µl was pipetted out to culture glass tubes containing 1.5 ml isoctane:isopropanol mixture (3:1) to extract oil. It was then vortexed and centrifuged for 4 min at 2000 rpm. 200 µl of the upper layer was added in another culture tubes containing 2.8 ml methanol: butanol mixture (2:1) which was followed by 30 µl of Ferrous solution: thiocyanate mixture (1:1). It was vortexed to allow complete mixing. Absorbance was measured at 510 nm after 20 min. Concentration is calculated by computing it against cumene hydroperoxides calibration curve (Nuchi et al., 2001; Shantha & Decker, 1994)

Reagents

Ferrous solution: 0.132 M $BaCl_2$ was prepared in 0.4M HCl and 0.144 M $FeSO_4$ in distilled water. They were mixed in the ratio 1:1 and then centrifuged at 5000 rpm for 5 min. Upper clear layer was ferrous solution.

Thiocyanate soln.: 3.94 M Ammonium thiocyanate was prepared in distilled water.

Calibration

A dilution series of cumene hydroperoxides was prepared by serial dilution. First a stock solution was prepared from Cumene hydroperoxides: A stock solution of 6570 µM of cumene hydroperoxides was prepared by dissolving 1 g in 1000 ml water and was serially diluted as shown in table 4. Then, the procedure mentioned in section 4.6.1 was followed to draw a calibration curve.

Table 4. Preparation of dilution series of Cumene hydroperoxides for calibration.

Dilution	*Concentration (µM)*
1:20	*276.356*
1:30	*184.969*
1:60	*92.4845*
1:120	*46.2425*
1:240	*23.121*
1:480	*11.560*
1: 960	*5.780*

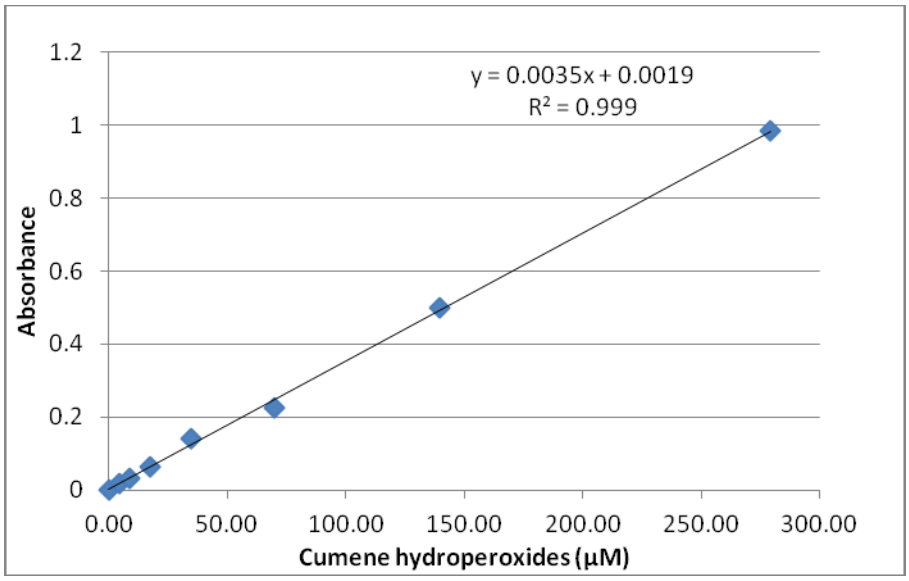


Figure 17. Calibration curve of cumene Hydroperoxides (absorbance at 517 nm) (Inset: regression equation).

4.6.2. Measurement of secondary volatile products

Samples were stored at -80 ˚C until the day of analysis. Within each experiment, the samples were analyzed for headspace volatiles in a completely randomized order. Headspace analysis was conducted using a Varian 3800 gas chromatograph equipped with a Combi-Pal autosampler with pre-heating unit and an FID (Flame Ionisation Detector). Samples were preheated to 45 ˚C for 5 min while rotating at 250 rpm to equilibrate, then rotation was stopped and the septum was pierced with a SPME (Solid Phase Micro-extraction) needle with a retractable 50/30 µm divinylbenze/Carboxen™ on polydimethylsiloxane coated fiber (Supelco, Bellefonte, PA). The fiber was exposed to the headspace for 15 min, then retracted and immediately injected and desorbed at 260 ˚C for 7 min in a Varian 3800 GC with helium as the carrier gas (1 ml/min), and an FID detector (280 ˚C). Volatiles were separated on a DB-WAX (30m x 0.25 mm i.d. 0.25 µm) column (Agilent, Santa Clara, CA) using a temperature program of 60 °C for 1 min, 2 ˚C/min to 76 ˚C, then 20 ˚C/min to 240 ˚C and held for 2 min. Injection was splitless until complete desorption (7 min) followed by split (1:50). Hexanal was identified by retention time compared to a commercial standard. Peaks were integrated and analyzed using Varian Galaxy Chromatography Software. A new fiber was used for each emulsion storage study. Hexanal standard (10 µg/g) in canola oil was used to verify the performance of the GC system and of the SPME fiber. A vial containing this standard was measured after every 8 experimental samples.

4.7. Particle size measurement

Distribution of the oil droplet samples was determined by a light scattering analyser (Malvern Mastersizer 2000). The principle is that "particles passing through a laser beam will scatter light at an angle that is directly related to their size" (www.malvern.com). Particle size is inversely related to the observed scattering angle logarithmically. Light scattering data was captured by the detectors. Fig. 18 shows the schematic diagram of Mastersizer 2000.

Samples incubated at 30 °C were taken out every five days and their droplet sizes were measured. The procedure for oil droplets in O/W emulsion followed was as follows (Kim et al., 2004): The emulsion was mixed with distilled water in the ratio 1: 1000 to get a better resolution in the measurement by preventing blockage from too many oil droplets. The diluted samples were added drop by drop to the sample inlet chamber. The size was measured as surface average mean diameters (d_{32}) by Malvern 2000 software. Instrument stirring was set at 50%. All measurements were carried out for duplicate samples.

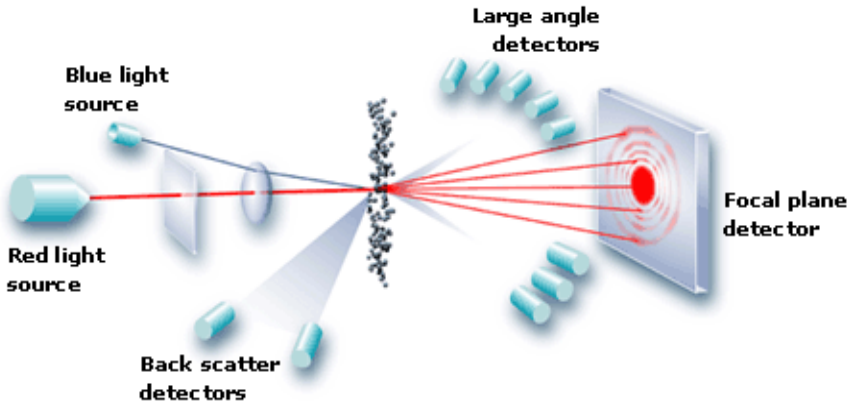


Figure 18. Schematic diagram showing principle for measuring particle size in emulsion. (*Copyright Permission taken from:* Malvern)

4.8. Rheological measurement

The shear flow curve of the emulsion was obtained by measuring shear stress against shear rate. Analysis was performed in a shear rheometer (AR 2000 rheometer; TA Instrument). Viscosity was calculated from the slope of the shear flow curve. About 22 ml of emulsion was added to the concentric cylinder (cup radius - 15 mm; DIN conical rotor radius of 14 mm and height of 42 mm). Pre-shearing was done for 2 min at 1000 s^{-1}.Temperature was maintained at 30 °C. Viscosities reported were measured in mPa.S. All measurements were carried out in duplicates.

4.9. Statistical analysis

Statistical treatment was performed by one way ANOVA followed by Tukey's multiple comparison tests to determine significant difference. Significance level was set up at $P < 0.05$. GraphPad Prism software was used.

5. Results and discussion

5.1. Comparison of the anti-oxidant activity of antioxidants

In-vitro assays, viz. DPPH, reducing power and iron chelating assays, were carried out to assess the anti-oxidative ability of canola derived antioxidants. These tests enabled a comparison of the anti-oxidative potential of all the anti-oxidants and may help to predict how they maybehave in the oxidation experiment of o/w emulsion.

5.1.1 Ferric Reducing Antioxidant Power Assay (FRAP)

Absorbance of the anti-oxidants at 100 µM showed that sinapine had the highest absorbance value which indicated its highest reducing power among the extracts tested (Fig. 19). Absorbance value of sinapine (0.16) was significantly higher ($p<0.05$) than the absorbance value of WE, which showed the second highest value of absorbance (0.12). However, the reducing powers of WE, SA and SAE were very close to each other and showed no significant difference. ASC showed significant difference from WE but not with SAE and BHT. CAN showed the lowest value of absorbance and was significantly different from all other antioxidants. Anti-oxidants at 100 µM showed the following order:

$$\mathbf{SP^{a} > WE^{b} = SA^{b} = SAE^{bc} > ASC^{c} = BHT^{c} > CAN^{d}}$$

A similar pattern was observed at 350 µM as indicated in Fig. 20. The values were, however, increased by almost 3.5 times in accordance with the concentration of anti-oxidants. SP showed the highest value of absorbance (0.4). This indicated that SP is comparatively better than all other anti-oxidants in terms of reducing power. SAE, ASC and BHT gave equally effective results.

Similar to concentration at 100 µM, CAN had the lowest reducing power at 350 µM. The efficacy of these antioxidants at the concentration of 350 µM showed the following order:

SP^{a}> WE^{b}> SA^{c}>SAE^{d}=ASC^{d}=BHT^{d}>CAN^{e}

Reducing power determined the capacity of the compound to reduce Ferric (Fe^{3+}) to Ferrous (Fe^{2+}) ions. Ferric ions are the initiator of the oxidation reaction. Higher reducing power of antioxidants reflects the ability of antioxidants to control initiation reaction of oxidation. The reason for the highest reducing power of sinapine is not clearly understood. However, being a larger molecule esterified with a choline group might have played an important role. Synergistic activity of phenolic compounds was also postulated by Wanasundara et al. (1996). It was found that ethanolic extract was given better antioxidative property than phenolic extract fractions (Wanasundara et al., 1996). The high reducing power of WE, being a mixture of different phenolic compounds, could be a result of synergism. An interesting observation from this study was that reducing power of ASC and BHT were lower than all antioxidants except CAN. This is in agreement with Wanasundara and Shahidi (1994) which found that antioxidative property of ethanolic extract of rapeseed meal was higher than that of synthetic antioxidant like BHT and BHA.

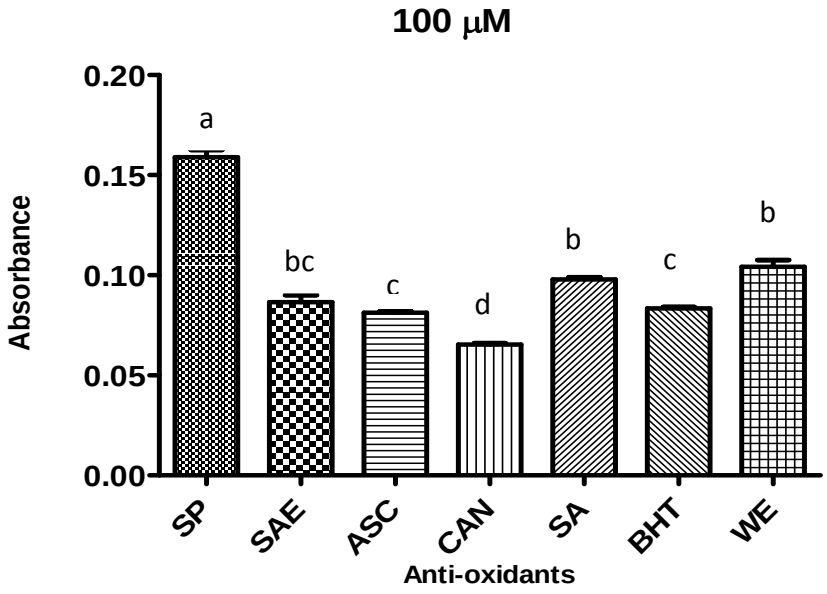


Figure 19. Reducing power of antioxidants at 100 µM (Mean±SD; n=3)

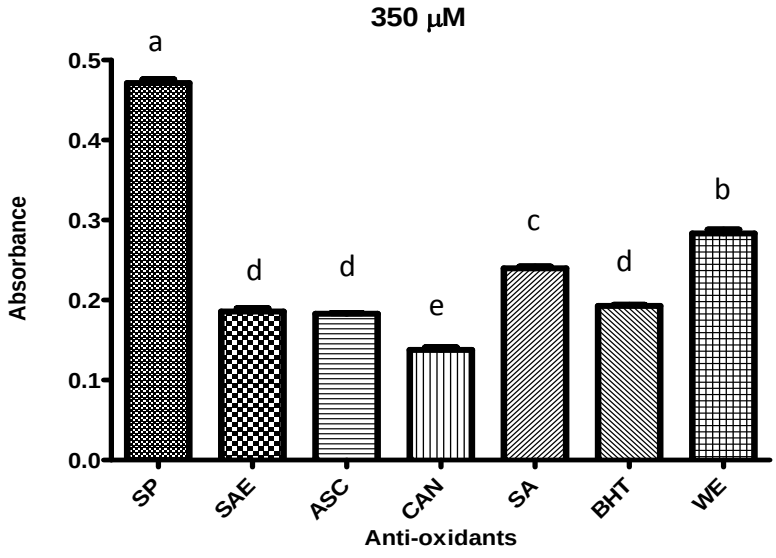


Figure 20. Reducing power of antioxidants at 350 µM (Mean±SD; n=3)[1]

[1] Letter a, b, c, d and e in the Fig. 19 and the following figures denote the level of significance. The same letter between each treatment signify that they are not significant ($p<0.05$).

5.1.2. Radical Scavenging assay (DPPH· assay)

DPPH· radical scavenging assay is a common yet important anti-oxidant assay (Sharma and Bhat, 2009) used in determining the anti-oxidative capacity of a compound. 1, 1-Diphenyl-2-picryl- hydrazyl (DPPH·) is a stable free radical which has an unpaired valence electron at one atom of a nitrogen bridge (Eklund et al., 2005). Unpaired electron of DPPH· can be shared with another unpaired electron of an anti-oxidant leading to stabilisation of DPPH·.

At 100 µM level, SP was found to possess the highest capacity to scavenge DPPH which was followed by WE and SA (Fig. 21). Inhibition % of SP was significantly higher than that of WE and thus SA ($p<0.05$). WE and SA showed no significant difference ($p<0.05$). SAE was much lower than SA and was significantly different. BHT was found to have lowest inhibiting capacity compared to all the anti-oxidants tested. At this concentration (100 µM), the pattern of inhibition percentage of antioxidants was similar to that of reducing power of antioxidants. The inhibition % was in the following order:

$$\mathbf{SP^{a}>WE^{b}=SA^{b}>SAE^{c}>CAN^{d}>BHT^{e}}$$

At 350 µM also, SP was still found to have the highest inhibition % as indicated in Fig. 22. However, no drastic increase was observed. From 85% at 100 µM, it slightly increased to 88% at 350 µM. Thus, by increasing the concentration more than thrice (from 100 µM to 350 µM), no proportionate increase in scavenging capacity was observed. It could be due to the saturation of its scavenging potential. SP showed no significant difference from SA at 350 µM. The inhibition percentage of SAE, CAN and BHT were almost increased by double when the

concentration increased from 100 to 350 μM. The pattern of radical scavenging of antioxidants was similar at both concentrations. The inhibition % at 350 μM indicated the following order:

SP^{a} =SA^{a}>WE^{b}>SAE^{c}>CAN^{d}>BHT^{e}

Radical scavenging is considered to be the main mechanism of action of phenolic antioxidants (Frankel, 1998) and therefore, DPPH radical scavenging assay is important from the point of its oxidation controlling capacity. Antioxidative property of a compound is also dictated by the characteristic group at the end of the side chain. Side chains containing electron donating groups like $–CH_3$ and –OH increase the ease of hydrogen abstraction while electron donating groups like –COOH and –CHO have the opposite effect (De Proft et al, 1995). Isoeugenol and coniferyl alcohol were found to be more than 30 times more effective than ferulic acid, coniferyl aldehyde and ethyl ferulate on the basis of AE (antiradical efficiency) values (Nenadis, 2003). Here, sinapic acid, sinapine and canolol also contained $-CH_3$, –OH and $-OCH_3$ side chains and they could have contributed their radical scavenging property. It could also be the result of esterification of choline group on the side chain. It was also very likely that presence of three methyl groups would assist in hydrogen abstraction and thus, increase radical scavenging capacity. Chain length could also contribute to antioxidative capacity of compounds (Kikuzaki et al., 2002). SA and SP which have longer side chain than CAN showed higher radical scavenging capacity. It was observed that BHT showed lowest radical scavenging capacity. Since antioxidant property is relative to the media of reaction, this might have contributed to the low activity of BHT as compared to other antioxidants. Elsewhere other studies on O/W emulsion gives insight into the BHT (Lea and Ward, 1959)

WE contained some components other than the principal component sinapine and could have affected radical scavenging leading to lower radical scavenging capacity. Similar reason could also for having low radical scavenging property in SAE where other components other than sinapic acid might have contributed to the lower activity of sinapic acid.

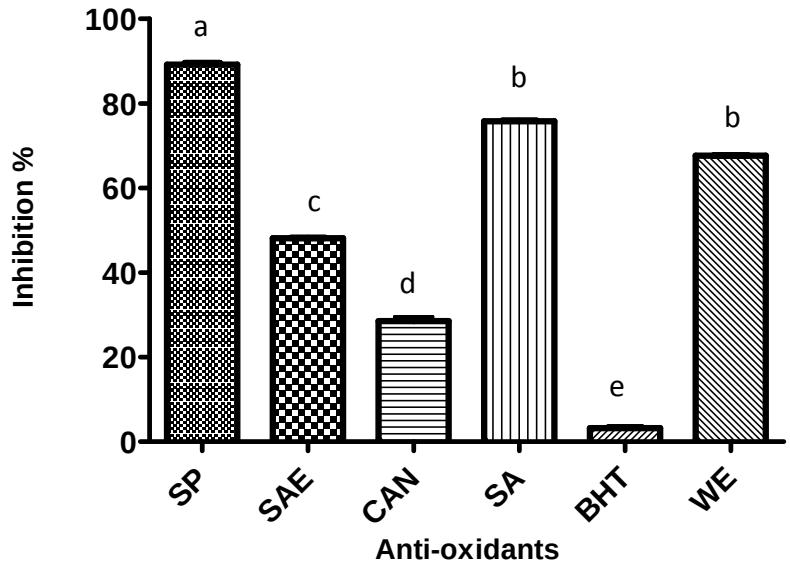


Figure 21. Radical scavenging capacity (inhibition percentage) of antioxidants at 100 µM (Mean±SD; n=3).

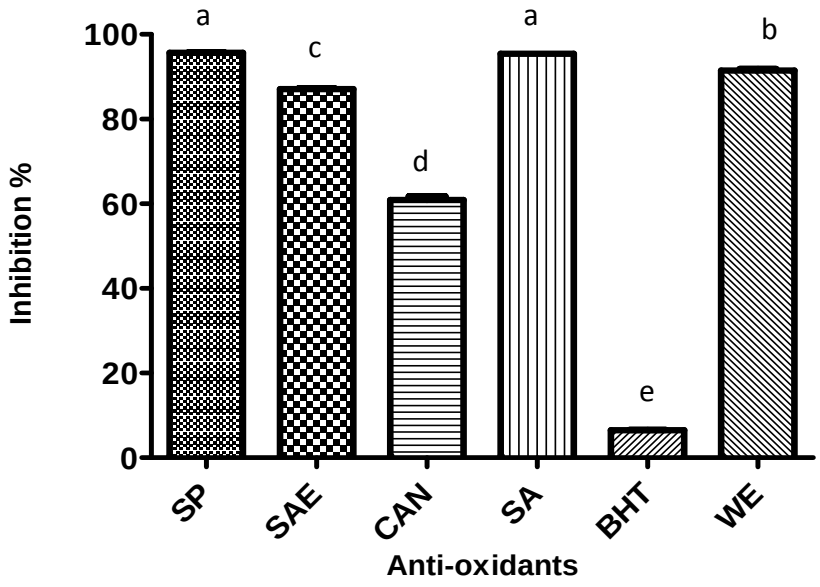


Figure 22. Radical scavenging capacity (inhibition percentage) of antioxidants at 350 µM (Mean±SD; n=3).

5.1.3. Iron chelating activity

Iron chelating assay is based on the principle that a highly coloured magenta coloured complex is formed when ferrous iron (Fe^{2+}) is chelated with ferrozine. A lower absorbance value indicated that lesser amounts of free ferrous iron (Fe^{2+}) are available after chelation by the anti-oxidants. Thus, absorbance value is inversely related to chelating activity. Metal ions like Iron (II) are a powerful initiator of oxidation. Many of the phenolic anti-oxidants are capable of chelating with metal ions (Saskia, 1998) to inhibit initiation of lipid oxidation. Metal chelating activity of EDTA was well known (Lindsay, 1976).

At 100 µM, EDTA showed the highest chelating activity percentage, far ahead of all other anti-oxidants and almost close to 100% (Fig. 23). WE also showed the promising result among all other anti-oxidants, in addition to EDTA. SAE, CAN and SA showed no significant difference ($p<0.05$) among them. Interestingly SP, which had high scavenging activity and reducing power exhibited a low chelating activity. This seemed to be not in accordance with study of Saskia et al. (1998) who stated that anti-oxidant good at scavenging were not good iron chelators. EDTA is a metal ion chelator and therefore, its metal ion chelating activity was expected. The iron chelating activity was in the following order:

$EDTA^{a} > WE^{b} > SP^{c} > BHT^{d} > SAE^{e} = CAN^{e} = SA^{e}$

The chelating activity of the antioxidants at a concentration of 350 µM is displayed at Fig. 24. As shown, the chelating activity of WE becomes almost equal to EDTA (close to 100%), which is almost a 5 times increase from its value 20% at a concentration of 100 µM. At a concentration of 350 µM, there is no significant difference between the two antioxidants namely WE and

EDTA. Chelating activity of SP also increased up from 10% to 60%. The iron chelating activity percentage followed the order:

$$\mathbf{EDTA^{a}=WE^{a}>SP^{b}>SAE^{c}>CAN^{d}=SA^{d}=BHT^{d}}$$

The increase in concentration from 100 µM to 350 µM, did not affect the chelating activity of the three antioxidants namely CAN, SA and BHT values, wherein their chelating activity (%) remains similar (less than 10%).

5.1.3. Comparison of reducing power, radical scavenging and iron chelating properties of antioxidants

In all the antioxidant assays, with the exception of iron chelating assay, it was very clear that SP possessed the highest antioxidative properties. The unique structural feature, i.e. presence of choline group as discussed earlier in section 5.1.1., might be the contributing factor for high antioxidative property. WE is a combination of different phenolic compounds with SP as major constituent given the high content of sinapine in canola meal. Therefore, high reducing power and radical scavenging activity of WE might be due to the presence of sinapine. This was further supported by the result that SP and WE displayed agreeable antioxidant activity in all assays.

The principle compound responsible for the activity of SAE and SA is sinapic acid. This seemed to be reflected in scavenging assay and iron chelating activity assay where SA and SAE showed similar values (with no significant difference) at both the concentrations (100 µM and 350 µM). However in reducing power assay, the SA showed more reducing power than SAE and therefore, not consistent with the result of scavenging assay and iron chelating activity assay. Higher reducing power of SA could be the result of higher level of purity of sinapic acid in SA.

While the presence of other minor components that could reduce the content of this principal component as well as interfere with the reducing activity. In all the assays, CAN showed comparatively lower antioxidative property. Theoretical study by Galano et al. (2011), however, showed that canolol is a potent radical scavenging antioxidant. According to this study, medium of reaction plays an important role in antioxidant activity. The low antioxidant activity of canolol in the methanolic DPPH medium could attribute to the low activity of canolol.

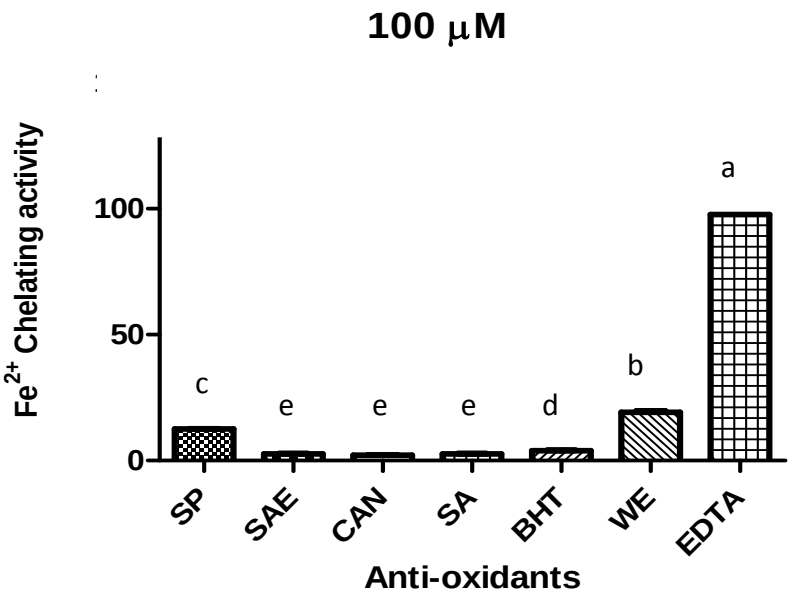


Figure 23. Chelating activity (percentage) of antioxidants at 100 µM (Mean±SD, n=3)

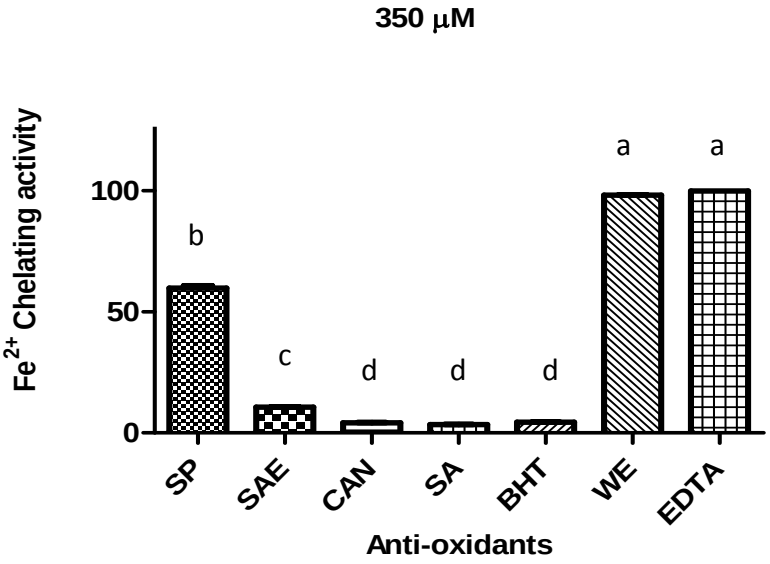


Figure 24. Chelating activity (percentage) of antioxidants at 350 µM (Mean±SD, n=3)

5.2. Storage study: Effect of anti-oxidants on lipid oxidation

The lipid oxidation in the O/W emulsion was monitored by primary and secondary oxidation markers. Hydroperoxides are the important primary by-product of lipid oxidation and they are also important and effective markers to track the degree of oxidation. Hexanal, propanal, pentanal and 2, 4-heptadienal are the volatile secondary oxidation products formed after complex reactions involving β scission of hydroperoxides (Frankel, 1998). Oxidative stability of canola oil was studied using headspace volatiles (2-pentanal, hexanal, 2-heptenal, 2,4-heptadienal, etc.) by Richards et al. (2005). A direct correlation was found between the peroxides values calculated from the formula (derived from volatiles contents) and those determined by standards chemical method.

The experiments were divided into three groups. Each group constituted two anti-oxidants and a control (without anti-oxidant). Selection of anti-oxidants was done randomly. Since the aim of the experiment was to examine relative difference of the effects of anti-oxidants on oxidation compared to control, grouping of antioxidants were immaterial.

5.2.1. Experiment I [SA, CAN and control]

In the first batch of experiment (Experiment I), Sinapic acid standard (SA) and canolol (CAN) were added to the emulsion at two level such as 100 µM and 350 µM respectively. SA is a synthetic one whereas CAN was extracted from canola meal extract (in the procedure described in section 4.2.). Tracking of peroxides showed that for the first 50 h (lag phase), no significant difference ($p<0.05$) was observed between the control and all other emulsions containing SA

and CAN (at both concentrations). However, after 50 h, a new trend in which the effects of the anti-oxidants become prominent was observed (Fig. 25). Interestingly, though significant difference was observed at different time points after 50 h increase hydroperoxides in control over time is paralleled to other treatments. Difference in concentration did not show any significant effect. *In-vitro* antioxidant assays also showed comparatively lower anti-oxidative property by these antioxidants. This observation, therefore, was perhaps a reflection of the *in-vitro* antioxidant assays of CAN and SA where they showed lower reducing power and iron chelating activity. But SA which showed high radical scavenging property at both concentrations seemed to have no effect in the emulsion system.

Effect of anti-oxidant on the lipid oxidation in O/W emulsion system was investigated by following the secondary marker i.e. hexanal, at different time intervals. The results from the SPME-GC analysis of volatiles showed steady increase hexanal over time. CAN and SA at both concentrations seemed to have no effect on oxidation. The control, however, already had high hexanal content and it did not show any significant increase over time. These results were in accordance (except control) with the results from the hydroperoxides. Hydroperoxides also increased with time and no treatments had inhibitory effect on oxidation. The results of hexanal formation were expected since secondary oxidation products are formed from lipid hydroperoxides. Given the low antioxidative capacity shown in various *in-vitro* antioxidant assays, CAN result was also expected. However, SA which showed high radical scavenging capacity did not effectively control oxidation as reflected in peroxide and hexanal formation. In other studies in bulk oil, SA was shown to be effective in controlling oxidation (Thiyam et al., 2006). Galano et al. (2011) also showed that from a theoretical perspective CAN and SA

possessed high antioxidative potential but no comparison was done with other antioxidants like sinapine.

Sinapic acid was predicted to react about 32.6 times and canolol 3.6 faster in aqueous solution than in lipid media (Galano et al. 2011). This suggested that low activity of sinapic acid and canolol in O/W emulsion. One probable reason for such anomalous behaviour may also be attributed to 'polar paradox' theory which stated that polar anti-oxidants are more effective in no-polar media (Porter, 1993). Compared to sinapine, sinapic acid and canolol are less polar. Therefore, SA and CAN might not be as effective as SP in O/W emulsion.

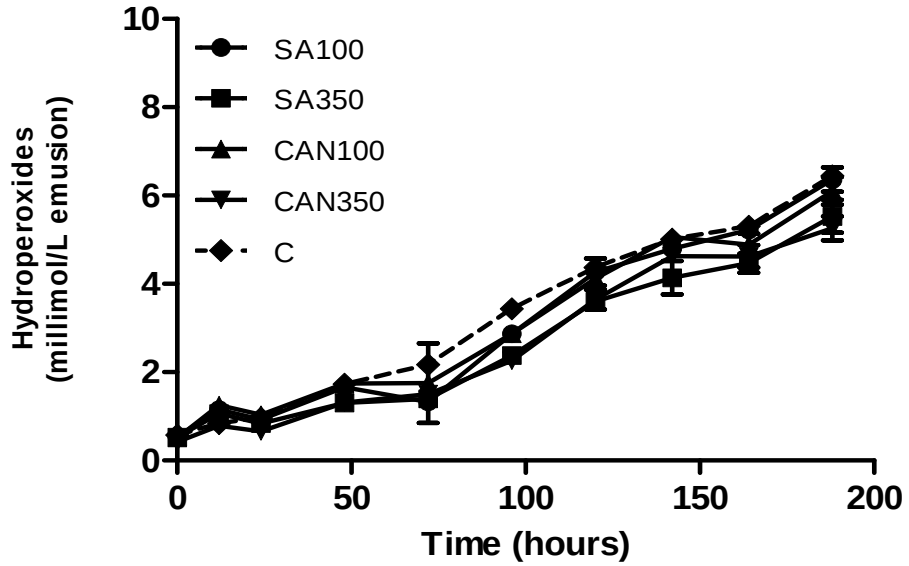


Figure 25. Formation of lipid hydroperoxides in whey protein stabilised 10% canola oil-in-water emulsion (pH 7) with added antioxidants (SA, Sinapic acid standard and CAN, Canolol extract at 100 and 350 µM emulsion) during oxidation at 30˚C (Mean ± SD, n=2)

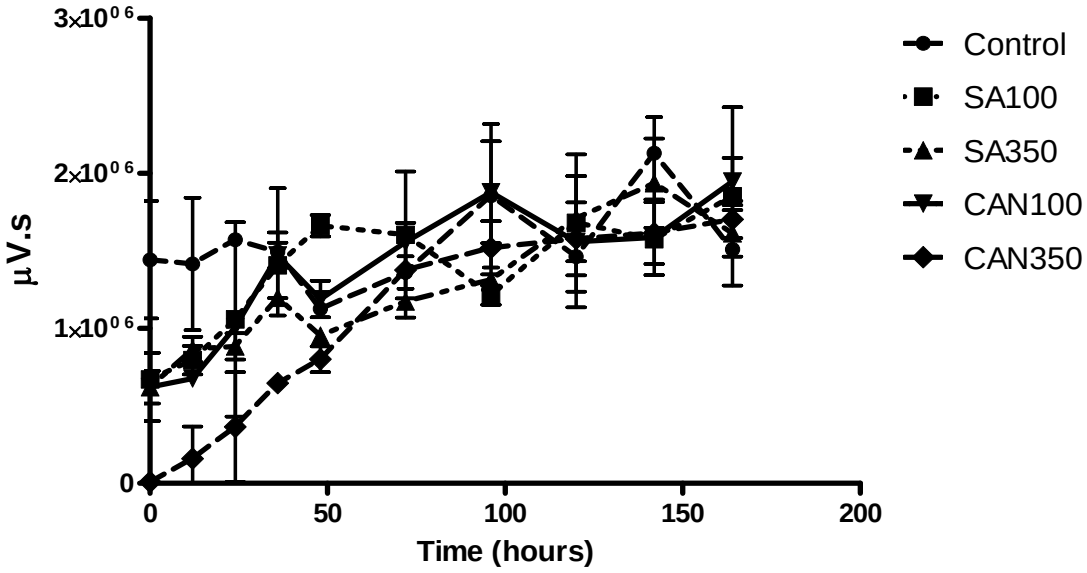


Figure 26. Development of hexanal as measured by SPME-GC of 10% canola oil-in-water emulsion (pH 7) with added antioxidants (SA, Sinapic acid standard and CAN, Canolol extract at 100 and 350 µM emulsion) during oxidation at 30˚C (Mean ± SD, n=2)

5.2.2. Experiment II [SP, BHT and control]

Sinapine is a choline ester form of sinapic acid. It is the most abundant phenolic compound present in canola meal. BHT is one of the most commonly used anti-oxidants in the food industry because of its effectiveness, economy and convenience. In the case of these anti-oxidants, concentration was found to play an important role in checking the oxidation. It was observed from the Fig. 27, after 24 h, the difference between the hydroperoxides content of all antioxidants (BHT and SP) and control became significant ($p<0.05$). However, SP and BHT at higher concentration (350 μM) did not show a significant rise after 50 h, signifying a widening gap compared to control. BHT at 350 μM gave the best result when compared with the control followed by SP at 350 μM. One interesting observation in this study was that after around 125 h, peroxide value of sample with BHT added at 100 μM became more than that of control. However, the difference was not significant and therefore, possibility of BHT being a pro-oxidant at lower concentration cannot be confirmed.

Similar to what we observed in hydroperoxides formation, hexanal was also developed in a similar pattern (Fig. 28). The inhibitory effect of SP and BHT (at 350 μM) on oxidation was clearly observed. This study showed that concentration plays a key role in controlling oxidation. At 100 μM, both SP and BHT were not effective. But interestingly at 350 μM, both were effective at inhibiting hexanal formation.

Given the widespread application of BHT in food emulsions for controlling lipid oxidation, the result is not surprising. However, in all three *in-vitro* antioxidants assays, BHT showed

comparatively low antioxidant capacity. The result, therefore, is in total contradiction to antioxidant assays (reducing power, radical scavenging and iron chelating). In earlier study (Lea, 1959), BHT was shown to be effective in emulsions rather than in bulk oil but α-, β-, γ-, and δ-tocopherols showed opposite trends. However, SP anti-oxidative capacity as shown by antioxidant assays seemed to be truly reflected in the O/W emulsion. The trend displayed by these antioxidants followed the order: BHT350>SP350>SP100>BHT100. Similar result was also observed by Vuorela (2005) in which sinapine was found to be the best antioxidant of all the rapeseed phenolic extracts in liposome oxidation. According to Vuorela et al. (2005), the presence of anion in choline group makes sinapine more polar than other antioxidants. Antioxidants which can electrostatically attract to the surface of the emulsion droplets can be effective in controlling lipid oxidation (Mei et al., 1999). Such an interface could be O/W or W/O interface. Lipid oxidation occurs primarily in lipid medium or at the water-lipid interface (Yuchi et al., 2007). Therefore, sinapine anti-oxidative property could also be attributed to its highly polar nature. Alkyl chain length of the antioxidant was also found to play a critical in the antioxidative property of antioxidant in emulsion (Laguerre et al., 2009; Laguerre et al., 2010). It was found that antioxidant activity increases as the chain length was increased but decrease after a certain length. Given the longer alkyl chain length of SP as compared to other antioxidants, higher antioxidant acivity of SP in emulsion could be expected.

As it was observed from this study, concentration played an important role in being pro-oxidant or antioxidant. At 100 µM, SP and BHT did not show any antioxidative effect. This is in agreement with the findings of Huang et al. (1994) wherein observed a maximum anti-oxidative activity for α-tocopherol at 250-500 ppm in 10% O/W emulsion as indicated by hydroperoxides

formation. However at 100 ppm, it did not show significant antioxidative activity. Concentration sensitivity of antioxidant was also studied by Zhong and Shahidi (2011) which found that 'polar paradox was applicable upto a certain range. After a critical concentration, antioxidant activity of a compound decreased.

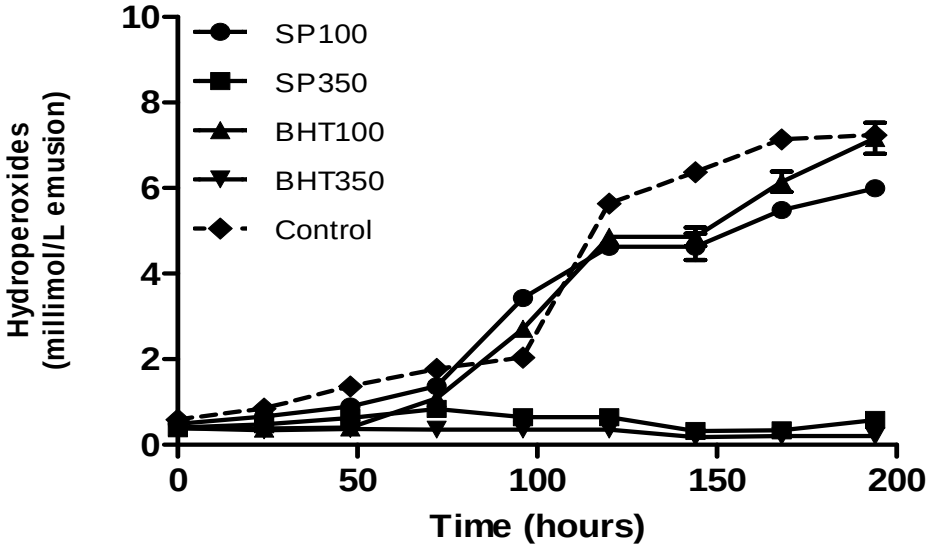


Figure 27. Formation of lipid hydroperoxides in whey protein stabilised 10% canola oil-in-water emulsion (pH 7) with added antioxidants (SP, Sinapine and BHT, Butylated hydroxytoluene at 100 and 350 µM emulsion) during oxidation at 30° C (Mean ± SD, n=2).

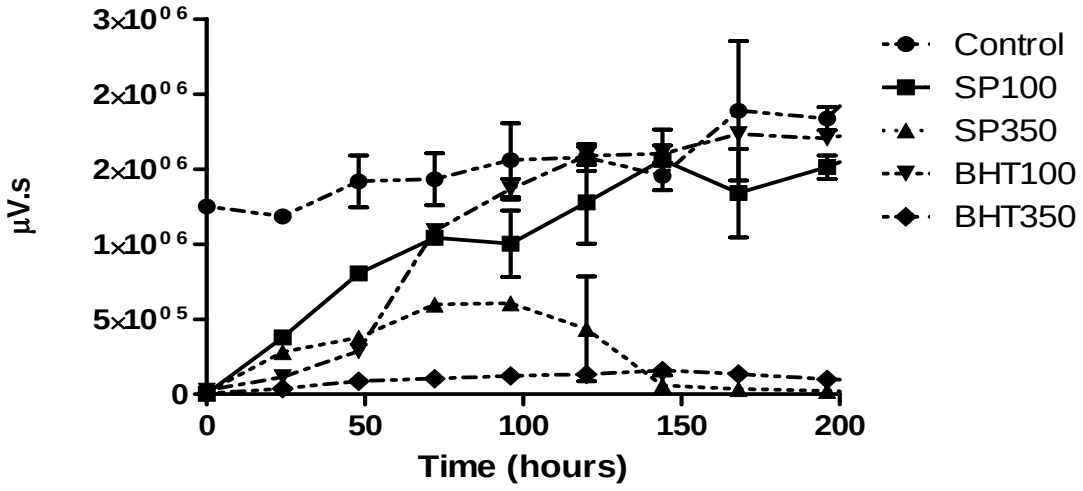


Figure 28. Development of hexanal as measured by SPME-GC of 10% canola oil-in-water emulsion (pH 7) with added antioxidants (SP, Sinapine extract and BHT, Butylated hydroxyanisole standard at 100 and 350 µM emulsion) during oxidation at 30° C (Mean ± SD, n=2).

5.2.3. Experiment III [WE, SAE and control]

WE (whole extract) includes all major phenolic anti-oxidants. In principle, anti-oxidative effect of WE would be a combinatorial result of all the individual phenolic compounds. SAE (Sinapic acid extract) was obtained after purification (see section 4.1) and identified by HPLC. SAE contained some other minor components which could not be identified by HPLC.

As observed from the Fig. 29, the amount of hydroperoxides in the control sharply increased as the storage time increases. Though a significant difference ($P<0.05$) was shown by SAE (compared to control) after 50 h, SAE was shown to have little effect on the oxidation (Fig. 31). Peroxide value of SAE added emulsion consistently increased and ran in parallel to the control. Moreover, an increase in concentration also had no effect. SAE, at both concentrations, gave the closely similar results. Interestingly, WE**,** at both 100 and 350 µM, were shown to strongly inhibit the oxidation in the emulsion. It was clearly evident that concentration played a critical role in controlling oxidation. Just a few hours after storage, WE at 350 µM showed a significant shift from the control. Peroxides values did not change significantly over time and thus indicated that oxidation could be effectively control. WE at 100 µM also showed effective oxidation controlling ability. Up until 50 h, no significant difference (from control) ($P<0.05$) was observed but after 50 h, the effect of the WE became prominent as it was shown by insignificant change of peroxides values over time while widening the value from control. Role of concentration in control of oxidation was also shown by the significantly different value between the two concentrations. Lower peroxide value reflected better effectiveness of the anti-oxidants; thus, SAE350 was better compared to SAE100.

Hexanal was also increased with increase in oxidation time (Fig. 30). The hexanal formation followed a pattern similar to hydroperoxides formation. Whole extract (WE) at both concentration were better than SAE in terms of hexanal formation inhibiting capacity. Higher concentration gave higher oxidation controlling capacity. Until 50 h, there was no significant difference between the two treatments i.e. WE100 and WE350 but after 50 h only WE350 could retard oxidation as shown by its absolute values (in μV.S).

As discussed in section 5.1., WE possessed high anti-oxidative property as indicated by - reducing power, free radical scavenging and iron chelating assays. Therefore, this storage study further supported the results antioxidant assays. The component responsible for the potent anti-oxidant activity in WE was not known. However, as mentioned in section 5.2, sinapine which had already shown to be an effective anti-oxidant in *in-vitro* antioxidant assays, must have played a key role. An interesting observation from the study was that WE was effective at both the concentrations (100 μM and 350 μM). Even SP could not effectively control oxidation at lower concentration (100 μM).Therefore, the synergistic activity of the other minor components can't be rule out. Sinapic acid is the principle component of both SA and SAE. In section 5.2.1. (experiment I), SAE was shown to be ineffective in controlling oxidation. Since sinapic acid is the principle component of SAE, the result could be expected. Moreover, the reasons applicable to SA for ineffectiveness in controlling oxidation in O/W emulsion (section 5.2.1.) could also be applied to SAE. These results also reflected the *in-vitro* antioxidant assays which shown low anti-oxidative property.

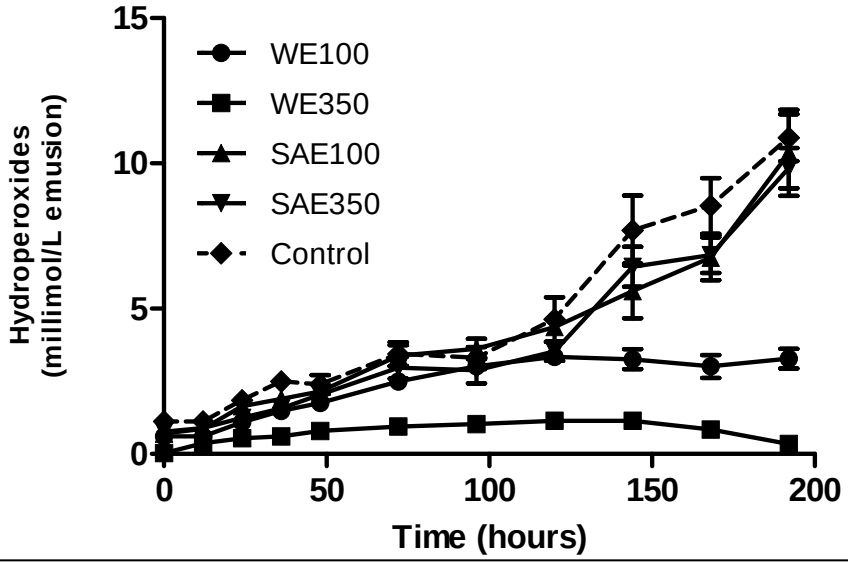


Figure 29. Formation of lipid hydroperoxides in whey protein stabilised 10% canola oil-in-water emulsion (pH 7) with added antioxidants (WE, Whole extract and SAE, Sinapic acid extract at 100 and 350 µM emulsion) during oxidation at 30˚ C (Mean ± SD, n=2).

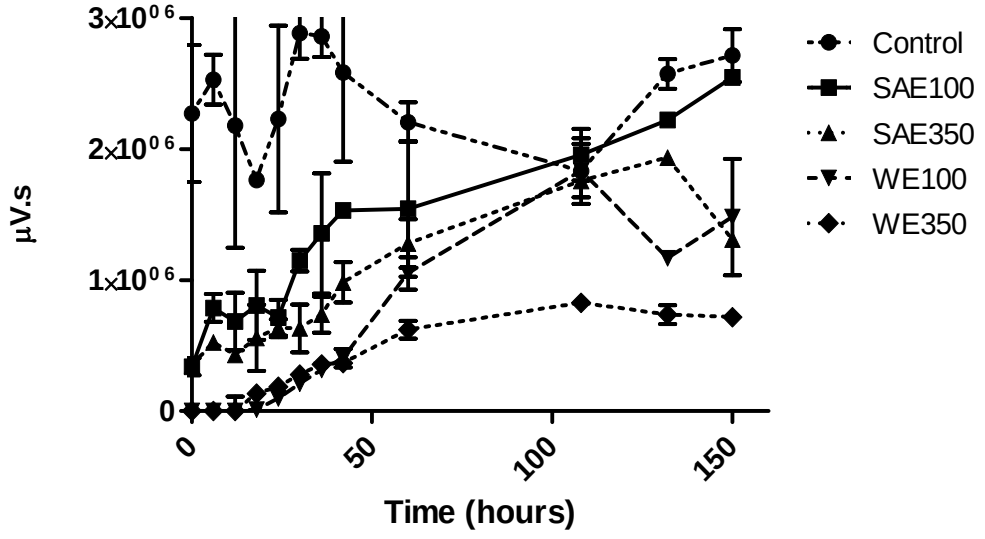


Figure 30. Development of hexanal as measured by SPME-GC of 10% canola oil-in-water emulsion (pH 7) with added antioxidants (SA, Sinapic acid standard and CAN, Canolol extract at 100 and 350 µM emulsion) during oxidation at 30˚ C (Mean ± SD, n=2).

5.3. Particle size distribution of canola oil-in-water emulsion

An objective of the present study was to create an O/W emulsion of similar oil droplet diameter so that there was no significant difference in the rate of oxidation due to surface area effect. Particle size is an important factor in determining the stability of the emulsion. The particle size was measured in the control emulsion (no anti-oxidants). Previous investigation showed that addition of anti-oxidants did not significantly affect the particle size (Appendix: Fig. 1 and 2). Based on this observation, all the particle size measurements were done only in the control. The average particle was in the range between 1.625 – 1.88 µm over a 15 day period (Fig. 31-34). No significant change in droplet diameter over the 15 days period indicated that the emulsion was stable and there was no coalescence or breaking down of the emulsion system. Moreover, it was also very unlikely that small difference in particle sizes would significantly affect oxidative deterioration (Kiokias, 2007). Since the amount of emulsifier and homogenisation parameters remained the same, the results should not have been significantly different.

Table 5. Particle size of oil droplets in canola oil-in-water emulsion during 15 days (Mean±SD, n=2).

Day	d_{32}µm	Coefficient of variation
1	0.1625 ±0.0007	0.4%
5	0.1615±0.0021	1.3%
10	0.18±0.0028	1.6%
15	0.188±0.0014	0.8%

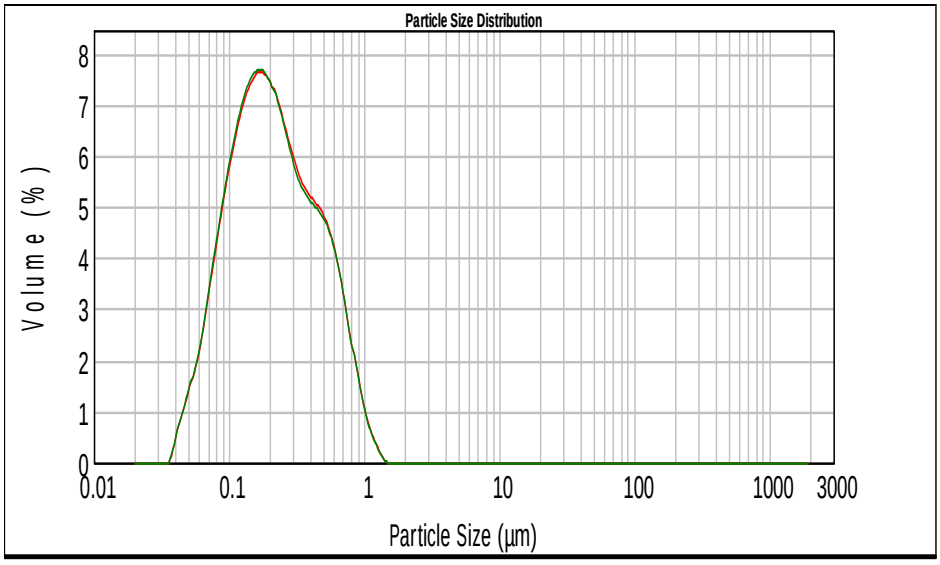


Figure 31. Particle size distribution of 10% canola O/W emulsion on day 1.

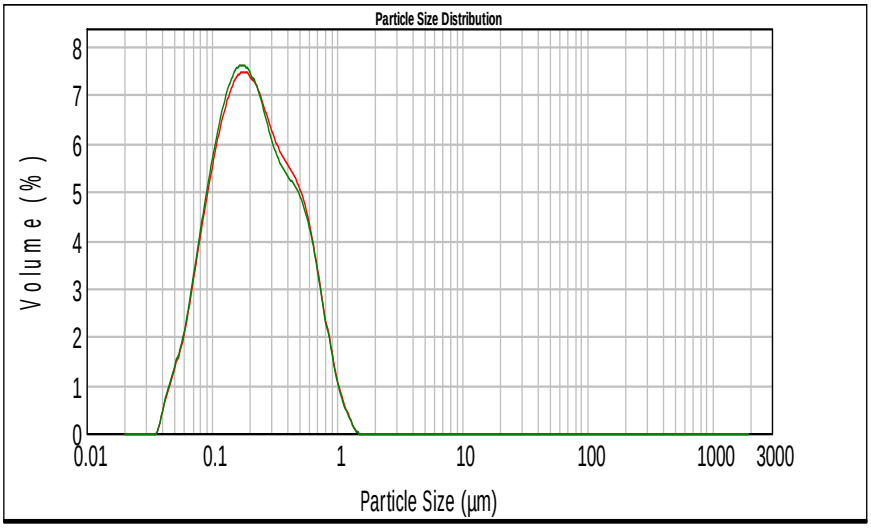


Figure 32. Particle size distribution of 10% canola O/W emulsion on day 5.

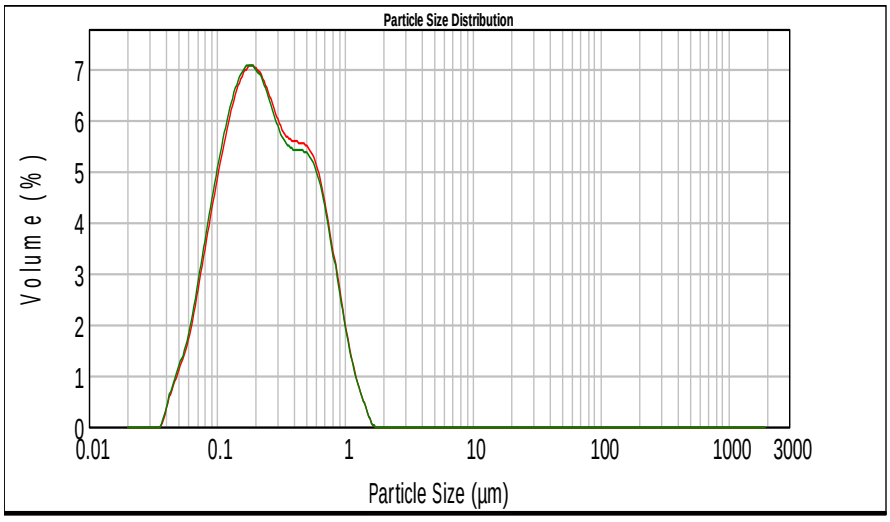


Figure 33. Particle size distribution of 10% canola O/W emulsion on day 10.

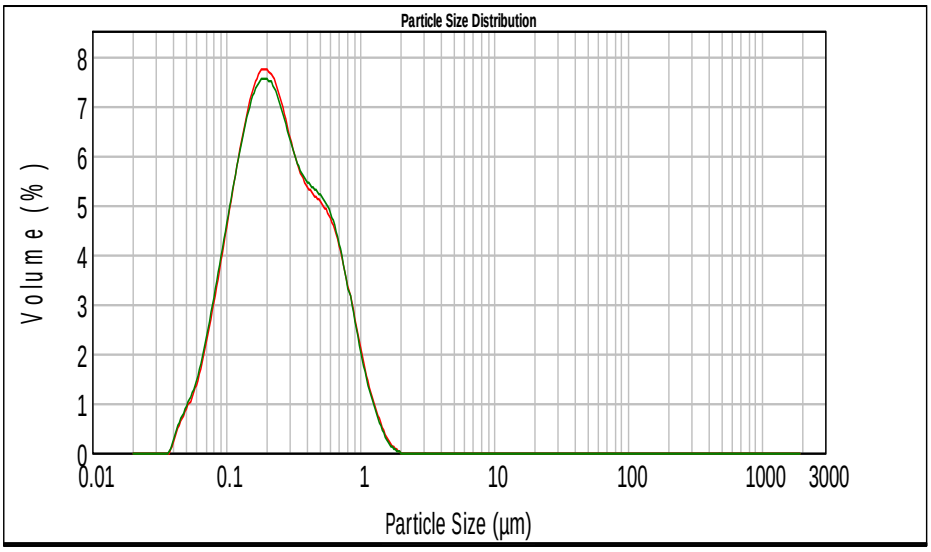


Figure 34. Particle size distribution of 10% canola O/W emulsion on day 15

5.4. Rheological study: Viscosity measurement of canola oil-in-water emulsion

A rheological parameter viz. viscosity was studied by measuring the shear stress against shear rate. Shear flow was measured periodically over the period of 15 days to determine if there were any significant changes in viscosity. In all the measurements over 15 days, shear rate was linearly related to shear stress. Thus, 10% canola oil-in-water emulsion behaved as a Newtonian fluid. Viscosity was measured from the slope of the shear-flow curve. From the computed values of viscosities (7.0 – 8.55 mPa.s), it was found that viscosity is not significantly affected by oxidation. This may be due to the physical stability achieved by the canola-oil-in water emulsion. Rheological parameters like viscosity give a fundamental insight of components within the emulsion. Data from the particle size study also showed that that over this period of time, the emulsion was stable and there was no coalescence of oil droplets. Therefore, the stability of the emulsion is further confirmed by the rheological study.

Rheology plays a key role in dictating the processing parameters of food emulsion. Understanding rheology, therefore, is critical in designing food emulsions. For example, viscosity of the aqueous phase dictates the creaming of the oil droplets in O/W emulsion (McKenna and Lyng, 2003) which in turn determines the shelf life of a food emulsion. Information on the rheology of food emulsions is also important for designing process parameters like product flow through pipes, stirring and packaging in containers. It is also one of the factors in determing sensory properties e.g. creaminess, spreadability, pourability, and flowability (Jellema et al., 2005; Kiosseoglou and Sherman, 1983).

Table 6. Viscosities canola oil-in-water emulsion during 15 days (Mean±SD, n=2) measured in mPa.S).

Time	Mean±SD (mPa.s)	Coeffecient of variation
Day 1	8.26±0.64	8%
Day 5	7.6±0	NA
Day10	8.55±0.22	2.5%
Day 15	7.0±0.77	10%

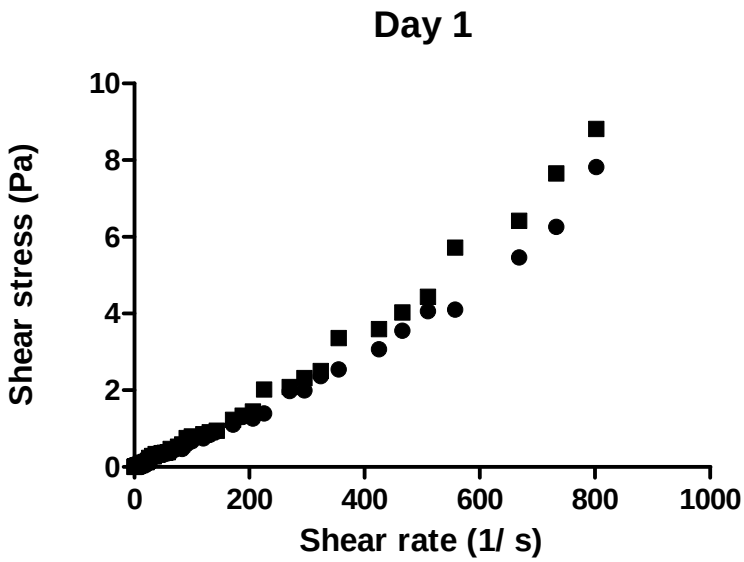


Figure 35. Shear flow curves for 10% canola oil-in-water emulsions on day 1 (n=2).

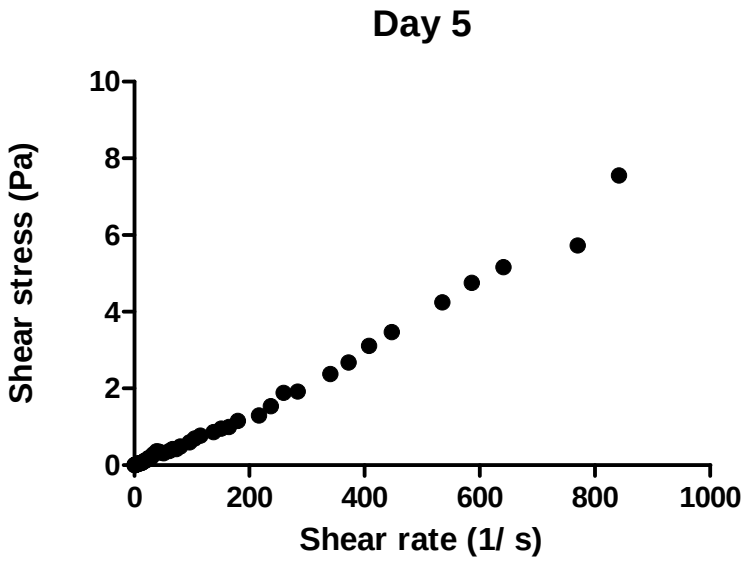


Figure 36. Shear flow curves for 10% canola oil-in-water emulsions on day 5 (n=1).

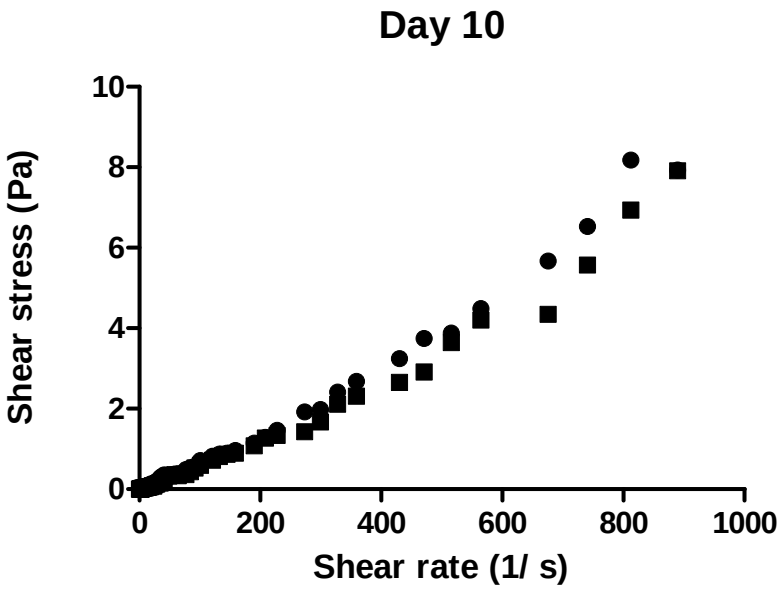


Figure 37. Shear flow curves for 10% canola oil-in-water emulsions on day 10 (n=2).

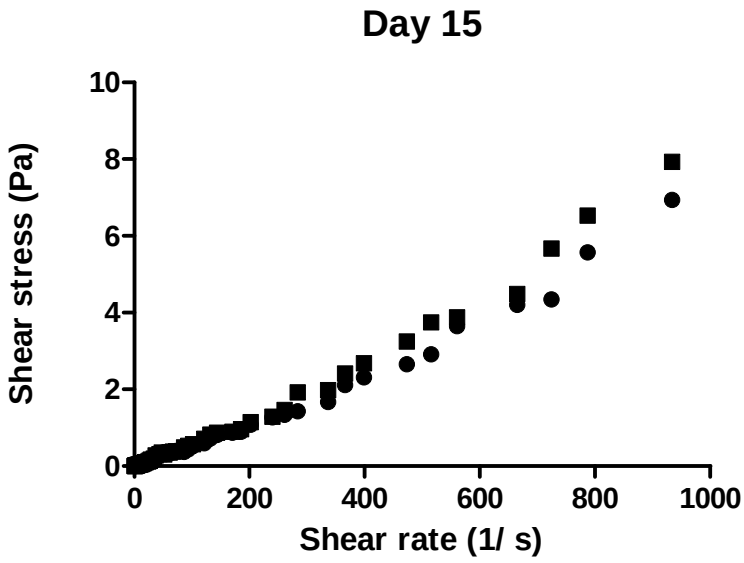


Figure 38. Shear flow curves for 10% canola oil-in-water emulsions on day 15 (n=2).

6. Conclusion

Anti-oxidants assays gave a useful clue to extrapolate their lipid oxidation controlling capacity in real system either in bulk oil or in emulsion systems which can either be oil-in-water or water-in-oil. Our studies showed that sinapine and whole canola meal extract had very strong reducing power and radical scavenging capacity and were also able to control lipid oxidation in an oil-in-water emulsion at higher concentration as shown by their ability to inhibit formation of primary (hydroperoxides) and secondary oxidation markers (hexanal). It is very likely that the antioxidative property of whole extract might be due to the presence of sinapine. Comparable results of sinapic acid standard and sinapic acid extract could be expected given the same component present. Both showed comparatively weak antioxidative capacity in all three antioxidative assays and also they didn't strongly inhibit lipid oxidation in the emulsion.

BHT, a common synthetic antioxidant in lipid containing food, in the experiment showed a good result in controlling lipid oxidation in the oil-in-water emulsion but concentration played an important role. Only at 350 µM, BHT could inhibit oxidation significantly. However, interestingly, BHT showed a very weak antioxidative capacity in *in-vitro* antioxidant assays. Earlier studies also showed BHA and BHT are more effective in emulsions rather than in bulk oil. To understand if a 'polar paradox' comes into play, we need a closer look in future studies. Yanishlieva-Maslarova (2001) stated that besides chemical structure, other factors like 'concentration, temperature, type of oxidation substrate, and physical state of the system

media, as well as the presence of antagonists and synergists' can also play an important role in determining the effectiveness of antioxidants.

From these studies, we also found that canolol and sinapic acid were not effective inhibitors of lipid oxidation in an O/W emulsion system.

These studies further proved that canola meal is a good source of powerful phenolic antioxidants and may lead to commercial exploitation for canola by-products like canola meal.

Though sinapine and whole extract were found to be effective in controlling formation of primary and secondary oxidation products, it would be too early to presume they can be used as alternatives to other standard antioxidants like BHT. Phenolic compounds often have astringent flavour and may render unfavourable taste. So, sensory analysis would be recommended besides assessment of antioxidative properties.

Given a fairly consistent droplet size, over the experimental period, it could be presumed that oxidation kinetics was not significantly changed. Moreover, no significant change in viscosity indicated that there was no structural change.

The study supported our hypothesis that addition of canola derived phenolic antioxidants can inhibit lipid oxidation in oil-in water-oxidation.

Appendix

Table 1. Reducing power

	Concentration (μM)	
	100 μM	350 μM
Antioxidant	Absorbance (Mean±SD)	Absorbance (Mean±SD)
SP	0.158967±0.006335	0.471404±0.007783
SAE	0.086567±0.006117	0.185567±0.0072
ASC	0.0813±0.001249	0.182767±0.001656
CAN	0.065367±0.001343	0.137733±0.005559
SA	0.0979±0.002066	0.239633±0.00394
BHT	0.083433±0.001137	0.1927±0.002621
WE	0.104167±0.005962	0.283333±0.00824

Table 2. Radical Scavenging

	Concentration (μM)	
	100 μM	350 μM
Antioxidant	Inhibition % Mean±SD	Inhibition % Mean±SD
SP	89.17072±0.741474	95.67381±0.098632
SAE	48.15185±0.069279	87.05945±0.182555
CAN	28.5543±1.602853	60.90202±1.486443
SA	75.76735±0.219306	95.39173±0.038855
BHT	3.212404±0.209191	6.462457±0.140558
WE	67.64302±0.07131	91.4523±0.6469

Table 3. Iron Chelating activity

	Concentration (μM)	
	100 μM	350 μM
Antioxidant	Chelating activity % Mean±SD	Chelating activity % Mean±SD
SP	12.53344±0.289309	59.79135±1.809528
SAE	2.663668±0.171097	10.68721±0.269448
CAN	2.177685±0.195439	4.212387±0.125476
SA	2.696675±0.122855	3.482544±0.093915
BHT	3.916213±0.236641	4.384905±0.086661
WE	19.14687±0.853917	98.23343±0.051941
EDTA	97.67975±0.041252	99.8844±0.009007

Table 4. Data of peroxide values of Experiment I

Time	Treatments				
	SA100	SA350	CAN100	CAN350	Control
0	303.6±28.2	295.5±31.1	283.4±13.4	232.9±5.3	320.4±19.3
12	639.3±0.9	590.6±14.8	697.9±35.3	438.0±40	467.4±20
24	513.9±5.0	465.1±18.1	575.6±42.0	366.4±28.2	532.8±26.0
48	923.4±29.0	723.5±26.3	971.0±14.1	726.9±70.1	1090.3±184.7
72	740.7±59.5	775.7±3.5	974.7±8.7	835.5±12.1	1203.9±62.3
96	1592.8±78.9	1320±25.0	1596.4±9.2	1254.6±98.5	1908.5±59
120	2373.6±170.5	2000.7±90.5	2290.4±69.6	2024±125.8	2430.3±99.0
144	2664.6±62.8	2300.3±209.1	2810.±53.1	2570.7±89.3	2785.8±3.2
168	2900.7±85.2	2484.3±118.9	2715.7±138.3	2568.5±140.1	2946.6±82.7
192	3535±154.7	3073.5±204.3	3392.8±172.4	2921.4±149.0	3579.0±63.6
216	3217.2±39.9	3053.5±43.4	3205.7±221.7	3043.9±186.9	3328.5±30.5
240	4519.6±213	4000.7±273.9	3786.8±77.7	3918.2±252.1	4479.1±91.2

Table 5. Data of peroxide values of Experiment II

Time (h)	Treatments				
	SP100	SP350	BHT100	BHT350	Control
0	272.1±7.3	226.1±3.3	216.9±5.2	213.7±3.2	324.2±3.9
24	369.8±6.9	264±3.2	210.1±3.7	188.3±1.9	469.6±10.4
48	498.0±19.5	347.8±13.2	224.7±4.9	204.9±17.8	758.5±75.0
72	762.4±42.2	466.3±72.6	602.2±11.5	199.0±24.2	982.0±81.8
96	848.1±7.0	469.7±30.0	870.8±12.2	197.4±14.8	1038.9±78.6
120	1029.0±79.5	356.5±48.9	1080.1±63.7	195.4±20.9	1131.9±44.8
144	1218.6±171.0	180.3±35.4	1366.1±119.9	100.2±8.3	1253.2±49.7
168	1332.1±25.0	189.6±30.6	1592.8±132.4	114.3±11.5	1415.3±70.2
192	1595.357±47.9	320.7143±40.7	1828.6±200.9	112.6±13.9	1586.1±47.9
216	1629.3±43.5	112.1±18.4	1914.6±60.6	82.6±12.4	1609±36.2

Table 6. Data of peroxide values of Experiment II

Time	Treatments				
	WE100	WE350	SA(E)100	SA(E)350	Control
0	336.4±10.8	211.9±14.4	393.8±19.3	427.9±41.1	622.6±27.2
6	336.5±24.7	206.6±13.9	484.7±61.7	499.4±22.1	621.4±36.7
12	605.8±20.5	302.9±38.7	954.8±77.2	714.7±62.1	1022.2±107.8
24	821.4±80.2	338.8±32.3	1052.5±102.2	1191±195.5	1386.7±128.3
27	978.1667±76.4	439.7619±44.4	1198.8±121.5	1133.1±109.8	1330.2±179.6
33	1382.1±146.5	526.5±86.3	1878.2±198.3	1649.3±204.9	1910.3±223.1
42	1678.9±155.9	570.8±135.7	2012.1±195.6	1600.3±251.4	1833.5±215.0
48	1860.3±118.6	638.2±91.2	2427.1±58.1	1961.8±179	1949.643±757.8
72	2921.8±191.8	745.3±44.6	4226.8±523.9	3746.1±379.9	4828.9±664.3
96	1676.4±218.3	465.9±12	4309.3±432.3	3799.3±339.6	4743.3±528.3
120	1821.8±185.8	191.4±37.4	5756.4±821.9	5465.7±387	6047.1±449.6
144	1686.429±251.9	212.8571±10.1	7362.143±751	5880.357±786.9	6443.929±539.5

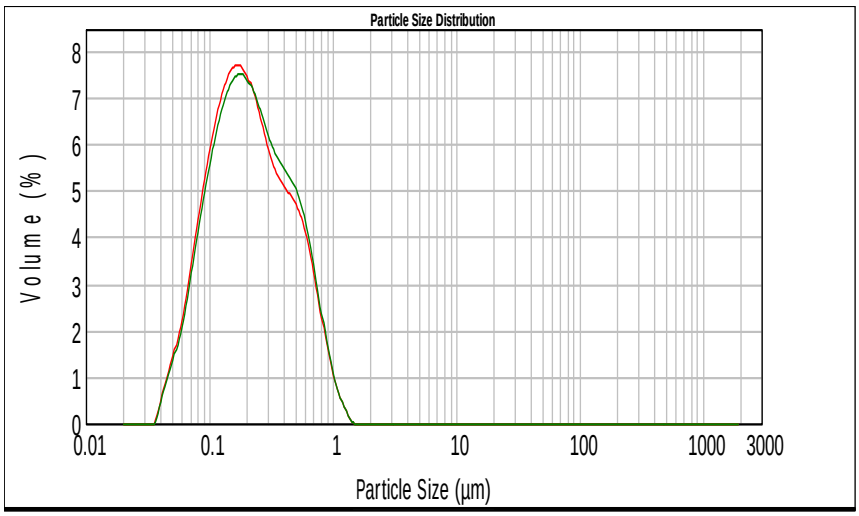


Fig. 1. Particle size distribution of 10% canola O/W emulsion added with 350 µmol WE on day 1 (Mean d_{32} = 0.1625± .003 µm; n=2)

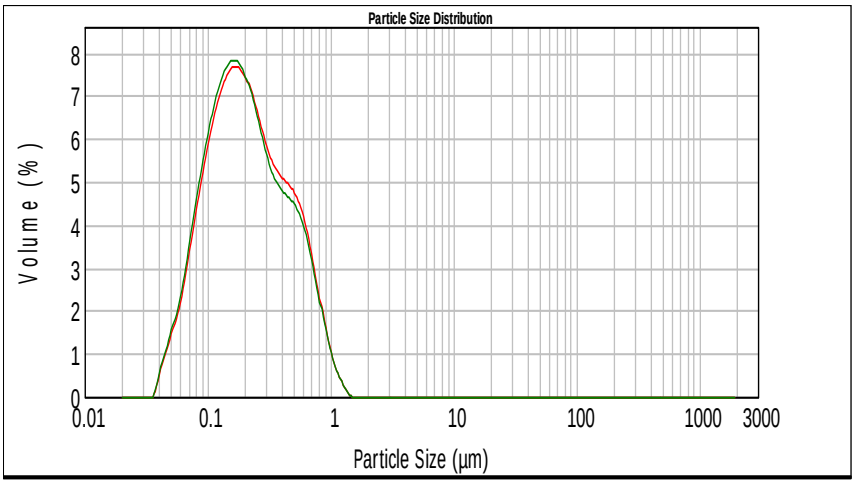


Fig. 1. Particle size distribution of 10% canola O/W emulsion added with 350 µmol WE on day 15 (Mean d_{32} = 0.163± .0028 µm; n=2)

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