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Inhibiting thermo-oxidative degradation of oils during frying

Department of Chemistry and Biochemistry

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INHIBITING THERMO-OXIDATIVE DEGRADATION OF OILS DURING FRYING

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B.Sc., M.Sc., University of Ibadan

A Thesis
Submitted to the School of Graduate Studies
of the University of Lethbridge
in Partial Fulfillment of the
Requirements for the Degree

DOCTOR OF PHILOSOPHY

Department of Chemistry and Biochemistry
University of Lethbridge
LETHBRIDGE, ALBERTA, CANADA

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To those who, in any capacity, are giving their best towards a global community devoid of racial, gender, ethnic, and any other form of discrimination.
ABSTRACT

The present study sought for practical ways to improve the frying performance of oils without compromising the availability of the essential fatty acids and nutraceuticals. To this end, the influence of temperature, oxygen concentrations, and compositions of minor components on frying performance was investigated.

A novel frying protocol, utilizing carbon dioxide blanketing, was developed and found to significantly improve the performance of the frying oil. Optimizing both the amounts and the compositions of endogenous minor components also improved the performance of the frying oil.

Twenty one novel antioxidants were synthesized and evaluated under frying and storage conditions. Antioxidant formulations consisting of a combination of endogenous and synthesized antioxidants were developed and tested in an institutional frying operation.

A rapid and effective frying test was developed to assess the frying performance of oils and applied antioxidants. Furthermore, a novel procedure for direct hydroxynonenal analysis in frying oil was developed.
I wish to express my profound gratitude to my supervisor, Dr. Roman Przybylski, for his invaluable guidance, patience and suggestions throughout the course of this study. I appreciate having had the opportunity to share in his wealth of experience in various aspects of lipid chemistry.

I would like to thank the other members of my committee, Drs. James Thomas, Peter Dibble, Paul Hazendonk, and Olga Kovalchuk for their interest, investment of time, and advice during the course of this work.

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Finally, I give my immense thanks to the God of heaven, the Maker of all things for His unfailing love, for unusual favour, and for stabilizing my home throughout the course of this study.
LIST OF ORIGINAL PUBLICATIONS AND MANUSCRIPTS

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<tr>
<td>AAPH</td>
<td>2,2'‐Azobis (2‐amidinopropane) dihydrochloride</td>
</tr>
<tr>
<td>APCI</td>
<td>atmospheric pressure chemical ionization</td>
</tr>
<tr>
<td>AV</td>
<td>para anisidine value</td>
</tr>
<tr>
<td>BHA</td>
<td>butylated hydroxyanisole</td>
</tr>
<tr>
<td>BHT</td>
<td>butylated hydroxytoluene</td>
</tr>
<tr>
<td>BOP</td>
<td>benzotriazole-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate</td>
</tr>
<tr>
<td>BSTFA</td>
<td>N,O-bis(trimethylsilyl)trifluoroacetamide</td>
</tr>
<tr>
<td>CA</td>
<td>caffeic acid</td>
</tr>
<tr>
<td>CDB</td>
<td>carbon dioxide blanketing</td>
</tr>
<tr>
<td>CO</td>
<td>canola oil</td>
</tr>
<tr>
<td>CSTEE</td>
<td>Scientific Committee on Toxicity Ecotoxicity and Environment</td>
</tr>
<tr>
<td>δ</td>
<td>chemical shift</td>
</tr>
<tr>
<td>d</td>
<td>doublet</td>
</tr>
<tr>
<td>DCC</td>
<td>N,N'-dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DHC</td>
<td>dynamic headspace analysis</td>
</tr>
<tr>
<td>DMAP</td>
<td>dimethylaminopyridine</td>
</tr>
<tr>
<td>DNPH</td>
<td>2,4-dinitrophenylhydrazine</td>
</tr>
<tr>
<td>DP</td>
<td>declustering potential</td>
</tr>
<tr>
<td>DPPH</td>
<td>2,2-diphenyl-1-picrylhydrazyl</td>
</tr>
<tr>
<td>DSC</td>
<td>differential scanning calorimetry</td>
</tr>
<tr>
<td>EF</td>
<td>ethyl ferulate</td>
</tr>
<tr>
<td>EI</td>
<td>electron ionization</td>
</tr>
<tr>
<td>ELSD</td>
<td>evaporative light scattering detector</td>
</tr>
<tr>
<td>FA</td>
<td>ferulic acid</td>
</tr>
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<td>FAME</td>
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<td>free fatty acids</td>
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<td>FP</td>
<td>focus potential</td>
</tr>
<tr>
<td>FTIR</td>
<td>fourier transformed infrared spectroscopy</td>
</tr>
<tr>
<td>FTNIR</td>
<td>fourier transformed near-infrared spectroscopy</td>
</tr>
<tr>
<td>GA</td>
<td>gallic acid</td>
</tr>
<tr>
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</tr>
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<td>HCA</td>
<td>dihydrocaffeic acid</td>
</tr>
<tr>
<td>HNE</td>
<td>4-hydroxynonenal</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HPSEC</td>
<td>high performance size exclusion chromatography</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>50% inhibition concentration</td>
</tr>
<tr>
<td>μg/g</td>
<td>microgram per gram</td>
</tr>
<tr>
<td>MHE</td>
<td>multiple headspace extraction</td>
</tr>
<tr>
<td>NMR</td>
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</tr>
<tr>
<td>ORAC</td>
<td>oxygen radical absorbance capacity</td>
</tr>
<tr>
<td>OSI</td>
<td>oxidative stability index</td>
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<tr>
<td>PDA</td>
<td>photodiode array</td>
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NUMBERING SCHEME FOR SYNTHESIZED ANTIOXIDANTS

1a

1b

1c

1d

1e

2a

2b

2c

3
Chapter 1 – Introduction

Fried foods, whether deep-fried, pan-fried or stir-fried are one of the most popular culinary delights enjoyed by consumers throughout the world (Gupta, 2004). Institutionally, frying oils are commonly reused for several frying cycles before being discarded, unlike in the home frying where fats are usually used a few times. Such prolonged frying results in diminishing acceptability and nutritive value of the fried product owing it to the oxidative, hydrolytic and thermal degradation in the oil.

At frying temperature, a large number of volatile and nonvolatile compounds is formed. Not only do these compounds adversely affect the stability of the frying oil, but the food fried in deteriorated oils also acquires decomposition products that may have adverse effects on food safety, flavour and stability. For instance, 4-hydroxy-2-nonenal (HNE), the oxidation product formed mainly from the n-6 fatty acid, linoleic acid, has been established to possess cytotoxic and mutagenic activities (Esterbauer, 1982; Esterbauer et al., 1991). Acrylamide, also a frying by-product formed by the reactions between sugars and specific amino acids, has been classified by the International Agency for Research on Cancer as “carcinogenic to humans and animals”. Acrylamide has also been considered as “neurotoxic to humans” (Granda and Moreira, 2005; Pedrischi et al., 2006). Furthermore, the economic loss resulting from degradation of frying oil cannot be ignored.

The various factors affecting frying stability and performance of oil can be broadly categorized into two groups: (1) The external factors which include; frying temperature, frying time, presence of oxygen, and the type of fryer, and these factors can easily be manipulated by a frying operator. (2) The internal or endogenous factors are oil-
specific and include fatty acid composition and their distribution on triacylglycerols, and the amounts and composition of the minor components. The external factors are as important as the internal factors in the optimization of frying performance of oils.

The influence of fatty acid and triacylglycerol composition on frying stability has been variously reported, and the search for more stable oil has led to several modifications of the fatty acid compositions (Fuller et al., 1966; Eskin et al., 1989; Wilson et al., 1989; Neff et al., 1992; Neff et al., 1993; Warner and Mounts, 1993; Neff et al., 1994; Neff and El-Agaimy, 1996; Martin et al., 2010). Warner and Mounts (1993) evaluated the frying stability of soybean and canola oils with fatty acid compositions modified by breeding and/or hydrogenation. They reported that modified oils with reduced linolenic acid contents ranging from 3.7 to 0.4% had less room odour intensity; lower free fatty acid, polar compounds contents, and foam heights; lower intensity of off-odours; and they produced better quality fried food than the corresponding unmodified oils containing linolenic acid at contents ranging from 6.2 to 10.1%. According to Neff and El-Agaimy (1996), triacylglycerols containing linoleic acid located on glycerol position 2 (e.g., sn-1,3-dipalmitoyl-sn-2-linoleoyl, PLP) had lower oxidative stability than triacylglycerols containing linoleic acid located on glycerol position 1 or 3 (e.g. sn-1,2(2,3)-dipalmitoyl-sn-1(3)-monolinoleoyl, PPL) as measured by the amounts of hydroperoxide and conjugated diene formed during heating at 60°C in the dark. In a related study, Hoshina et al. (2004) reported that PPL and the mixture of PPP/PLL (1:1, mol/mol) exhibited higher thermo-oxidative stability than PPP/LLL (2:1, mol/mol) as measured by the amounts of total polar compounds, polymeric compounds, carbonyl compounds, and free fatty acids formed during heating at 150 and 180°C for 12 h.
Similarly, PPO was more stable than the mixture of PPP/OOO (2.1, mol/mol) (Hoshina et al., 2004).

However, the relative frying stability of an oil cannot always be accurately predicted based only on the composition of fatty acids (Normand et al., 2001), and a body of research attested to the significant roles of minor components on the frying stability of oils (Lampi et al., 1997; Abdalla, 1999; Normand et al., 2001; Abuzaytoun and Shahidi, 2006; Shahidi et al., 2006; Romero et al., 2007). Normand et al. (2001) compared the frying stability of regular and three modified canola oils. At the end of a 72-h frying operation, regular canola oil with a linolenic acid content at 10.2% showed a significantly higher stability than low linolenic acid canola oil containing 3.0% linolenic acid, as measured by the rate of formation of free fatty acids and total polar components. Furthermore, no significant difference was observed in the frying stability of regular canola oil and a high oleic low linolenic canola oil despite the improved fatty acid composition of the latter. The authors concluded that the frying stability of the oils was affected far more by the rate of tocopherol degradation than by any changes in fatty acid composition.

This thesis reviews the literature related to chemistry of oil components during deep fat frying, the various factors affecting frying stability of oils and the methods used to assess frying performance. The experimental part is a summary of the procedures used and described in published or accepted papers, being an integral part of this thesis and covers studies on: (1) The effect of temperature; (2) a procedure for limiting oxygen availability during frying; (3) synthesis and evaluation of novel antioxidants; and (4) optimization of minor components to improve frying performance of PUFA oils. The
data from the various studies allowed design of a canola oil with significantly improved frying stability.
Chapter 2 – Literature review

2.1 The frying operation

Deep fat frying is defined as the process of cooking foods by immersing them in edible fat or oil at a temperature above the boiling point of water, usually 150 – 200°C (Farkas et al., 1996). It is one of the oldest means of preparing food known to man. For instance, Egyptian wall paintings show dough being fried in oil, indicating that Europe and North Africa were using frying as a method of food preparation well before the new era (Stier, 2004). Although an ancient food preparation technique, frying has grown exponentially over the last fifty years, and the consumption of fried food continues to grow even in the midst of various campaigns against dietary fat consumption. For instance, more than 500,000 institutional and commercial restaurants are involved in deep fat frying in the U.S alone (Brooks, 1991).

Although deep fat frying is a relatively simple food preparation technique, the physical and chemical changes occurring during frying are very complex. Fritsch (1981) outlined the principal events and the mechanisms of the frying process (Figure 1). Basically, heat is transferred from the heat source to the food through the frying oil. Heat transfer from frying oil involves convective heat transfer while conductive heat transfer occurs through the food being fried (Orthoefer and List, 2006). The heat transfer usually instigates extensive mass transfer: Water in the interior of the food is heated and pumped from the food to the surrounding oil where it is transferred into steam; extraction and leaching of components from food to frying oil occurs; frying oil and all the components dissolved in it are also absorbed/adsorbed into the fried food. Ultimately, reactions between the frying oil and the food components are promoted. The leaching of food
components into the oil, the breakdown of the oil compounds, and oxygen absorption at the oil-air interface all contribute to changes in the make-up of the oil from almost pure triacylglycerols (>96%) to a mixture of literally hundreds of compounds (Blumenthal, 1987).

The intimate contact between the food and the oil, competently described by the popular Blumenthal’s Surfactant Theory of Frying (Blumenthal, 1987), makes frying a more efficient process than the dry oven or wet steam method. Absorption/adsorption of oil, surface dehydration of food with the consequent crust formation, development of surface colour, and generation of flavour cumulatively account for the universally desirable taste of fried food (Orthoefer and List, 2006).
Figure 1. Principal events during frying. Adapted from Fritsch, 1981
2.2 Chemistry of frying

Although deep fat frying is a relatively simple way of food preparation, the complex chemical reactions involved are yet to be fully understood. During repeated frying, such as is happening in institutional frying operations, the oil is continuously and repeatedly used at elevated temperature, often topped-up with fresh oil regularly. The high temperature, continuous exposure to oxygen, coupled with the presence of water from the food result in a series of chemical reactions with consequent degradation of the frying oil and food components. More than 500 different chemical compounds have been detected as a result of the complex reactions occurring during frying (Gertz, 2001).

The major chemical reactions occurring during frying can be classified into three groups, namely; hydrolytic, oxidative, and oligomerization reactions (Gutierrez et al., 1988; Warner, 2004). The various chemical compounds arising from these reactions are responsible for the unique flavour, colour, texture, taste, and of course off-flavour of frying oil and food.

2.2.1 Hydrolytic reaction

The main components of edible oils are esters of fatty acids with glycerol. During deep fat frying, water and steam hydrolyze triacylglycerols producing free fatty acids (FFA), diacylglycerol, monoacylglycerol, and eventually glycerol. A typical hydrolytic reaction is depicted in Figure 2. At the high temperature employed during frying, glycerol and free fatty acids will partially evaporate and the reaction equilibrium is shifted in favour of other hydrolysis products (Warner, 2004). Because short and unsaturated fatty acids are more soluble in water than long and saturated fatty acids, they
become more accessible to water from food (Nawar, 1969). Consequently, oils with short and unsaturated fatty acids are more susceptible to hydrolytic reactions. It has been reported that frequent top-up of frying oil with fresh oil during frying slows down the prevalence of the hydrolytic reaction (Romero et al., 1998), while the presence of alkali used for cleaning the fryer increases hydrolysis of oil (Choe and Min, 2007). Being an ionic reaction, hydrolysis can also be accelerated by cations and anions present in both the frying oil and the fried food (Pokorny, 1989).

Products from hydrolytic reactions seem to have no positive contribution to either the stability of the frying oil or the desirability of the fried food. Free fatty acids and their oxidized compounds produce off-flavour in both the oil and food fried in it. They can catalyze further oxidation of oils by solubilisation and activation of metal catalysts (Frankel, 2005). Additionally, as a surface-active substance, FFA lowers the surface tension of the oil thereby increasing oxygen accessibility during frying, promoting oxidative degradation of oil (Choe and Min, 2006; Choe and Min, 2007). The products from hydrolytic reactions are lower in molecular weight but higher in polarity than the original triacylglycerol (Dobarganes and Marquez-Ruiz, 2006). The level of the FFA in the oil is a measure of the degree of hydrolytic reaction.

From a nutritional or physiological point of view, the products from a hydrolytic reaction are of no consequence as they are also produced in the small intestine by pancreatic lipases prior to absorption (Dobarganes and Marquez-Ruiz, 2006).
2.2.2 Oxidative reaction

The elevated temperature used during frying accelerates the reactions between atmospheric oxygen and the frying oil resulting in the formation of various degradation products (Peer and Swoboda, 1982; Houhoula et al, 2003). Thermo-oxidation, like autooxidation proceeds by a free radical mechanism, which can be described in terms of initiation, propagation, and termination processes. These processes often consist of a complex series of reactions (Frankel, 2005)
At the initiation step, the hydrogen atom from the fatty acid is removed and a lipid alkyl radical is produced. The initiation step is usually catalyzed by an energy carrier such as temperature, and light, and by metal catalysis (Artman, 1969; St Angelo, 1996). The energy required to remove hydrogen atoms from fatty acids and initiate radical formation depends on the position of the hydrogen on the molecule. For instance, the energy required to abstract the bis-allylic hydrogen at C11 of linoleic acid is at 50 kcal/mol while 75 kcal/mol is required to remove the hydrogen at C8 or C14 of the same fatty acid. On the other hand, the homolytic dissociation energy between a carbon-hydrogen bond on the saturated carbon such as C17 or C18 of linoleic acid is about 100 kcal/mol (Min and Boff, 2002). Thus, an oil’s propensity for oxidative reaction depends on how easy it is to initiate the formation of free radicals.

In the propagation step, a lipid radical reacts with triplet oxygen to produce peroxy radicals, which in turn abstract hydrogen from another lipid molecule to form hydroperoxide and next lipid radical (Choe and Min, 2006). Thus a chain reaction occurs at this stage when free radicals are continually produced. Oxidation reaction is terminated when free radicals react to form nonradical products. Lipid hydroperoxides are the primary products of the oxidative degradation. However, due to their instability, lipid hydroperoxides rapidly decompose at frying temperature into secondary products, such as aldehydes, ketones, alcohols, esters, lactones, acids, and hydrocarbons (Figure 1). Many of the secondary oxidation products are volatile and evaporate from the oil mainly by steam distillation, while others accumulate in the oil and are absorbed in the fried food. A typical example depicting each of these three steps in frying oil is presented in Figure 3.
Figure 3. Oxidative reaction and representative products
Volatile oxidation products contribute significantly to the flavour of the oil and the fried food. For example, unsaturated aldehydes such as 2,4-decadienal, 2,4-nonadienal, 2,4-octadienal, 2-heptenal, or 2-octenal, contribute to the desirable, characteristic deep fried flavour in oils and fried products (Warner, 2004). On the other hand, many saturated and unsaturated aldehydes are known to produce distinctive off-flavour in the frying oil. For instance, the grass-like off-flavour in oxidized soybean oil has been attributed to the presence of 2t-hexenal while 2t,4c,7t-decatrienal and 1-octen-3-one are responsible for its fish-like off-flavour (Min and Bradley, 1992). A list of common volatile oxidation products and the corresponding characteristic flavour impacted on oxidized frying oil and fried food are presented in Table 1 (Malcolmson et al., 1996).

Hexanal, pentanal, pentane, and 2,4-decadienal have been suggested and used as indicators for the degree of oxidative degradation (Jarvi et al., 1971; Warner et al., 1978; Warner and Frankel, 1985; Choe, 1997; Heinonen et al., 1997; Shiozawa et al., 2007; Toyosaki, et al., 2008; Toyosaki, 2010). In their study of the flavour stability of soybean, cottonseed and peanut oils oxidized for 16 h at 60°C, Warner et al. (1978) reported a significant correlation between hexanal and pentanal contents of oils and the flavour scores reported by a 20-member experienced panel. The authors then suggested the use of hexanal and pentanal contents for flavour stability of the oils. Jarvi et al. (1971) monitored pentane formation as an index of oxidative stability in soybean oil. The soybean was aged at 60°C for 24 h and analyzed by direct injection GC with a packed column, using n-octanol as internal standard. While it was not possible to separate pentane cleanly, the area under the pentane peak was measured, and the ratio of this area
to the height of the n-octanol peak was used to compute the so-called “Oxidative Value” (OV). The researcher found an excellent correlation between OV, peroxide value, and flavour. Warner and Frankel (1985) studied the oxidative and flavour stability of soybean oil by measuring the induction periods based on the time required for rapid formation of volatile compounds during storage at 60°C in a forced-air draft oven. The study showed that measurements of pentane and 2,4-decadienal were best related to deteriorative changes and could be used to predict flavour stability of oils.

Besides affecting the flavour, colour and nutritive value of frying oil and fried food, many oxidation products are known to possess detrimental health effects (LoPachin et al., 2008; Niki, 2009; Gueraud et al., 2010; Singh et al., 2010). Shiozawa et al. (2007) evaluated the cytotoxicity of several volatile oxidation products against two Chinese hamster cell lines (CHL/IU and CHO-KI) and two human cell lines (HeLa and MCF-7). The volatile oxidation products were added to the culture medium at a final ethanol concentration of 1%, and cytotoxicity was evaluated using the Pre-Mix WST-1 cell-proliferation assay system. Although no significant cytotoxic activity was observed for the saturated alcohol and carboxylic acids (pentanol, pentanoic acid, hexanoic acid), and the saturated aldehydes (pentanal, hexanal, octanal, nonanal, decanal), significant cytotoxicity towards all the tested cell lines were reported for the unsaturated aldehydes (2-heptenal, 2-octenal, 2-decenal, 2-undecenal, and 2,4-decadienal). Kimura et al. (2008) reported the cytotoxicity of oil fumes against rat hepatocytes. The oil fumes generated during a model frying by a blend of soybean and canola oils were trapped and the cytotoxicity was evaluated by the MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. The researchers further analyzed the composition of the oil
fumes using TLC and HPLC and ascribed the observed cytotoxicity to the volatile carbonyl compounds, primarily 2,4-decadienal.

Table 1. Characteristics of individual volatiles formed from oxidative degradation of oils. Adapted from Malcolmson et al. (1996)

<table>
<thead>
<tr>
<th>Volatile</th>
<th>Reported odour threshold in oil (mg/kg)</th>
<th>Reported odour descriptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentane</td>
<td>340</td>
<td>-</td>
</tr>
<tr>
<td>Hexane</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Butanal</td>
<td>0.025</td>
<td>-</td>
</tr>
<tr>
<td>Pentanal</td>
<td>0.070</td>
<td>Painty, herbal</td>
</tr>
<tr>
<td>Hexanal</td>
<td>0.120</td>
<td>Fatty, green, fruity, cut grass, herbal, rancid, painty, crushed weeds</td>
</tr>
<tr>
<td>Heptanal</td>
<td>0.055</td>
<td>Weeds, green, sour, sweaty, herbal, painty, rancid</td>
</tr>
<tr>
<td>Octanal</td>
<td>1.50</td>
<td>Lime, grassy, citrus, sharp, heavy, candle-like, crushed weeds</td>
</tr>
<tr>
<td>Nonanal</td>
<td>1.00</td>
<td>Green, soapy, rubbery, beany</td>
</tr>
<tr>
<td>Decanal</td>
<td>-</td>
<td>Fruity, candle-like</td>
</tr>
<tr>
<td>2-pentenal</td>
<td>1.00</td>
<td>-</td>
</tr>
<tr>
<td>3-Hexenal</td>
<td>0.003</td>
<td>Green, apple-like</td>
</tr>
<tr>
<td>2-Heptenal</td>
<td>1.50</td>
<td>-</td>
</tr>
<tr>
<td>2-Nonenal</td>
<td>0.15</td>
<td>Green, fatty, tallow</td>
</tr>
<tr>
<td>2-Decenal</td>
<td>2.10</td>
<td>Metallic</td>
</tr>
<tr>
<td>2,4-Hexadienal</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2,4-Heptadienal</td>
<td>0.04</td>
<td>Fatty, nutty</td>
</tr>
<tr>
<td>2,4-Octadienal</td>
<td>2.40</td>
<td>-</td>
</tr>
<tr>
<td>2,4-Decadienal</td>
<td>0.135</td>
<td>Waxy, fatty, green</td>
</tr>
</tbody>
</table>
Recent epidemiological studies revealed a positive relationship between lung cancer and exposure to cooking fumes (Metayer et al., 2002; Yu et al., 2006; Wang et al., 2009; Lee et al., 2010). In 2006, based on available evidence in both humans and experimental animals, emissions from frying were classified as Group 2A carcinogens (“probably carcinogenic to human”) by a working committee of the International Agency for Research on Cancer (IARC) (Straif et al., 2006). While further studies are required to establish the toxicity and carcinogenicity of volatile oxidation products to humans, the available data indicate that the development of methods to reduce their formation during frying is warranted.

2.2.3 Oligomerization reaction

The initiation and propagation stages of an oxidative reaction can generate several alkyl radicals as previously noted. The alkyl radical, formed during the initiation reaction, the alkyperoxy radicals formed by the addition of oxygen, and the alkoxy radicals formed by the decomposition of hydroperoxides are precursors of oligomers produced at the termination stage (Dobarganes and Marquez-Ruiz, 2006).

At the high temperature employed during frying, the solubility of oxygen decreases dramatically. At this reduced amount of oxygen, the initiation reaction becomes more important where the ratio between alkyl radicals (R’) and alkyperoxy radicals (ROO’) increases. Accordingly, oligomers are formed through reactions involving mainly alkyl and alkoxy radicals (Scott, 1965). Dimers are formed between two fatty acids, either between or within triacylglycerols, and oligomers with high molecular weight are obtained as these molecules continue to cross-link.
Oligomers can be polar or nonpolar depending on whether the monomers are connected by a -C-C-, -C-O-C, or a –C-O-O-C linkages. Formation of polar oligomers during deep frying is a free radical reaction (Figure 4). Nonpolar oligomers on the other hand, can be formed by both a free radical mechanism and Diels-Alder reactions as shown in Figures 4 and 5 (Perkins, 1992; Dobarganes and Marquez-Ruiz, 2006). Brüttting and Spitteller (1994) also proposed a nonradical, cation initiated reaction mechanism for the dimerization of unsaturated fatty acids (Figure 6). A number of dehydrodimers, bicyclic, tricyclic, and Diels-Alder nonpolar dimers has been reported in fried or heated oils (Scharman et al., 1969; Christopoulou and Perkins, 1989). Ottaviani et al. (1979) reported the isolation of acyclic dimers with C-O-C linkages and tetrahydrofuran substituted dimers from soybean oil.

The amount and type of oligomers formed during frying depends mainly on the type of oil, concentration of oxygen, frying temperature and number of frying cycles (Choe and Min, 2006). For instance, oil rich in linoleic acid oligomerises easier during deep frying than oil rich in oleic acid (Takeoka et al., 1997; Tompkins and Perkins, 2000; Bastida and Sanchez-Muniz, 2001). Most of the physical changes observed in the frying oil during prolonged frying are related to the formation and accumulation of oligomers. For instance, increase in the amount of oligomers increases viscosity, darkening, and foaming of frying oil. Since amounts of oligomers steadily increase in the frying oil due to their nonvolatility, they have become reliable indicators of the fat abuse (Paradis and Nawar, 1981).
Figure 4. Formation of polar and nonpolar dimers by free radical mechanism

\[
R^1 - \text{alkyl residues} + R^3 - \text{alkyl residues} \rightarrow R^1\text{-alkyl residue}\text{-alkyl residue}R^3
\]

\[
\text{non-polar dimer (C-C bridge)} \quad \text{Polar dimer (C-O-O-C bridge)}
\]

Figure 5. Formation of nonpolar dimers by Diels-Alder reaction

\[
R^1, R^2, R^3, R^4 = \text{alkyl residues}
\]
Figure 6. Formation of nonpolar dimers by cationic mechanism. Adapted from Brütting and Spitteller (1994)

The nutritional and physiological effects of thermo-oxidized oils have been reviewed by Billek (2000) and Dobarganes and Marquez-Ruiz (2006). In a recent study, Leong et al. (2010) associated the elevated blood pressure and impaired vasorelaxation in experimental rats to the consumption of soybean oil heated to 180°C and up to 10 batches of potatoes intermittently fried in it. Sprague-Dawley rats were fed with commercial rat chow supplemented with the thermo-oxidized oil for 6 months. The researchers observed a significant elevation in blood pressure in all the rats fed with the thermo-oxidized oil compared to rats fed with fresh soybean oil. Similar results were reported earlier using
heated palm oil (Leong et al., 2008; Leong et al., 2009). A study by David et al. (2010) demonstrated that the ingestion of thermally oxidized sunflower oil by rats resulted in a significant increase of intestinal oxidative stress. The observed effect was attributed to the increased amounts of nonvolatile degradation products formed in the oil during frying.

Shuid et al. (2007) indicated that the level of toxicity observed in thermo-oxidized oils is dependent on the inherent stability of the oils. Palm oil and soybean oil were subjected to the same frying conditions. A mixture of the thermo-oxidized oils and rat chow (15:100, w/w) were fed to ovariectomised rats for 6 months and the effects on the bone histomorphometric parameters were assessed. The researchers observed that while no effect was seen in rats fed with thermo-oxidized palm oil, those that consumed the less stable soybean oil showed significantly deteriorated histomorphometric parameters. Indeed, the susceptibility of polyunsaturated oils such as soybean and canola oils to the various thermo-oxidative reactions discussed above explains why they are considered unsatisfactory for extended frying operation (Stevenson et al., 1984). It becomes important therefore that measures be taken to improve the frying performance of such oils.

2.3 Factors affecting frying stability of oil

The frying stability of an oil is a measure of its resistance to several of the degradative reactions occurring during frying. The various factors influencing the stability and performance of a frying oil can be categorized into external and internal factors depending on whether they are oil-dependent or operator-dependent.
2.3.1 External factors

As earlier mentioned, external factors are operator-dependent and are independent of the inherent quality of the frying oil. External factors include: frying temperature; accessibility to oxygen; duration of frying; size, dimension, and composition of food; design of fryer (shape, size relative to production requirements, surface to volume ratio, responsiveness of heating element, and accuracy of temperature controllers); and frying management (debris removal and oil replenishment). Of these various factors, the influence of oxygen accessibility, frying temperature and time are the most significant and warrant a closer look.

2.3.1.1 Oxygen

Lipid oxidation is arguably the single most important factor affecting the life of edible oil. As previously stated, the alkyl radical formed at the initiation step of oxidation reacts very rapidly with molecular oxygen to form peroxy radical. At oxygen pressures greater than 100 mm Hg, such as usually present in fats and oils at room temperature, the rate of oxidation is independent of the oxygen concentration (Frankel, 2005). However, during deep frying, when the oxygen supply is limited due to its poor solubility at high temperature, and a steam blanketing, the rate of oxidation becomes highly oxygen-dependent (Andersson, 1998; Frankel, 2005).

Oxidation of oil increased with the amount of dissolved oxygen in the oil (Min and Wen, 1983). Przybylski and Eskin (1988) reported that the amount of oxygen dissolved in oil is sufficient to provide a peroxide value of 10 meq/kg. The total amount of volatile aldehydes emitted during thermal treatment of high oleic safflower oil was
found to increase with increased oxygen concentration in the oil (Fujisaki et al., 2002). Besides affecting the extent of oxidative degradation, concentration of oxygen also affects the type of degradation products formed during frying. For instance, in an atmosphere containing 2% oxygen, acetaldehyde was the dominant volatile carbonyl compound formed during oxidative degradation of high oleic safflower oil whereas hexanal and nonanal were the most abundant when 20% oxygen was present (Fujisaki et al., 2002). Because individual volatile carbonyl compounds possess characteristic flavour and threshold values, the concentration of oxygen during deep frying will also affect the flavour of fried food.

Despite the poor solubility of oxygen at frying temperatures and steam blanketing, several factors are known to increase the availability of oxygen in the frying medium. Introduction of fresh food and oil into a frying medium increases the level of oxygen in the oil (Warner, 2004). Thermal agitation accompanying boiling during frying of food breaks the oil surface and enhances accessibility of atmospheric oxygen by enlarging the surface area (Mezouari and Eichner, 2006). Furthermore, surface-active compound naturally occurring in oil or formed during frying can also enhance access of atmospheric oxygen during frying. Mistry and Min (1987) reported that free fatty acids decrease the surface tension of oil, thereby increasing the diffusion rate of oxygen into the oil to accelerate oil oxidation. A frying system with a large surface-to-volume ratio also increases the oxygen availability during frying.

Approaches aimed to reduce availability of oxygen during frying have been reported as ways of protecting oil from thermo-oxidative degradation. Przybylski and Eskin (1988) evaluated the efficacy of nitrogen and carbon dioxide flushing to prevent
canola oil oxidation under frying conditions. The oil samples were heated at 195°C in a variety of containers of differing dimensions through which the flow of nitrogen and carbon dioxide were regulated. Peroxide value, thiobarbituric acid value, and formation of volatile compounds were used to assess the level of thermo-oxidation in the oils. It was observed that oils heated without prior nitrogen or carbon dioxide flushing underwent more rapid oxidation compared to oils with prior nitrogen or carbon dioxide flushing. Based on their findings, the authors suggested that to prevent oxidation of oils and fats during thermal treatment, the following should be considered: (1) Flush oil with carbon dioxide rather than nitrogen; (2) the oil should be flushed with nitrogen for 15 minutes or carbon dioxide for 5 minutes prior to heating to eliminate any dissolved oxygen; (3) the linear flow of gas in the container should be at 50 cm/min; and (4) the vessel should not be filled to more than 70% of its height. The superior protection offered by carbon dioxide over nitrogen was attributed to its higher density and greater solubility in oil. Although this study indicated that nitrogen and carbon dioxide could inhibit thermo-oxidative degradation of oils, their effectiveness during actual deep frying still remains to be verified.

Shyu et al. (1998) reduced the content of oxygen by conducting the frying under vacuum. For six consecutive days, carrot slices were fried in palm oil, lard and soybean oil at 105°C for 20 minutes each hour in an 8-hour shift. Peroxide value, acid value, viscosity, fatty acid composition, and total polar components were used to evaluate the extent of thermo-oxidative degradation. The authors concluded that vacuum frying imparted a lower thermo-oxidative degradation on the oils than the typical atmospheric frying. Although, the better stability of the oils during vacuum frying was attributed to
the lower content of oxygen, the lower frying temperature in the study (105°C) makes comparison with typical frying (temperature ~ 180°C) unrealistic. Thus, a study that investigates the effect on thermo-oxidative degradation of vacuum frying under a similar temperature to that used for standard frying is required to better evaluate effectiveness of this method.

2.3.1.2 Temperature

The various reactions threatening oxidative stability of an oil require some level of energy to proceed. For instance, 50 kcal/mol of energy is required to break the carbon-hydrogen bond on the carbon 11 of linoleic acid and to initiate free radical formation (Min and Boff, 2002). The oxygen-oxygen bond of alkyl hydroperoxide requires 44 kcal/mol to break it (Hiatt et al., 1968). This energy requirement is more than fulfilled at the temperature employed during frying. Nawar (1984) reported a peroxide value of 1777 meq/kg when ethyl linolenate was heated at 70°C for 6 h. However, only 13.3% of this value remained when the same oil was heated at 180°C for 5 h. At 250°C, tremendous degradation of hydroperoxide occurred and only 2.5% of the peroxide value was left after 3 h of thermal treatment. Therefore, apart from accelerating the initiation step of oxidative degradation, elevated temperature enhances thermal degradation of alkylhydroperoxides, the primary oxidation product. Consequently, oxidative degradation proceeds more rapidly during deep frying than at room temperature (Fedeli, 1988; Blumenthal, 1991; Tyagi and Vasishtha, 1996).

Increase in frying temperature increases thermal oxidation and oligomerization reactions not only of the fatty acids or triacylglycerol molecules, but also of the
unsaponifiable minor components. Thus, antioxidant minor components in oil are either thermally inactivated during frying or have their levels severely reduced (Allam and Mohamed, 2002; Rêblova, 2006; Rennick and Warner, 2006; Marmesat et al., 2010). While tocopherol in rice bran oil heated to 100°C in the absence of air showed a reduction by 29% at the end of 432 h of heating, its reduction was 100% when the oil was heated at 180°C for 240 h (Bruscatto et al., 2009). Oxidation and polymerization of phytosterols, consequent to increase in temperature has also been studied, and the formation of oligomers arising from thermo-oxidation of phytosterols has been reported (Rudzińska et al., 2009; Rudzińska et al., 2010).

The effect of temperature on isomerisation reactions has also been reported. Wolff (1993) studied the formation of linolenic acid geometrical isomers in flaxseed oils heated under vacuum at different temperatures. After 16 h of heating at 190, 220, and 245°C, the total trans linolenic acid increased from an initial 0.2% in fresh oil to 1.3, 9.5, and 28.8%, respectively. Tyagi and Vasishtha (1996) reported an increase in trans fat from 1.7 to 2.6% in soybean oil when the frying temperature was increased from 170 to 190°C. Moreno et al. (1999) utilized FTIR spectroscopy to monitor the formation of trans isomers during heating of olive oil, sunflower oil, corn oil, and lard over a wide temperature range of 80 – 300°C. Their results showed that the amount of trans isomers consistently increased as a function of temperature irrespective of the type of oil. Fournier et al. (2006) evaluated the effect of deodorization temperature on the isomerisation of polyunsaturated fatty acids in fish oil. They reported that the amount of trans fatty acids increased from 0.2% in fresh oil to 0.3, 4.2, and 7.6% during deodorization at 180, 220, and 250°C. In a recent study, Tsuzuki et al. (2010) compared
the amounts of trans isomers formed when canola oil was heated at different temperatures (160, 180, and 200°C). They reported a statistically significant increase in the isomerisation rate of linolenic acid when the heating temperature was increased from 180 to 200°C. In a similar experiment, 10 batches of potatoes were fried in canola oil every 10 minutes, and the amounts of trans fatty acids formed at the end of the frying period (100 minutes) were analyzed by gas chromatography. It was observed that at all tested temperatures, the amounts of trans isomers were higher in oils used for deep frying than the heated samples (Tsuzuki et al., 2010). Although the levels of trans isomers observed in the fried potatoes might not have any practical implications related to nutritional claims about “zero trans” content in a serving portion, the duration of the frying operation (1 hour, 40 minutes) was too short and can not be representative of a typical institutional frying operation.

2.3.1.3 Duration of frying operation

Although deep frying is a fast method of food preparation and the contact time between food and oil is relatively short, the length of time spent using a frying oil is usually long, especially during continuous and repeated frying such as in industrial and institutional operations. All things being equal, thermo-oxidative degradation increases with increase in length of time for which the oil is used (Xu et al., 2000; Houhoula, 2003; Farhoosh and Tavassoli-Kafrani, 2010; Rani et al., 2010). In a study by Bansal et al. (2009), three different frying oils; palm olein, sunflower oil, and a blend of palm olein, sesame and peanut oils, were used to prepare French fries. The oils were heated for 6 h daily for 4 days and 10 batches of French fries were fried each day without oil
replenishment. The extents of isomerisation of the fatty acids were quantified by gas chromatography and infrared spectroscopy. Irrespective of the type of oil and the quantification method, the authors reported a direct relationship between the extent of isomerization and the number of frying cycles.

According to Rennick and Warner (2006), the content of α-tocopherol in sunflower oil decreased from 829 ppm at 0 h to 183 ppm at 5 h and to 0 ppm by 10 h of heating at frying temperature, indicating that the thermal stability of an oil’s natural antioxidants also depends on the duration of frying.

2.3.2 Internal factors

Unlike the external factors affecting frying stability of an oil, internal factors arise from the inherent composition of the frying oil. Edible oils are composed of triacylglycerols (>96%) and endogenous minor components. It is generally agreed that the inherent composition of edible oils exerts significant influence on their frying stability (Shahidi, 2003; Shahidi and Zhong, 2010).

2.3.2.1 Fatty acid composition and distribution

The influence of fatty acid composition of oils on stability has been variously reported (Warner et al., 1989; Neff et al., 1992; Neff et al., 1993). In general, oils that are more unsaturated oxidize more readily than less unsaturated (Parker et al., 2003). Worded differently, as the number of double bonds in a fatty acid increases, both the rate of formation and the amount of primary oxidation compounds accumulated at the end of the induction period increase (Martin-Polvillo et al., 2004). This observation correlates with
the relative rate of the fatty acid alkyl radical formation (Table 2). On the basis of oxygen uptake, linoleate was 40 times more reactive than oleate; linolenate was 2.4 times more reactive than linoleate, and arachidonate was 2 times more reactive than linolenate (Holman and Elmer, 1947; Min and Bradley, 1992). The oxidizability of linoleic (18:2), linolenic (18:3), arachidonic (20:4) and cervonic (22:6) was linearly correlated to the number of bis-allylic bonds present in the fatty acid ester (Cosgrove et al., 1987).

Table 2. Relative rates of formation of fatty acid alkyl radicals. Adapted from Frankel (2005)

<table>
<thead>
<tr>
<th>Fatty esters</th>
<th>Number of allylic –CH₂–</th>
<th>Moles O₂ per 100 h</th>
<th>Relative rates</th>
<th>Oxidizability M⁻¹/₂ sec¹/₂</th>
<th>Relative rates</th>
</tr>
</thead>
<tbody>
<tr>
<td>18:1</td>
<td>0</td>
<td>0.04</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2</td>
<td>1</td>
<td>1.63</td>
<td>41</td>
<td>0.020</td>
<td>1</td>
</tr>
<tr>
<td>18:3</td>
<td>2</td>
<td>3.90</td>
<td>98</td>
<td>0.041</td>
<td>2.1</td>
</tr>
<tr>
<td>20:4</td>
<td>3</td>
<td>7.78</td>
<td>195</td>
<td>0.058</td>
<td>2.9</td>
</tr>
<tr>
<td>22.6</td>
<td>5</td>
<td></td>
<td></td>
<td>0.402</td>
<td>5.1</td>
</tr>
<tr>
<td>Trilinolein</td>
<td>1 x 3</td>
<td>1.99</td>
<td>50</td>
<td>0.080</td>
<td>4.0</td>
</tr>
</tbody>
</table>

The search for a more stable oil has led to several modifications to the fatty acid composition of edible oils. The newest and the most recent approach has been to modify fatty acid compositions of oilseeds to produce oils with greater frying stability, usually by decreasing the contribution of linoleic and linolenic acids and increasing that of oleic acid (Fuller et al., 1966; Eskin et al., 1989; Wilson et al., 1989). Almost all conventional oils
now have counterparts with modified fatty acid compositions (Table 3). Various high oleic sunflower oils containing from 75% to 90% oleic acid are now on the market. The amount of linolenic acid in conventional canola and soybean oils has been significantly reduced in low linolenic canola and soybean oils. Soybeans containing increased levels of oleic acid with reduced linoleic and linolenic acid levels have been developed or are being developed for commercialization (Wilkes, 2008).
### Table 3. Oils with modified fatty acid compositions.
Adapted from Hazebroek, 2000

<table>
<thead>
<tr>
<th>Oil type</th>
<th>Crops</th>
<th>Developers</th>
</tr>
</thead>
<tbody>
<tr>
<td>High oleate</td>
<td>Sunflower</td>
<td>Dow, DuPont, Instituto de la Grasa</td>
</tr>
<tr>
<td></td>
<td>Canola</td>
<td>Cargill, DuPont, Dow</td>
</tr>
<tr>
<td></td>
<td>Soybean</td>
<td>DuPont, Monsanto</td>
</tr>
<tr>
<td></td>
<td>Corn</td>
<td>DuPont</td>
</tr>
<tr>
<td></td>
<td>Peanut</td>
<td>Mycogen, Univ. FL</td>
</tr>
<tr>
<td>Low linolenate</td>
<td>Canola</td>
<td>Cargill, DuPont, Dow</td>
</tr>
<tr>
<td></td>
<td>Soybean</td>
<td>DuPont</td>
</tr>
<tr>
<td></td>
<td>Linseed</td>
<td>CSIRO</td>
</tr>
<tr>
<td>Low saturate</td>
<td>Canola</td>
<td>Cargill, DuPont</td>
</tr>
<tr>
<td></td>
<td>Soybean</td>
<td>DuPont, NC St. Univ.</td>
</tr>
<tr>
<td>High Palmitate and /or stearate</td>
<td>Sunflower</td>
<td>Instituto de la Grasa</td>
</tr>
<tr>
<td></td>
<td>Canola</td>
<td>Cargill, Monsanto</td>
</tr>
<tr>
<td></td>
<td>Soybean</td>
<td>Monsanto</td>
</tr>
<tr>
<td>High Laurate</td>
<td>Canola</td>
<td>Monsanto</td>
</tr>
<tr>
<td>High medium-chain</td>
<td>Canola</td>
<td>Monsanto</td>
</tr>
<tr>
<td>High erucate</td>
<td>Rapeseed</td>
<td>Various</td>
</tr>
</tbody>
</table>

Notable methods for lowering unsaturation in oils include: fractionation, hydrogenation, interesterification (chemical and enzymatic), conventional seed breeding, and genetic engineering. Several reviews up to 2011 covering some aspects of these
methods are available (Dijkstra, 2006; Holm and Cowan, 2008; Clemente and Cahoon, 2009; Dijkstra, 2009; Jhala et al., 2009; Martin et al., 2010; Hayes and Pronczuk, 2010; Kontkanen et al, 2011). The review by Dijkstra (2006) covered available modifications to the hydrogenation process which were geared towards reducing the formation of trans fat. The oxidative stability of vegetable oils with a modified fatty acid composition by interesterification, technically called structured lipids, compared to conventional oils was reviewed by Martin et al. (2010).

Blending of polyunsaturated oils with a more saturated or monounsaturated oils is also being used as a cost-effective way of reducing the amounts of linoleic and linolenic acids (Gupta et al., 2001; Su and White, 2004; Mariod et al., 2005; Farhoosh et al, 2009; Panghal et al., 2010; Khan et al., 2011). In the recent study by Khan et al. (2011), the contribution of linoleic acid to the total fatty acids was 50% lower in a blend of sesame and coconut oil, compared to the pure sesame oil. Panghal et al. (2010) prepared blends of soybean oil and palm oil lowering linolenic acid to 3.1%, compared to the 9.8% in soybean oil. In the same study, a blend of sunflower and palm oil contained 32.2% linoleic acid, lowered from 73.6% in pure sunflower oil. Although, oxidative and frying stability of vegetable oil blends are reported to be higher than the stability of the oils used in blending (Su and White, 2004; Farhoosh et al., 2009; Alireza et al., 2010; Serjouie et al., 2010), it is preposterous to attribute the increase in stability entirely to changes in fatty acid composition since the endogenous minor components from the constituent oils may also contribute.

Some of the methods currently used to modify fatty acid compositions of oils are also known to negatively affect the nutritional, functional and oxidative stability of the
oils. In a study by Lichtenstein et al. (2006), thirty subjects were fed experimental diets in random order for 35 days. The diets contained the same foods and provided 30% of energy as fat, of which two-thirds was either regular soybean oil or modified soybean oils. Evaluation of cardiovascular disease risk factors showed than LDL-cholesterol level was significantly higher in subjects fed with hydrogenated soybean oil, compared to subjects that consumed regular soybean oil. Furthermore, the ratio of total cholesterol to HDL-cholesterol was higher in subjects fed with hydrogenated soybean oil than those that consumed regular soybean oil. Hamam and Shahidi (2006) reported a significant reduction in tocopherol content during the synthesis of structured lipids by interesterification. Consequently, the modified oils were less stable than their unmodified counterparts despite an increase in the degree of saturation in the products. In a more recent study by Wang et al. (2010), sea blubber and menhaden oils were modified through chemical interesterification using sodium methoxide. The oxidative stability of the oils during accelerated storage at 60°C for 4 days was assessed by measuring conjugated dienes and thiobarbituric acid value. The authors reported a significant reduction in oxidative stability attributable to loss of endogenous minor components during the modification process. Their observations agreed with studies by Turan et al. (2007), Lee et al. (2008a), Maduko et al. (2008), and Jennings and Akoh (2009).

Apart from reducing the availability of essential fatty acids (linoleic and linolenic), the use of genetic engineering for modifying edible oil is still plagued by suspicious attitudes of consumers towards GMO products (Matthäus, 2007). Therefore, research into development of methods to improve the stability of oils without compromising their nutritional and functional qualities becomes imperative.
The high frying stability of some conventional oils such as olive and palm oils, and the modified oils such as high oleic sunflower and soybean oils, high oleic low linolenic soybean and canola oils are often attributed to their fatty acid compositions (Smith et al., 2007; Matthäus, 2007; Ryan et al., 2008). However, as previously mentioned, the frying stability of an oil cannot be accurately predicted based only on composition of the fatty acids (Normand et al., 2001).

2.3.2.2 Minor components

The minor components, also referred to as unsaponifiable matters, are the non-triacylglycerol constituents of an oil and constitute up to 5% of the total lipid composition (Abuzaytoun and Shahidi, 2006). Different classes of compounds belonging to this important group are summarized in Table 4.

<table>
<thead>
<tr>
<th>Class of compounds</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrocarbons</td>
<td>Squalene</td>
</tr>
<tr>
<td>Phytosterols</td>
<td>β-Sitosterol, sigmasterol</td>
</tr>
<tr>
<td>Tocochromanols</td>
<td>α-, β-, γ-, δ-Tocopherol / Tocotrienols</td>
</tr>
<tr>
<td>Ubiquinones</td>
<td>Ubiquinone 9, ubiquinone 10</td>
</tr>
<tr>
<td>Phenolic compounds</td>
<td>Phenolic acids, flavonoids, and isoflavonoids</td>
</tr>
<tr>
<td>Carotenoids</td>
<td>Carotenes, xanthophylls</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>Phosphatidylcholine, phosphatidylethanolamine</td>
</tr>
</tbody>
</table>

Table 4. Classes of minor components and examples.
Adapted from Shahidi, (2003)
The composition of edible oil minor components, especially chromanols, sterols, phenolics and hydrocarbons is of great importance to oil stability both during storage and at frying. For instance, a statistical study showed that the phenolic compounds in olive oil contributed approximately 51% to its storage stability as compared to a 24% contribution arising from its fatty acid composition (Aparicio et al., 1999). Zambiazi and Przybylski (1998) estimated that only about half of the storage stability of an oil can be adequately explained by the fatty acid composition. Lampi et al. (1997) concluded that in canola oil, a concentration of γ-tocopherol as low as 11µg/g was sufficient to reduce hydroperoxides and secondary oxidation product formation by 46 and 39%, respectively. Even though there was no significant difference in their fatty acid profiles, a modified high oleic sunflower oil containing γ-tocopherol as the most abundant natural antioxidant minor component was more thermally stable under frying conditions than a sample containing α-tocopherol as the most abundant antioxidant, suggestive of the importance of the minor components (Marmesat et al., 2008a).

Individual edible oil is naturally endowed with its own unique composition of minor components. For instance, soybean oil is rich in γ- and δ-tocopherol; sunflower oil contains predominantly α-tocopherol; palm oil is rich in tocotrienols and β-carotene; rice bran oil and sesame oil uniquely contain γ-oryzanol (a group of sterol ferulates) and lignans (sesamin and sesamolin), respectively. However, the stability-enhancing capacity of minor components differs one from another. Accordingly, research is being carried out to study the performance of some minor components in oils in which they are naturally absent. For instance, Lee et al. (2007) studied the effects of lignans (sesamol, sesamin, and sesamolin) isolated from roasted sesame seed oil on the oxidative and frying stability
of sunflower oil. In their study, Hemalatha and Ghafoorunissa (2007) evaluated the effect of sesame lignans on the thermal stability of sunflower, soybean and rice bran oils. The effect of \( \gamma \)-oryzanol on the Oxidative Stability at Elevated Temperature (OSET) index of sunflower and canola oils was studied by Gertz et al. (2000). Nystrom et al. (2007) assessed the effect of sitostanyl ferulate (a component of \( \gamma \)-oryzanol) on thermo-oxidative degradation of high oleic sunflower oil heated at 100 and 180\(^\circ\)C. Goulson and Warthesen (1999) evaluated the antioxidant activity of \( \beta \)-carotene in conventional and high oleic canola oil. Attempts are also being made by plant breeders to enhance the amount and composition of some minor components in oilseeds in which they are either naturally absent or are present in negligible amounts (Wang et al., 2007; Warner et al., 2008).

Although endogenous minor components are important to the stability of frying oils, the applied processing steps such as degumming, refining, bleaching and deodorization often result in a significant portion of them being removed from the oils. For example, loss of sterols, tocopherols, carotenoids and related minor compounds during processing may range from 35 to 95\% (Shahidi, 2003). In a recent study, Naz et al. (2011) reported an overall loss of 38\% in tocopherol content during the neutralization, bleaching and deodorization of sunflower oil.

2.4 Antioxidants

As the name implies, antioxidants are compounds possessing the ability to inhibit oxidation when present in food or biological systems. Depending on their structural features, antioxidants can scavenge free radicals, inactivate prooxidant metals, quench singlet oxygen, and inactivate sensitizers (Figure 7). Besides their structural
features, the effectiveness of antioxidants also depends on the concentration used, the applied temperature, exposure to light, and type of substrates (Yanishlieva and Marinova, 2001). For frying applications, radical scavengers and metal inactivators are the most interesting group of antioxidants. A list of some approved antioxidants and their E numbers in the EU is presented in Table 5.

The standard one-electron reduction potentials of major lipid and tocopheryl radicals are presented in Table 6. Any compound with reduction potential lower than that of a free radical can quench the radical by proton donation to the radical, unless the reaction is kinetically unfavourable (Choe and Min, 2006). Radical scavenging antioxidants possess reduction potentials considerably lower than those of the free radicals generated during thermo-oxidation, thus can convert them to more stable nonradical products (Decker, 2002). The major radical scavenging antioxidants are monohydroxy or polyhydroxy phenolic compounds with various aromatic substitutions (Frankel, 2005). The antioxidant radical produced from the reaction with lipid alkyl or peroxy radicals are effectively stabilized by resonance (Shahidi and Wanasundara, 1992) (Figure 8).

Transition metals such as iron and copper are excellent initiators of free radicals; they react directly with lipids to produce alkyl radicals, and also catalyze the decomposition of alkyl hydroperoxides into the alkoxy or peroxy radicals (Benjelloun et al., 1991; Jadhav et al., 1996). Metal inactivators function by sequestering metals ions through chelation or by blocking the formation of complexes between metals and alkyl hydroperoxides, thus preventing hydroperoxide decomposition (Frankel, 2005).
Antioxidants in this group include citric acid, phosphoric acid, ethylenediaminetetraacetic acid (EDTA), and ascorbic acid.

A number of antioxidants occur naturally in food where they offer protection against oxidative damage. However, due to shortcomings in their performance and applicability, natural antioxidants are being modified and synthetic antioxidants are emerging. A number of synthetic (or semi-synthetic) antioxidants have been approved by appropriate authorities in several nations (Torres et al., 2008). For instance, fatty acid esters of vitamin C (ascorbyl palmitate, stearate, oleate and linoleate; E304) have been synthesized to enhance the lipophilicity of vitamin C for stabilization of lipids (LoNostro et al., 2000; Song, 2004).
Table 5. Approved antioxidants most commonly used in food.
Adapted from Torres et al. (2008)

<table>
<thead>
<tr>
<th>Antioxidants</th>
<th>E number*</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Ascorbic acid</td>
<td>E300</td>
</tr>
<tr>
<td>Sodium L-Ascorbate</td>
<td>E301</td>
</tr>
<tr>
<td>Calcium L-Ascorbate</td>
<td>E302</td>
</tr>
<tr>
<td>Potassium L-Ascorbate</td>
<td>E303</td>
</tr>
<tr>
<td>Ascorbyl Palmitate, Ascorbyl Stearate</td>
<td>E304</td>
</tr>
<tr>
<td>Mixed tocopherol concentrate (natural)</td>
<td>E306</td>
</tr>
<tr>
<td>Alpha-tocopherol (synthetic)</td>
<td>E307</td>
</tr>
<tr>
<td>Gamma-tocopherol (synthetic)</td>
<td>E308</td>
</tr>
<tr>
<td>Delta-tocopherol</td>
<td>E309</td>
</tr>
<tr>
<td>Propyl gallate</td>
<td>E310</td>
</tr>
<tr>
<td>Octyl gallate</td>
<td>E311</td>
</tr>
<tr>
<td>Dodecyl gallate</td>
<td>E312</td>
</tr>
<tr>
<td>Erythorbic acid</td>
<td>E315</td>
</tr>
<tr>
<td><em>tert</em>-Butylhydroquinone (TBHQ)</td>
<td>E319</td>
</tr>
<tr>
<td>Butylated hydroxyanisole (BHA)</td>
<td>E320</td>
</tr>
<tr>
<td>Butylated hydroxytoluene (BHT)</td>
<td>E321</td>
</tr>
<tr>
<td>Lecithins</td>
<td>E322</td>
</tr>
<tr>
<td>Citric acid</td>
<td>E330</td>
</tr>
<tr>
<td>L-Tartaric acid</td>
<td>E334</td>
</tr>
</tbody>
</table>

*E numbers are number codes for food additives that have been assessed for use within the European Union
Table 6. Standard one-electron reduction potentials for common free radicals. Adapted from Kim and Min (2008)

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Standard reduction potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘OH (hydroxyl radical)</td>
<td>2310</td>
</tr>
<tr>
<td>RO’ (alkoxy radical)</td>
<td>1600</td>
</tr>
<tr>
<td>ROO’ (peroxy radical)</td>
<td>1000</td>
</tr>
<tr>
<td>R’ (alkyl radical)</td>
<td>600</td>
</tr>
<tr>
<td>α-Tocopheryl’</td>
<td>270</td>
</tr>
<tr>
<td>β-Tocopheryl’</td>
<td>345</td>
</tr>
<tr>
<td>γ-Tocopheryl’</td>
<td>350</td>
</tr>
<tr>
<td>δ-Tocopheryl’</td>
<td>405</td>
</tr>
</tbody>
</table>
Type of Antioxidants:
OS - Oxygen Scavengers;
CB - Chain Reaction Breakers;
S - Synergists;
Q - Quenchers;
CA - Chelating Agents;
RS - Radicals Scavengers/Blockers

LH - Lipid Component;
L· - Lipid Radical;
LOO· - Lipid Peroxy Radical;
LOOH - Lipid Hydroperoxide

Figure 7. Type of antioxidants and respective point of antioxidant activity

Figure 8. Resonance stabilization of an antioxidant radical.
(Shahidi and Wanasundara, 1992)
2.4.1. Natural antioxidants

2.4.1.1 Tocochromanols

A number of naturally occurring minor components in edible oils possess antioxidant activity, and are of great importance to both storage and frying stability of oils. The most important and often studied antioxidants are the tocopherols and tocotrienols, collectively referred to as tocochromanols. The structural difference between tocopherols and tocotrienols is in the unsaturation of the phytol side chain (Figure 9). Depending on the position and the number of a methyl substitution on the chromanol ring, four homologues, α, β, γ, and δ, are recognized for each tocopherol and tocotrienol. Of these, the α- and γ-tocopherols are the most abundant in frying oils (Seppanen et al., 2010).

More often than not, edible oils rely on tocochromanols for protection against oxidative degradation, mainly because of their excellent radical scavenging activity. It has been reported that lipid peroxy radicals react with tocopherols much faster ($10^4$ to $10^9$ M$^{-1}$s$^{-1}$) than with lipids ($10$ to $60$ M$^{-1}$s$^{-1}$) (Choe and Min, 2006). According to Kamal-Eldin and Appelqvist (1996), one tocopherol molecule can protect about $10^3$ to $10^8$ polyunsaturated fatty acid molecules at low peroxide value. A very strong positive correlation was reported between the radical scavenging capacity of different refined oils and the total content of tocochromanols (Rossi, 2007). The oxidative stability of soybean oil (Jung and Min, 1990; Jung et al., 1991), sunflower oil (Fuster et al., 1998), canola oil (Lampi et al., 1999), corn oil (Huang et al., 1995), fish oil (Kulas and Ackman, 2001), egg yolk lipid (Rocha et al., 2010), lard (Parkhurst et al., 1968; King et al., 2009), and
butter oil (Kanno et al., 1970; Lampi and Piironen, 1998; Chawla et al., 2003) were significantly enhanced in the presence of tocopherols.

![Structures of tocochromanols](image)

Figure 9. Structures of tocochromanols

Although there appear to be no controversy regarding the ability of tocopherols to protect oil from oxidative degradation, conflicting reports do appear in the literature on the relative effectiveness of the homologues. The relative antioxidant activity of tocopherol isomers against autoxidation in butter oil at 50°C was reported to be $\gamma > \delta > \beta > \alpha$ when applied at a concentration of 100 µg/g, while $\delta > \gamma > \beta > \alpha$ at 500 µg/g concentration (Kanno et al., 1970). In a study by Fuster et al. (1998), sunflower oil
triacylglycerols containing different concentrations of \( \alpha \)- and \( \gamma \)-tocopherol were autoxidized at 55°C in the dark for 7 days. The effectiveness of the tocopherols to inhibit oxidation was monitored by peroxide value. The authors concluded that \( \alpha \)-tocopherol was more effective than \( \gamma \)-tocopherol at concentrations below 40 µg/g, while \( \gamma \)-tocopherol was a better antioxidant at concentrations above 200 µg/g. Yanishlieva et al. (2002) reported a relative antioxidant effectiveness as \( \alpha \)- > \( \gamma \)-tocopherols at concentrations below 400 and 700 µg/g during oxidation of sunflower and soybean oils, respectively. However, a reversed order, \( \gamma \)- > \( \alpha \)-tocopherol, was observed above these concentrations. In a review of eight studies from the literature, Frankel (2005) reported that the most common order of relative antioxidant activity of tocopherol isomers was \( \gamma \) > \( \delta \) > \( \beta \) > \( \alpha \). Thus, the oxidative stability of oils is affected by the isomeric distribution of the tocopherols.

Apart from the composition of isomers, the applied concentration also exerts remarkable influence on the antioxidant activity of tocopherols. For instance, reduction of tocopherol content in soybean oil from 1500 to 544 µg/g markedly increased oxidative stability at 60°C (Frankel et al., 1959). Similarly, Evans et al. (2002) reported that the antioxidant activity of tocopherol significantly diminished at concentrations above 660 µg/g during accelerated storage of soybean oil at 60°C. Shishkov et al. (1979) studied the effects of tocopherol concentration on the oxidative stability of sunflower oil. They reported concentrations between 350 and 500 µg/g as the optimum levels for maximum stability of the oil. Above or below these levels, the observed antioxidant effect was significantly diminished (Shishkov et al., 1979).

Although much study has been done assessing activity of tocopherols at temperatures <120°C, the results cannot correctly depict their performance under a frying
temperature of 185°C. For instance, in a study by Réblová (2006), the antioxidant activity of α- and δ-tocopherol on the stability of lard was evaluated at temperatures ranging from 80 to 150°C. It was found that while both tocopherol isomers were active between 80 and 110°C, none of them was effective at 150°C. On the contrary, Wagner et al. (2001) reported an increase in the Oxidative Stability Index assessed at 160°C for coconut oil fortified with α, γ, and δ tocopherol compared to coconut oil without added tocopherols. Therefore, it is worthwhile to obtain data on tocopherol antioxidant activity in various oils under thermo-oxidative conditions. While the antioxidant activity of tocopherols under oxidative conditions is readily ascribed to the donation of their phenolic hydrogen to lipid free radicals, their mode of action at high temperatures is not that simple (Steel et al., 2005).

A number of studies has evaluated the ability of tocochromanols to protect oils under frying conditions. According to Nogala-Kalucka et al. (2005), the addition of 100, 500, or 1000 µg/g of δ-tocopherol to Planta, a commercial blend of hydrogenated canola oil and palm oil resulted in a significant decrease in the peroxide value, p-anisidine value, and hexanal formation during heating at 160°C for 2 h in a Rancimat. Supplementing technical triolein with 100 or 400 µg/g of γ-tocopherol significantly inhibited polymer formation during frying of potato chips at 190°C for 6 h (Neff et al., 2003). The effect of α-tocopherol and α-tocotrienol on the performance of antioxidant stripped canola oil at a high temperature was reported by Romero et al. (2007). The pure canola oil triacylglycerols were supplemented with α-tocopherol and α-tocotrienol at various concentrations: 155 and 432 µg/g tocopherol; 138 µg/g tocotrienol; and a mixture of 66 µg/g tocopherol and 72 µg/g of tocotrienol. The samples were heated at 180°C for 18 h in
a Rancimat apparatus, and performance assessed by measurement of the amount of total polar components. The authors reported a significant level of protection by both α-tocopherol and α-tocotrienol, although the later was significantly less effective. They also observed that increasing the level of α-tocopherol from 155 to 432 µg/g did not improve its antioxidative activity. In a similar study, Lampi and Kamal-Eldin (1998) evaluated the antioxidant activity of α- and γ-tocopherol in purified high oleic sunflower triacylglycerols. The oil with or without α- and γ-tocopherol was heated for 24 h at 185°C in an oven. Thermo-oxidative changes in oil were measured by analysis of polymerized materials, and both tocopherols significantly inhibited polymerization of the oil; however, the γ-isomer was more effective. The authors did not observe any synergistic relationship between the two isomers when they were added as a mixture. On the contrary, Warner and Moser (2009) observed that samples of purified mid-oleic sunflower oil triacylglycerols containing a mixture of α, γ, and δ tocopherols accumulated significantly lower amounts of the total polar components compared to effectiveness of individual isomers during frying of tortilla chips. Similarly, Barrera-Arellano et al. (2002) reported a synergistic interaction between α, β, γ, and δ tocopherols during thermal treatment of purified palm olein and soybean oil triacylglycerols at 180°C for 10 h in a Rancimat as assessed by the amounts of polymers formed.

Normand et al. (2001) compared the frying stability of regular and three modified canola oils during a 72-hour frying operation, and performance was assessed by the analysis of free fatty acids and total polar components. The rate of tocopherol degradation was reported as the major factor determining the frying stability of the oils. This was in
agreement with the conclusion by Petukhov et al. (1999) during frying of potato chips in regular, high oleic low linolenic, and hydrogenated canola oils.

Majority of the studies on the antioxidant activity of tocochromanols was conducted by heating the oils to a frying temperature rather than during actual deep fat frying (Lampi and Kamal-Eldin 1998; Steel et al., 2005; Romero et al., 2007). However, the chemical reactions taking place during actual frying of food are different from those during continuous heating (Chang et al., 1978; Fritsch, 1981; Kalogianni et al., 2010). Thus, the activity of tocochromanols can be better assessed during an actual frying or in a frying test that truly mimics actual deep fat frying conditions.

Tocochromanols are thermally unstable, and are known to be easily removed during frying by evaporation/distillation (Marmesat et al., 2010). The disappearance of tocopherols has been attributed to both oxidative and thermal degradation, with the rate being significantly slower in unsaturated oils than in saturated oils (Yuki and Ishikawa, 1976; Yoshida et al., 1991; Jorge et al., 1996; Normand, 2001). A number of tocopherol degradation products notably, α-tocopherolquinone, 4a,5-epoxy-α-tocopheroxyquinone, 5,6-epoxy-α-tocopheroxyquinone, and 7,8-epoxy-α-tocopheroxyquinone have been identified both in model systems and during actual deep fat frying (Murkovic et al., 1997; Verleyen et al., 2001; Rennick and Warner, 2006). However, despite the prevalence of oligomerization during frying, oligomers of tocopherols are rarely encountered (Marmesat et al., 2010), presumably because coupling of tocopheroxyl radicals with lipid peroxy and alkyl radicals predominates over the formation of tocopherol oligomers through self-coupling (Kamal-Eldin and Appelqvist, 1996). The prooxidant effect observed for tocopherols under thermo-oxidative conditions has been attributed to their
oxidation products. Rietjens et al. (2002) suggested that increased levels of oxidized α-tocopherol could result in increased levels of intermediate radicals, which can initiate lipid oxidation. Jung and Min (1992) reported that the oxidative stability of soybean oil at 55°C was significantly reduced in the presence of oxidized α, γ, and δ tocopherols. The observed prooxidant effect was more pronounced with oxidized α-tocopherol. According to Pokorny et al. (1973), tocopherol oxidation products can react with other food components such as protein to generate several other products.

Apart from their antioxidant/prooxidant activity, little is known about biological activities of tocopherol degradation products. Therefore, to ensure high nutritional quality and the safety of fried foods, methods must be developed to discourage tocopherol degradation during deep frying.

2.4.1.2 Phytosterols

Phytosterols are the major constituents of unsaponifiables present in edible oils (Rudzińska, 2009). They are triterpenes, structurally different from cholesterols only in the side chain configuration. The most common phytosterols in edible oils are β-sitosterol, campesterol, stigmasterol, Δ⁵-avenasterol, and brassicasterol (Figure 10). In vegetable oils, phytosterols are the dominant class of minor components and occur primarily as free sterols or steryl fatty acid esters (Piironen et al., 2000).

There is a general agreement in publications that phytosterols offer no protection to oils under storage conditions or low temperature applications (<120°C) (Kochhar, 2001; Cercaci et al., 2007). However, under frying conditions, phytosterols have been reported to inhibit thermo-oxidative alterations of frying oils. Sims et al. (1972)
investigated the ability of some phytosterols to inhibit thermo-oxidation of safflower oil by heating it to 180°C, and the extent of thermo-oxidation was assessed by iodine value. Fucosterol, Δ⁷-avenasterol, and vernosterol offered significant protection to the oil, while ergosterol, β-sitosterol, and sigmasterol were either ineffective or slightly prooxidant. In a similar study, Gordon and Magos (1983) reported that the addition of fucosterol and Δ⁵-avenasterol to technical triolein and heating it to 180°C inhibited thermo-oxidation as measured by iodine value. According to Kochhar and Gertz (2004), a mixture of phytosterols isolated from canola or sunflower oils significantly increased value of the Oxidative Stability at Elevated Temperature (OSET) index of canola oil heated at 170°C, indicating antioxidant activity. In a recent study, Winkler and Warner (2008a) observed an oil dependent activity of phytosterols. A mixture of phytosterols was added to purified soybean and high oleic sunflower oil triacylglycerols. The oils were heated at 180°C for up to 12 h, and formation of polymers was quantified by high performance size exclusion chromatography (HPSEC). The authors reported that the added phytosterols significantly decreased thermal polymerization of soybean oil triacylglycerols; however in high oleic sunflower oil triacylglycerols polymerization was significantly increased. Thus, the phytosterol mixture was effective in unsaturated oil but ineffective in the more saturated oil.

White and Armstrong (1986) compared the antioxidant activity of pure β-sitosterol and a purified oat sterol containing a mixture of Δ⁵-avenasterol and β-sitosterol using soybean oil heated to 180°C. Thermo-oxidative degradations were followed by changes in fatty acid, conjugated diene formation and polymerization. At all tested concentrations, samples containing Δ⁵-avenasterol were more stable than the control -
soybean oil without additives. In contrast, samples containing β-sitosterol were altered at the same rate as the control. Because observed antioxidant activity appeared to be restricted to sterols with ethylidene side chain configuration, such as avenasterol, fucosterol, vernosterol and citrostadienol, the mechanism of phytosterol antioxidant activity has been ascribed to the formation of an allylic free radical at C-29 followed by isomerisation to a relatively stable tertiary free radical at C-24 (Figure 11) (Sims et al., 1972; Gordon and Magos, 1983; White and Armstrong, 1986). However, there is a lack of supporting experimental data, showing presence of phytosterol side chain oxidation products to support this mechanism. Furthermore, since the phytosterol mixtures evaluated by Kochhar and Gertz (2004), and Winkler and Warner (2008a) were mainly composed of β-sitosterol, stigmasterol, and campesterol, phytosterols without ethylidene side chain configurations, a more plausible explanation is required for their observed inhibitory activity.
Figure 10. Common phytosterols

beta-sitosterol

stigmasterol

campesterol

Δ5-avenasterol

brassicasterol

Figure 11. Formation of tertiary free radical at C24 of the sterol containing ethyldiene configuration in the side chain
A further study of the effect of structure on phytosterol antioxidant activity was undertaken by Winkler and Warner (2008b). Endogenous minor components were removed from soybean oil by molecular distillation. Various concentrations of pure phytosterols mixture containing β-sitosterol, sitostanol, stigmasterol, fucosterol, brassicasterol, and ergosterol were added back to the stripped oil, and the samples heated at 180°C for 8 h. As measured by the amounts of polymers formed at the end of the heating period, all phytosterols with two or more double bonds, regardless of the presence of an ethylidene group in the side chain, provided protection against polymerization of the oil. The best protection was offered by ergosterol with three double bonds. The authors concluded that the degree of phytosterol unsaturation was more important for its antioxidant activity than the presence of an ethylidene group.

Like tocopherols, phytosterol can undergo thermo-oxidative degradation under the conditions employed during deep frying, leading to a variety of polar and nonpolar compounds. The formation of phytosterol oxidation products have been studied both in model heating systems and under actual deep frying conditions (Ghavami and Morton, 1984; Dutta and Appelqvist, 1996; Dutta, 1997; Oehri et al., 2001; Soupas et al., 2004; Soupas et al., 2005; Tabee et al., 2008). Dutta et al. (1997) assessed the contents of phytosterol oxides in a hydrogenated canola/palm blend, sunflower, high oleic sunflower oils, and the French fries fried in the various oils. 7α-, and 7β-hydroxysterols, 7-ketosterols, 5α,6α-epoxysterols, and dihydroxysterols were the major phytosterol oxides identified. Soupas et al. (2004) evaluated the effects of the degree of unsaturation of both the phytosterols and the lipid medium on the formation of phytosterol oxides under different temperatures (60 – 180°C). Stigmasterol (unsaturated phytosterol) and sitostanol
(saturated phytosterols) were added as model compounds to tripalmitin and canola triacylglycerols. The authors reported a significant influence of lipid matrices and temperature on the level and reaction pathway of phytosterol oxidation. For instance, after 3 h of heating at 180°C, the stigmasterol oxide contents were 24.2 and 7.4% in tripalmitin and purified canola oil, respectively. However, heating at 100°C yielded 0.3 and 26.5% stigmasterol oxide in palmitin and canola oil, respectively. It was also observed that the level of oxidation products from the unsaturated phytosterol was significantly higher than the level from the saturated phytosterol. In a similar study, Oehri et al. (2001) monitored the formation of phytosterol oxidation products in canola, coconut, peanut, and soybean oils during heating at 100, 150, and 180°C for 20 h. The authors reported that the amounts and varieties of phytosterol oxidation products were significantly higher at 100 and 150°C than at 180°C, presumably due to their participation in oligomerization reactions at frying temperatures.

Oxidized phytosterols have been identified as the precursors for oligomers at frying temperatures (Lampi et al., 2009; Rudzińska et al., 2009; Rudzińska et al., 2010). Rudzińska et al. (2009) heated phytosterol standards at 60, 120, and 180°C for up to 24 h in the presence of pure oxygen, and the level of oligomers monitored by HPSEC. It was observed that at frying temperatures, oligomers were the main products, accounting for 60-74% phytosterol transformation. Several volatile degradation products of phytosterols were identified and quantified in the study. In a recent study, Struijs et al. (2010) reported a broad range of dimers with different polarity during thermo-oxidation of stigmasterol at 180°C for 3 h and the most abundant dimers were found to be linked by carbon-carbon bonds.
Unlike tocopherol oxidation products, phytosterol oxidation products have been shown to exhibit negative biological effects such as cytotoxicity (Adcox et al., 2001; Maguire et al., 2003). Thus, with the growing use of phytosterols as functional ingredients in foods, it is important that measures be taken to inhibit their degradation during high temperature processing such as deep frying. Alpha-tocopherol has been reported to inhibit phytosterol thermo-oxidation (Rudzinska et al., 2004; Tabee et al., 2008). A recent study by Kmiecik et al. (2009) showed that the formation of phytosterol oxidation products in canola oil heated for 4 h at 180°C was significantly inhibited by BHT and ethanolic extracts of rosemary and green tea. However, all of these studies were conducted by heating the oils in a Rancimat or an Oxidograph, where high levels of oxygen are available, and not by actual deep frying of food.

2.4.1.3 Gamma-Oryzanol

Gamma-oryzanol, a mixture of ferulic acid steryl esters is a major antioxidant found in rice bran oil. At least 16 steryl ferulates have been identified (Nakayama et al., 1987; Xu and Godber, 1999; Akihisa et al., 2000; Collins et al., 2002; Fang et al., 2003; Parrado et al., 2003; Gopala-Krishna et al., 2006; Miller and Engel, 2006). Major components in Figure 12 are presented.
The antioxidant activity of γ-oryzanol has been demonstrated by various studies. Xu and Godber (2001) reported that cycloartenyl ferulate, 24-methylene-cycloartenyl ferulate, and campesteryl ferulate, the major components of γ-oryzanol, significantly inhibited hydroperoxide formation in a linoleic acid model. Similar results were reported for linoleic acid in the presence of γ-oryzanol during storage at 40°C for 10 days (Cho et al., 2006). According to Wang et al. (2002), the addition of γ-oryzanol at a concentration of 6 µmol/5g significantly increased the OSI of both soybean FAME and soybean oil at 100°C, and inhibited polymerization in both substrates as measured by viscosity of samples after the OSI end point. Steryl ferulates from rice, wheat and rye bran significantly inhibited hydroperoxide formation in methyl linoleate incubated at 40°C in the dark (Nyström et al., 2005). According to a recent study, the oxidative stability of soybean oil stored at 60°C for 15 days was significantly improved in the presence of a γ-
oryzanol fraction isolated from rice bran, as measured by peroxide value, conjugated diene and p-anisidine value (Devi et al., 2007).

Studies on the activity of γ-oryzanol during frying are scarce, and are conducted by heating the oils rather than assessing during actual deep frying. It was reported that the addition of γ-oryzanol to refined canola and sunflower oils resulted in lower accumulation of dimers and polymers during an OSET test at 170°C (Gertz et al., 2000; Kochhar and Gertz, 2004). Sitostanyl ferulate prevented polymerization in antioxidant stripped high oleic sunflower oil during heating at 180°C for 6 h (Nyström et al., 2007). The capacity of rice bran oil to improve the thermo-oxidative stability of its blends with other frying oils is usually attributed to the γ-oryzanol component (Kamal-Eldin et al., 1998; Chotimarkorn and Silalai, 2008; Farhoosh and Kenari, 2009). However, rice bran oil also contains a number of other minor components like tocopherols and tocotrienols, and their influence cannot be neglected. Furthermore, the resulting changes in fatty acid and triacylglycerols composition arising from such blending will also affect thermo-oxidative stability of the oils. According to Mezouari and Eichner (2007), raising the amount of rice bran oil from 10 to 50% in a sunflower/rice bran oil blend will increase OLO, OLP, PPL, OOO, OPO, oleic and palmitic acids, and decrease LLL, LLO and linoleic acid contents to a level that can significantly influence thermo-oxidative stability. Changes in the triacylglycerol composition have been reported to exert a strong influence on stability of frying oils (Kim et al., 1988; Neff et al., 1992; Neff et al., 1994; Neff and El-Agaimy, 1996). Indeed, according to Kim et al. (1988), the influence of changes in triacylglycerol composition on foaming of oils during frying exceeded that of the fatty acid composition.
There is no agreement in the available literature on the relative antioxidant effectiveness of γ-oryzanol and tocopherols in oils. According to Gertz et al. (2000), polymerization of canola and sunflower oils was better inhibited by γ-oryzanol than α-tocopherol during heating at 170°C for 2 h. During accelerated storage of rice bran oil triacylglycerols at 60°C for 5 days, tocopherol was a better antioxidant than γ-oryzanol as assessed by the peroxide value and the level of conjugated diene (Deepam et al., 2010). Nyström et al. (2007) also reported a superior activity of α-tocopherol over sitostanyl ferulate during heating of high oleic sunflower triacylglycerols at 180°C for 6 h. However, unlike studies regarding antioxidant activity, all available data agreed that γ-oryzanol possessed a higher thermal stability than tocopherols (Mezouari and Eichner, 2007; Nyström et al., 2007; Chotimarkorn and Silalai, 2008; Jennings and Akoh, 2009). Although, the antioxidant activity of steryl ferulates is attributed to the radical scavenging activity of the ferulic acid moiety, their high thermal stability makes them a better antioxidant under frying conditions than ferulic acid (Marinova and Yanishlieva, 1994; Nyström et al., 2007).

2.4.1.4 Lignans

Lignans are compounds with a dibenzylbutane skeleton formed by coupling of two coniferyl alcohol residues that are present in the plant cell wall (Touré and Xueming, 2010). Sesamin, sesamol, sesamolin, sesaminol and sesamolinol (Figure 13) are lignan compounds naturally present in sesame oil, and have been implicated in the oil’s high stability (Yoshida, 1994; Namiki 1995). The ability of sesamol, sesamin and sesamolin to inhibit lipid oxidation in model systems has been reported. Sunflower oil containing
sesamol, sesamin, and sesamolin extracted from roasted sesame seed oil was heated at 180°C for 10 h, and thermo-oxidative degradation assessed by conjugated diene contents, $p$-anisidine value, and fatty acid composition. Samples containing sesame lignans showed significantly higher stability compared to sunflower oil without them (Lee et al., 2007). The effects of sesame lignans on oxidation of methyl linoleate during accelerated storage at 60°C for 18 h, and under frying conditions during heating at 180°C for 1 h were assessed by Lee and Choe (2006) and Lee et al. (2008b). The contents of conjugated dienes and the $p$-anisidine value were significantly lower when lignans were added to oil than samples without lignans. The storage and frying stability of soybean oil also increased after addition of sesamin and sesamolin (Hemalatha and Ghafoorunissa, 2007). Sesamol and sesaminol acted as synergistic compounds with tocopherols during thermal oxidation of oils (Fukuda et al., 1994).

Due to the higher antioxidant activity and thermal stability of sesame lignans, conventional oils such as canola, soybean and sunflower are blended with sesame oil to improve their frying stability (Chung et al., 2004; Chung et al., 2006; Nasirullah and Rangaswamy, 2005; Farhoosh and Kenari, 2009; Alireza et al., 2010; Serjouie et al., 2010). For instance, Farhoosh and Kenari (2009), Alireza et al. (2010), and Serjouie et al. (2010) attributed the improved frying stability of blends of sesame and canola oils over pure canola oil to the activity of sesame oil lignans. Similarly, Chung et al. (2004, 2006) ascribed the superior frying performance of a blend of soybean and sesame oils over unblended soybean oil to the presence of sesame oil lignans.

Secoisolariciresinol diglycoside (SDG) and matairesinol (Figure 13) are the main flaxseed oil lignans (Touré and Xueming, 2010). Literature reports on the
antioxidant activity of flaxseed lignans in oils are rather scarce, and there is no data on their antioxidant activity under frying conditions. However, available data showed that SDG inhibited linoleic acid peroxidation during accelerated storage at 40°C for up to 48 h (Kitts et al., 1999). SDG, and its aglycone, secoisolariciresinol, significantly improved the Oxidative Stability Index of canola oil at 110°C (Hosseinian et al., 2006).

![Antioxidant lignan compounds from sesame and flaxseed oils](image)

**Figure 13.** Antioxidant lignan compounds from sesame and flaxseed oils

### 2.4.1.5 Carotenoids

Carotenoids are a group of naturally occurring tetraterpenoids, consisting of isoprenoid units (Choe and Min, 2006). They are lipid-soluble pigments that contribute to the yellow or deep orange colour of oils. Depending on source and variety, crude palm oil
may contain up to 0.5% carotenoids (Kochhar, 2001). β-Carotene is the most widespread carotenoid present in vegetable oils (Achir et al., 2010). Although the antioxidant activity of carotenoids against photo-oxidation has been recognized, their antioxidant activity during storage without light exposure or at elevated temperature remains controversial (Fakourelis et al., 1987; Lee and Min, 1988; Miller et al., 1996). Yanishlieva et al. (2001) observed a prooxidant effect when β-carotene was added to antioxidant free sunflower oil triacylglycerols during accelerated storage at 100°C. However, in the same study, an antioxidant activity was reported for regular sunflower oil. The observed activity was attributed to a synergistic action between β-carotene and the endogenous α-tocopherol in the sunflower oil. According to Schroeder et al. (2006), the addition of 100 – 1000 µg/g β-carotene to antioxidant depleted palm olein did not extend the induction period in a Rancimat stability test at 120°C. In a recent study, Zeb and Murkovic (2010) evaluated the effects of β-carotene on the oxidation of triacylglycerols. They observed that addition of β-carotene significantly increased the peroxide value of model triacylglycerols during oxidation at 110°C in a Rancimat apparatus. Procida et al. (2009) reported that β-carotene inhibited the formation of some deleterious carbonyl compounds such as pentanal during frying in olive oil. It was also reported that β-carotene protected α-tocopherol and tocotrienols during deep frying using palm olein, thus improving the frying performance of the oil (Schroeder et al., 2006). Edge et al. (1998) observed that a 7, 7’-dihydro-β-carotene derivative of β-carotene, was able to regenerate α-tocopherol from the tocopheroxyl radical.

With regard to antioxidant activity of carotenoids during high temperature processing of lipids, there are two major possible mechanisms proposed for their reaction
with lipid radical species: (1) According to Burton and Ingold (1984), a lipid peroxy radical (ROO') can add at any place across the carotenoid (CAR) polyene chain, resulting in the formation of a resonance stabilized carbon centered radical, ROO-CAR'. Because this reaction interferes with the propagation step of lipid oxidation, it is being used to explain some of the reported antioxidant effect of carotenoids (Palozza and Krinsky, 1992). However, it has been suggested that the carotenoid-peroxy addition radical, ROO-CAR' could react reversibly with molecular oxygen to form a new peroxy radical, ROO-CAR-OO' which is believed to be responsible for some of the reported prooxidant activity of carotenoids at high oxygen concentrations (Krinsky and Yeum, 2003); (2) Depending on the chemical structure of the carotenoids and the reduction potential of the free radicals, carotenoids can donate hydrogen to free radicals, exhibiting antioxidant activity (Woodall et al., 1997; Choe and Min, 2006). Lee et al. (2003) reported that β-carotene with a high reduction potential of 1060 mV had great difficulty donating hydrogen to alkyl and peroxy radicals with reduction potentials of 600 and 1000 mV, respectively (Table 6). However, β-carotene can donate hydrogen to hydroxyl and alkoxy radicals formed from the decomposition of hydroperoxides, because of their relatively higher reduction potentials.

2.4.1.6 Squalene

Squalene is a triterpene hydrocarbon widely distributed in vegetable oils, with olive (10 – 1200 mg/kg) and rice bran oils (100 – 330 mg/kg) containing the highest amounts. The capacity of squalene to protect oils against oxidative degradation has been evaluated by Rao and Achaya (1968), Boskou and Katsikas (1979), Psomiadou and
Tsimidou (1999), Shahidi and Wanasundara (1999), Dessi et al. (2002), Psomiadou and Tsimidou (2002a), and Mateos et al. (2003). Literature reports, however, on squalene antioxidant activity remain controversial. In an early study by Rao and Achaya (1968), addition of 0.02% squalene to methyl oleate and methyl linoleate significantly inhibited hydroperoxide formation during accelerated storage at 63°C for 10 days. At equivalent concentrations, the activity of squalene was reportedly higher than that of mixed tocopherols isolated from sunflower oil within the first 6 days of storage. According to Dessi et al. (2002), the addition of squalene decreased hydroperoxide formation by up to 50% in a model system containing polyunsaturated fatty acids incubated at 37°C for up to 14 h. Psomiadou and Tsimidou (1999) reported a concentration dependent moderate antioxidant activity when purified olive oil triacylglycerols containing squalene were stored at 40°C for 7 days. A lack of antioxidant activity, however, was observed at elevated temperatures of 100 and 120°C using the Rancimat assessment. In a similar study, Mateos et al. (2003) reported a negligible antioxidant effect for squalene during accelerated oxidation of olive oil triacylglycerols in a Rancimat apparatus at 100°C. On the other hand, Shahidi and Wanasundara reported a prooxidant effect of squalene during accelerated storage of purified corn, menhaden, and chicken oils when stored at 65°C for up to 7 days. Oxidative changes were assessed by the levels of conjugated dienes and thiobarbituric acid values.

Although all available data on the antioxidant activity of squalene under frying conditions are obtained by heating the oils and not during actual deep fat frying, it is generally agreed that squalene inhibits thermo-oxidative degradation of frying oils. Addition of squalene at 0.5% significantly reduced the thermo-oxidative degradation of
safflower oil during intermittent heating at 180°C for 7 h per day for 4 days (Sims et al., 1972). Malecka (1991, 1994) reported a significant increase in thermo-oxidative stability of canola oil heated at 170°C for 35 h in the presence of different concentrations of squalene (0.1 – 1.0%). Addition of squalene isolated from shark and olive oils at a concentration of 0.25% also increased the frying stability of sunflower and canola oils as evaluated by an OSET index (Gertz et al., 2000). A synergistic interaction between tocopherols and squalene has been suggested by Kochhar (2001) and Psomiadou and Tsimidou (2002a, 2002b).

2.4.1.7 Phospholipids

Phospholipids such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylycerine (PS), phosphatidylinositol (PI), and phosphatidic acid (PA) are endogenous minor components of oils. The addition of a soybean phospholipid mixture called soy lecithin, at concentrations of 0.25 to 1% significantly increased the oxidative stability of olive oil during a Rancimat test at 120°C (Koprivnjak et al., 2008). The formation of hydroperoxides and propanal was significantly inhibited in the presence of lecithin during storage of fish oil at 20°C for 28 days (Drusch et al., 2008). Ramadan (2008) reported that addition of soy lecithin at a concentration of 0.25% inhibited the formation of hydroperoxides and conjugated dienes in a triolein model system during accelerated storage at 60°C for 15 days. According to Murano et al. (2008), fish fed with diets containing soy lecithin at concentrations of 1 and 2.5% for 4 weeks contained more stable meat than fish raised without a lecithin diet when stored at 4°C under fluorescent lights for 3 days. Utilizing a higher purity soybean phospholipid mixture consisting of
26% PC, 25% PI, 23% PS, and 23% PA, Chu and Hsu (1999) reported a significant increase in the Oxidative Stability Index of peanut oil supplemented with it at concentrations of 0.05 to 0.35%.

The relative antioxidant activity of individual phospholipids such as PC, PE, and PI was assessed during accelerated storage of soybean oil at 60°C for 18 days. While protection by PI was marginal, PE and PC offered significant protection, with PC being the most effective (Hidalgo et al., 2005). Bandarra et al., 1999 reported that addition of 0.5% PC was more efficient than an equivalent amount of PE in preventing oxidation of refined sardine oil during storage at 40°C for 1 month. Similarly, Nwosu et al. (1997) and Boyd et al. (1998) reported a superior antioxidant activity of PC over PE during accelerated oxidation of salmon oil at 100°C in a Rancimat apparatus. On the other hand, Kashima et al. (1991) reported that oxidative stability of perilla oil was significantly enhanced by PS and PE, but not by PC during accelerated storage at 37°C. While both PC and PE protected soybean oil against oxidation during accelerated storage at 60°C, only PE offered protection during accelerated oxidation of olive oil at 110°C (Hidalgo et al., 2005; 2006). Khan and Shahidi (2000) evaluated the effects of PC and PE on the oxidative stability of borage and evening primrose triacylglycerols at 60°C for 168 h. PC was more effective than PE in protecting borage TAG while the reverse was observed for evening primrose TAG. Thus, the relative activity seems to depend on the type of oil and the applied temperature. It has also been reported that the antioxidant activity of phospholipids depends on the presence of trace metals such as copper, iron, and manganese in the oil where potentially they work as chelating agents (Yoon and Min, 1987; Pokorny et al., 1992).
Saito and Ishihara (1997) observed that while choline and ethanolamine inhibited hydroperoxide formation in sardine oil during storage, phosphatidic acid and glycerols showed no antioxidant activity. Thus, they concluded that the activity of PC and PE are attributable to the amine head groups. Pokorny et al. (1992) also reported that the decomposition of hydroperoxide was enhanced when nitrogen containing phospholipids were used as compared to phosphatidic acid. In a recent study, Pan et al. (2010) observed that choline, ethanolamine, and soybean phospholipids decompose linoleic and linolenic acid hydroperoxides to corresponding hydroxides at a higher rate than observed for α-tocopherol and BHT.

Unlike studies describing the antioxidant activity of phospholipids under accelerated storage conditions, rather fragmented information is available on their application under frying conditions, probably because of their adverse effects on colour and foaming of oils (Dobarganes et al., 2000a). The addition of 0.1% soy lecithin remarkably inhibited thermo-oxidative alteration of oils during frying (Chu, 1991; Kourimska et al., 1994; Gordon and Kourimska, 1995b). The thermo-oxidative stability of salmon oil heated at 180°C was significantly improved in the presence of a phospholipid fraction isolated from bluefish (King et al., 1992). The antioxidant effect of egg yolk during frying of flour dough containing different amounts of egg yolk powder was attributed to phospholipids present in this ingredient (Kim and Choe, 2008). According to Kourimska et al. (1994), addition of soy lecithin in an amount not higher than 0.2% had no negative effect on foaming of the oil and the quality of the prepared French fries.
The observed antioxidant activity of phospholipids has been attributed to: (1) their synergistic activity with phenolic antioxidants such as tocopherols (Dziedzic and Hudson, 1984; Weng and Gordon, 1993; Lambelet et al., 1994; Koga and Terao, 1995; Bandarra et al., 1999; Khan and Shahidi, 2000; Judde et al., 2003; Ramadan, 2008); (2) the ability of the phosphate group to chelate prooxidant metals (Pokorny, 1991; Pokorny et al., 1992; Drusch et al., 2008); (3) the formation of non enzymatic browning reaction products between amino phospholipids and sugar or lipid oxidation products (Husain et al., 1984; Hidago et al., 2005; 2006; 2007; Zamora et al., 2011); and (4) the ability of phospholipids to form an oxygen barrier between the oil and air interface (Porter, 1980; Calvo et al., 1994).

2.4.1.8 Polyphenolics

A recent trend in the search for natural antioxidants is the application of extracts and isolates from different plants. The most prominent compounds present in those extracts are polyphenols (Shahidi and Naczk, 1995). The structures of common simple phenolic and polyphenolic compounds are presented in Figure 14. The isolation of polyphenolic compounds, their antioxidant activities and applications in biological and food systems has been extensively reviewed by Moure et al. (2001), Schieber et al. (2001), Yao et al. (2004), Shi et al. (2005), Balasundram et al. (2006), Pandey and Rizvi (2009), Perron and Brumaghim (2009), Raederstorff (2009), Serrano et al. (2009), Valls et al. (2009), Nichols and Kaliyar (2010), and Xia et al. (2010). Polyphenols extracted from sea buckthorn protected soybean oil against thermal and photochemical oxidation (Papuc, 2010). Barley seed methanol extracts stabilized sunflower oil during extended
accelerated storage (Anwar et al., 2010). Addition of olive leaf extract enhanced the oxidative stability of olive, sunflower and palm oils, and the observed enhancement was attributed to the presence of polyphenols added to the oils (Salta et al., 2007). During deep frying of corn oil, a crude extract from tea inhibited thermo-oxidative degradation (Naz et al., 2005). Addition of oregano extract significantly enhanced the frying stability of cottonseed oil (Houhoula et al., 2004). Extracts from rosemary, sage and rosa mosqueta shell, to mention just a few, have been used to enhance the oxidative and frying stability of oils (Jaswir et al., 2000; Man and Jaswir, 2000; Romero et al., 2007). Nevertheless, a major set-back to the applications of polyphenolic compounds is their poor solubility in oils.

![Chemical structures](image)

**Figure 14.** Some phenolic and polyphenolic antioxidants
2.4.2 Synthetic antioxidants

A broader application of natural antioxidants in the protection of oils against thermo-oxidative degradation is hampered by some inherent shortcomings. For instance, the poor thermal stability of tocopherols and carotenoids limits their applications for extended institutional frying operations. The poor solubility of phenolic and polyphenolic compounds and variability in composition, and hence variable activity of the extracts limit their applications in fats and oils (Pokorny, 2007). Consequently, synthetic antioxidants have been developed, and a number of these have been approved for food applications (Table 5).

Synthetic antioxidants are often added to processed oils to retard oxidative degradation during storage and frying (Warner, 2004). The most widely used synthetic antioxidants include: butylated hydroxyanisole (BHA); butylated hydroxytoluene (BHT); propyl gallate (PG); and tert-butylhydroquinone (TBHQ) (Figure 15). Transition metal chelators such as citric acids are also used to counteract the deleterious effects of metals such as iron and copper. Although BHT and BHA are effective antioxidants at ambient and accelerated storage temperatures (Gordon and Kourimska, 1995a; Khan and Shahidi, 2001; Nenadis et al., 2003; Yeo et al., 2010), they offer no protection during frying of food. They are known to evaporate and thus do not remain in the frying oil long enough to provide protection against thermo-oxidative degradation (Peled et al., 1975; Augustin and Berry, 1983; Tsaknis et al., 2002; Warner, 2004). For instance, 75% of the added BHT and 86% of the original BHA were lost after 6 h of intermittent frying of potato chips in palm olein (Augustin and Berry, 1983). TBHQ, on the other hand, has been reported to possess better thermal stability and effectiveness during frying (Gordon and
Kourimska, 1995a). Che Man et al. (1999) found that TBHQ was more efficient than α-tocopherol in inhibiting thermo-oxidative degradation during frying in palm olein. Nevertheless, Asap and Augustin (1986) reported a 95% loss of the initial amount of TBHQ after 5 h of frying in palm olein. The loss of TBHQ was attributed to steam distillation, thermal decomposition, and absorption of TBHQ by the fried food.

Aside from poor protection, especially under frying conditions, use of common synthetic antioxidants has also been limited due to their perceived detrimental effect on human health (Frankel, 2007). Consequently, there is a growing interest in development of new antioxidants with improved antioxidant activity and thermal stability, but prepared from natural precursors. The new trend in this direction is leading to modification of existing natural antioxidants.

Figure 15. Common synthetic antioxidants
In addition to commercially available tocopherol derivatives such as α-tocopheryl acetate, α-tocopheryl succinate and racemic trolox (Figure 16), a number of tocopherol derivatives have been synthesized. These are excellently reviewed by Cerecetto and López (2007). Koufaki et al. (2004) reported the synthesis of a series of chromanol analogues of lipoic acid exhibiting strong inhibition of lipid peroxidation in rat liver microsomal membranes induced by ferrous ions and ascorbate. A novel “twin-chromanol” derivative (Figure 16) was reported to exhibit twice the radical scavenging activity and reducing power of α-tocopherol (Rosenau et al., 2002). The reported synergy between tocopherols and carotenoids prompted the development of synthetic antioxidants incorporating these antioxidant substructures (Larsen et al., 1998; Naalsund et al., 2001; Palozza et al., 2002). FeAOX-6 (Figure 16) is an example of such a group of antioxidants reportedly possessing potent radical scavenging activity (Palozza et al., 2002). Likewise, the reported synergistic activity between ascorbic acid and α-tocopherol prompted the development of a series of novel antioxidants derived from molecular combination of ascorbic acid and tocopherol analogues (Figure 16) (Morisaki and Ozaki, 1996; Manfredini et al., 2000). Although no improvement in inhibitory activities against tyrosinase-catalyzed melanin formation, active oxygen species, and free radicals were observed from these molecular combinations, the ascorbic acid-α-tocopherol hybrid exhibited significantly higher thermal stability than the parent components (Morisaki and Ozaki, 1996). Similarly, Voisin-Chiret et al. (2007) reported the synthesis of a series of thermally stable ascorbic-ferulic acid hybrids.

Available literature on the chemical and enzymatic syntheses and modification of polyphenolic compounds have been recently reviewed by Chebil et al. (2006), Boudet
(2007), Fernandez-Bolanos (2008), Viskupicova et al. (2009), and Augustyniak et al. (2010). Recently, Hamdi et al. (2008) reported the synthesis of a series of dicoumarol and epoxydicoumarin derivatives exhibiting good radical scavenging activity against the ABTS radical. Rajan et al. (2001) described the synthesis of new caffeic acid amides exhibiting good antioxidant activity. Jung et al. (2002) also reported the synthesis of 4-hydroxyphenylacetic acid amide possessing potent analgesic and antioxidant activities. The synthesis of caffeic acid amides possessing antimicrobial activities was recently reported by Fu et al. (2010). The syntheses of several gallic and ferulic acid derivatives with enhanced lipophilicity and radical scavenging activity have also been reported by Belin et al. (2003) and Zheng et al. (2010). In a recent study, Lorentz et al. (2010) reported a lipase catalyzed synthesis of two new 4-O- and 3-O-palmitoyl chlorogenic acids; however, the new chlorogenic acid derivatives exhibited weaker radical scavenging activities against the stable DPPH radical, compared to the original chlorogenic acid.

Although syntheses and performance evaluation of over a hundred (semi)synthetic antioxidants have been reported in literature, only a small fraction have been specifically designed and/or evaluated under frying conditions.
2.5 Assessing performance of oil during frying

Traditionally, the frying operator employs experience to decide when to halt frying and change oil and often it is based on physical changes such as colour, odour, excessive foaming and smoking. Assessing oil degradation using visual indicators, however, is inadequate and often unreliable due to its subjective nature. Thus, simple and objective parameters should be used to assess quality and performance of frying oil; with time, many reliable analytical methods based on quantification of a specific group of degradation products have been developed (Paradis and Nawar, 1981; Melton et al., 1994).
Standard methods based on quantification of primary oxidation products such as peroxide value, free fatty acid content, and conjugated dienes are usually unreliable because products measured are thermally unstable (Fritsch, 1981; Gertz and Matthäus, 2008). Generally, methods based on nonvolatile degradation products are more reliable and dependable indicators of oil degradation than those based on volatile or unstable degradation products (Table 7) (Melton et al., 1994; Gertz and Matthäus, 2008).

2.5.1 Total polar components (TPC)

As previously mentioned, chemical reactions occurring during frying generate several groups of compounds with higher polarity than the original triacylglycerols. Polar compounds formed in oils during frying include oxidized and oligomerized triacylglycerols, free fatty acids, mono- and diacylglycerols, oxidized and oligomerized sterols, and degradation compounds of antioxidants and other constituents of oil and food. These compounds are nonvolatile and their amounts in oil are usually steadily increasing as frying progresses.

The analysis of total polar components (TPC) offers the best indicator of frying oil degradation because it measures directly all the degraded products present in the oil. Thus, many European countries have set a maximum permissible level for TPC in frying oil as a way of regulating the level of abuse in commercial and institutional frying operations (Firestone, 2006). There are differences among the European countries, however, in the discarding level of TPC as follows: Austria at 27%; Belgium, France, and Spain at 25%; while Germany allows not more than 24% of TPC (Firestone, 2006).

According to Gertz (2000), the value of 24 – 25% of TPC should be taken as borderline
in the assessment of the quality of frying oils. Presently, there is no specific regulation in Canada or the US defining maximum level of TPC.

Official methods for assessing TPC involve a gravimetric procedure which utilizes chromatographic separation of polar and nonpolar components on silica gel with adjusted water content to 5% (AOAC 982.27; AOCS Cd 20-91; IUPAC 2.507). TPC represents the components remaining on the column after elution of unaltered triacylglycerols, and is expressed in weight percent of the starting sample. Collaborative tests conducted by IUPAC and AOCS showed that the method is exact and reproducible, with a coefficient of variation lower than 5% (Dobarganes et al., 2000b; Firestone, 2009). For rapid analysis, and to reduce consumption of solvents and silica gel, use of miniaturized columns requiring a small sample size has been proposed (Schulte, 2004; Marmesat et al., 2007). However, the level of accuracy and precision is expected to decrease for such small sample amounts, especially in samples with low levels of TPC. In a recent study, Zainal and Isengard (2010) suggested the use of an automated accelerated solvent extraction to replace the manual chromatographic step for quicker analysis and to reduce experimental effort.

To eliminate the use of chemicals, the determination of TPC by nuclear magnetic spectroscopy (NMR), near infrared spectroscopy (NIR), and differential scanning calorimetry (DSC) has been proposed as a substitute for the adsorption chromatographic method (Sun and Moreira, 1996; Hein et al., 1998; Tan and Che Man, 1999; Gerde et al., 2007). Hein et al. (1998) evaluated total polar components in thermo-oxidized soybean, peanut, and palm oils using a Fourier transformed near-infrared (FTNIR) spectroscopic method. The authors reported correlation coefficients from 0.990 to 0.998 between TPC
determined by adsorption chromatography and the values obtained by NIR spectroscopy at the wavenumber range specific for hydroxyl and carbonyl groups (4700 – 4940 cm\(^{-1}\)). Sun and Moreira (1996) reported a correlation coefficient of 0.985 for TPC determined by adsorption chromatography and NMR proton relaxation time of thermo-oxidized soybean oils. Similarly, TPC obtained by DSC linearly correlated at \( r^2 = 0.956 \) - 0.999 to values obtained using an adsorption chromatographic method for fresh and thermo-oxidized corn oil, palm olein, and soybean oil (Tan and Che Man, 1999). Although instrumental methods offer some advantages, it is unlikely that they will replace the adsorption chromatographic method for routine laboratory analysis of TPC; because besides its simplicity, materials required for adsorption chromatography are affordable and readily available in most laboratories.

As shown in Table 7, several of the observed changes in physical parameters occurring in oils during frying, including increase in conductivity, viscosity, specific heat, and decrease in surface tension and dielectric coefficient are caused by the formation of polar compounds (Gertz and Matthäus, 2008). The direct relationship between these physical changes and the amounts of total polar components are explored in a number of the available commercial quick test kits and equipment used for monitoring thermo-oxidative degradation (Table 8).
Table 7. Changes in physical and chemical parameters during deep frying; main causes and correlation with oil deterioration. Adapted from Gertz and Matthäus (2008)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Changes during deep frying</th>
<th>Mainly caused by</th>
<th>Correlation with oil deterioration</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV</td>
<td>Increases</td>
<td>Conjugated fatty acids</td>
<td>Yes</td>
</tr>
<tr>
<td>Refractive index</td>
<td>Increases</td>
<td>Polar compounds</td>
<td>Yes</td>
</tr>
<tr>
<td>Density</td>
<td>Increases</td>
<td>Polymerized TAG</td>
<td>Yes</td>
</tr>
<tr>
<td>Dielectric coefficient</td>
<td>Decreases</td>
<td>Polar-oxidized components – affected by FFA and water</td>
<td>Yes</td>
</tr>
<tr>
<td>Colour</td>
<td>Becomes more intensive and darker</td>
<td>Maillard reaction products of amino acids, protein, sugar, and carbonyl compounds</td>
<td>Yes</td>
</tr>
<tr>
<td>Conductivity</td>
<td>Increases</td>
<td>Polar compounds</td>
<td>Yes</td>
</tr>
<tr>
<td>Surface tension</td>
<td>Decreases</td>
<td>Polar compounds</td>
<td>Yes</td>
</tr>
<tr>
<td>Smoke point</td>
<td>Decreases</td>
<td>Volatile oxidized products</td>
<td>Yes</td>
</tr>
<tr>
<td>Specific heat</td>
<td>Increases</td>
<td>Polar compounds</td>
<td>Yes</td>
</tr>
<tr>
<td>Viscosity</td>
<td>Increases</td>
<td>Polymerized TAG</td>
<td>Yes</td>
</tr>
<tr>
<td>Anisidine value</td>
<td>Increases</td>
<td>Non-volatile aldehydes</td>
<td>Yes</td>
</tr>
<tr>
<td>Iodine value</td>
<td>Decreases</td>
<td>Formation of oxidized fat</td>
<td>Yes</td>
</tr>
<tr>
<td>Peroxide value</td>
<td>Fluctuates</td>
<td>Hydroperoxides</td>
<td>No</td>
</tr>
<tr>
<td>TPC</td>
<td>Increases</td>
<td>Oxidized and polymerized degradation products</td>
<td>Yes</td>
</tr>
<tr>
<td>Polymerized TAG</td>
<td>Increases</td>
<td>Oxidized and not oxidized polymerized TAG</td>
<td>Yes</td>
</tr>
<tr>
<td>Free fatty acid/Acid value</td>
<td>Fluctuates</td>
<td>Hydrolysis &amp; oxidation products with free carboxyl groups</td>
<td>No</td>
</tr>
<tr>
<td>Petroleum ether-insoluble oxidized fatty acids</td>
<td>Increases</td>
<td>Oxidized polymerization products</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Table 8. Commercial test providing high correlations with TPC. 
Adapted from Bansal et al. (2010a)

<table>
<thead>
<tr>
<th>Test Kit</th>
<th>Principle</th>
<th>Manufacturers Specifications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fri-check®</td>
<td>Viscosity</td>
<td>Measurement time: 5 min.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Operating temperature: 20 –</td>
</tr>
<tr>
<td></td>
<td></td>
<td>180°C</td>
</tr>
<tr>
<td>Capsens5000 (FOS)</td>
<td>Dielectric constant</td>
<td>Accuracy: ±1.4%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Resolution: 1%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Range of TPC: 0 – 35%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Operating temperature: 50°C</td>
</tr>
<tr>
<td>Food Oil Monitor (FOM 310)</td>
<td>Dielectric constant</td>
<td>Accuracy: ±2%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Resolution: 0.5%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Range of TPC: 0 – 40%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Measurement time: &lt;2 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Operating temperature: 50-200°C</td>
</tr>
<tr>
<td>Testo 265</td>
<td>Dielectric constant</td>
<td>Accuracy: ±2%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Resolution: 0.5%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Range of TPC: 0.5 – 40%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Measurement time: &lt;10 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Operating temperature: 40-200°C</td>
</tr>
<tr>
<td>TPM Very Fry</td>
<td>Patented gel reacts with the polar compounds to give specific colour</td>
<td>Range of TPC: 3 – 30%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Measurement time: 10 – 15s</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Operating temperature: 65°C</td>
</tr>
</tbody>
</table>
2.5.2 Composition of polar materials (CPM)

Although percent of TPC is an excellent indicator of oil degradation, it only determines the total level of polar materials without distinguishing specific compounds (Paradis and Nawar, 1981). A measure of the specific group of degradation products is, however, important as it provides information about the types and level of reaction occurring in the frying oil, and its potential toxicity. For example, the amount of diglycerides and FFAs are indicative of the level of hydrolytic alteration which has occurred, and although they contribute to the level of TPC, from a nutritional and physiological point of view, they are not as important as polar components arising from thermo-oxidative and oligomerization reactions (Dobarganes and Marquez-Ruiz, 2006).

High performance size exclusion chromatography (HPSEC) is usually used to separate and quantify molecules by their molecular weight (Christopoulou and Perkins, 1989). HPSEC method can be applied directly to used oil or isolated polar fractions (Dobarganes and Marquez-Ruiz, 2006). Polar materials are separated into several groups of compounds such as oligomers, oxidized monomeric triglycerides, diglycerides and free fatty acids (Dobarganes and Marquez-Ruiz, 2006). HPSEC is an invaluable technique and complements the TPC method. Lending credence to the importance of TPC and CPM analyses, the delegates at the 3rd International Symposium on Deep Fat Frying recommended these analyses as the best indicators of oil degradation (DGF, 2000). Regulators in European countries required that the total amount of oligomeric triglycerides be less than 10% (Firestone, 2006).
2.5.3  \textit{p}-Anisidine value (AV)

Thermal decomposition of hydroperoxides during frying generates a number of secondary oxidation products, with carbonyl compounds being the most prominent. Although some of the aldehydes produced are volatile and lost by evaporation during frying, a significant amount remains and is assessed by AV (Chang et al., 1978; Perkins, 1996). \textit{p}-Anisidine value is defined by convention as 100 times the optical density measured at 350 nm in a 1 cm cuvette of a solution containing 1.00 g of the oil in 100 mL of a mixture of solvent and reagent (Firestone, 2009). Official procedures for determining an anisidine value utilize the reaction between aldehydes, principally 2-alkenals and 2,4-dienal, and \textit{p}-anisidine reagent in glacial acetic acid solution (ISO 6885; AOCS Cd 18-90; IUPAC 2.502). The resulting Schiff bases possess a characteristic UV absorption at 350 nm, and the absorbance increases with the amount of relevant nonvolatile carbonyl compounds retained in the oil (White, 1995). The contribution of carbonyl compounds is expressed as absorbance units per 1 g of fat and is arbitrary.

The use of Fourier transformed infrared-analysis (FTIR) for the determination of AV was described by Dubois et al. (1996). Using a partial least square calibration (PLS) as the chemometric method, the authors reported a strong correlation ($r^2 = 0.998$) between the AV values for thermo-oxidized canola oil determined by FTIR and the official chemical method. In a recent study, Szabó et al. (2009) reported a very good correlation ($r^2 = 0.912$) between AV values determined for thermo-oxidized lard by the chemical method and the values obtained by NIR spectroscopy using a wavenumber range from 4000 to 5000 cm$^{-1}$. These spectroscopic analytical methods offer the
possibility for a rapid, automated, and solvent-free alternative to the current official chemical method.

2.5.4 Changes in fatty acid composition

Polyunsaturated fatty acids such as linoleic and linolenic are the main components affected by the various chemical reactions occurring during frying (Warner, 2004). The observed changes in the amount and configuration of the fatty acids increase with the temperature and length of frying. Thus, assessment of changes in fatty acid composition can be used to monitor thermo-oxidative degradation occurring during deep frying. Official methods for analyzing fatty acid composition involves transforming the fatty acids into the methyl esters, followed by separation and quantification using gas chromatography (AOCS methods Ce 1-62, Ce 1f-96, Ce 2-66, ISO method 5509, IUPAC method 2.301). Methods for preparing the methyl ester derivatives have been reviewed by Liu (1994), Eder (1995), Seppanen-Laakso et al. (2002), and Dijkstra et al. (2007).

For routine analysis, the gas chromatogram peak area is normally used to calculate the content of each fatty acid (Dijkstra et al., 2007). However, the gas chromatograms must be interpreted with caution since they only refer to standard fatty acids and exclude degradation products such as oxidized and oligomerized fatty acids. In addition, because in GC analysis the total fatty acid content is set by default to 100%, any decrease in the polyunsaturated fatty acids by thermo-oxidation is automatically counterbalanced by an increase in the more stable monounsaturated and saturated fatty acids to maintain the sum despite the fact that some materials are not eluted. Thus, the increase in oleic, palmitic, and stearic acid contents often reported by some authors
during deep frying gives an erroneous impression that these fatty acids are formed during thermal treatment of the oil. By using an internal standard (e.g. C17:1) the misconception can be overcome, and it becomes evident that the saturated fatty acid content does not increase and that the sum indeed falls below 100%.

The observed loss in fatty acids during frying is due to formation of several oxidized and oligomerized degradation products. Hydroxy, keto, and epoxy are the major functional groups identified in oxidized fatty acids of frying oils; GC methods for analyzing them have been reported by Schawrtz et al. (1994), Berdeaux et al. (1999), Velasco et al. (2002), and Marmesat et al. (2008b). Generally, methyl ester derivatives of the fatty acids (FAMEs) are obtained by base-catalyzed transmethylation of the triacylglycerols; the isolated FAMEs are then fractionated by adsorption chromatography on silica gel to separate the nonpolar FAMEs from the polar FAMEs containing fatty acids with at least one extra oxygen; polar FAMEs are subsequently subjected to GC analysis. Hydrogenation is often applied to the polar FAMEs in order to achieve a better GC separation of the keto- and hydroxyl-FAMEs (Marmesat et al., 2008b). For analysis of the various groups of thermo-oxidatively altered fatty acids, Márquez-Ruiz et al. (1995) utilized HPSEC to separate the nonpolar FAMEs into monomers and dimers; the polar FAMEs were further separated into oxidized fatty acid polymers, oxidized fatty acid dimers, and the oxidized fatty acid monomers.

2.5.5 Colour analysis

In actual practice, the colour of the oil becomes unacceptable first, well before the flavour and odour of the oil become objectionable (Paul et al., 1997). According to
Orthoefer (1988), the colour of the frying oil is one of the major parameters of acceptance to be evaluated on a daily basis. Indeed, regulations in many countries stipulate that colour must be one of the criteria for discarding frying oils (Bansal et al., 2010a). For instance, the Manufacturing Process Inspection document published by the U.S. Department of Agriculture, stipulates that the darkening of oil is evidence of unsuitability of a frying oil and requires rejection of the oil (USDA, 1985).

Many products arising from thermo-oxidative alteration of oil components contribute to colour change during frying. The colour intensity of a frying oil increases as the amount of polymeric materials increases (Stevenson et al., 1984; Blumenthal, 1991). Leaching of pigments from the food into the frying oil, and the presence of Maillard reaction products, formed during frying by the reaction of carbohydrates and some lipid oxidation products with amines, amino acids, and proteins also affects the colour development (Gutierrez et al., 1988; Lalas et al., 2006; Delgado-Andrade et al., 2010). Furthermore, particles from food being fried can become caramelized and release some fat-soluble pigments into the oil (Vijayan et al., 1996). According to Min et al. (1975), products with a molecular weight 300 – 551 Daltons and containing double bonds, carboxyl, ester, peroxide or hydroxyl functions contribute to the darkening of oil during frying.

Wesson (AOCS method Cc 13b-45), Lovibond (AOCS method Cc 13e-92) and spectrophotometric (AOCS method Cc-13c-50) procedures are official methods recognized for the measurement of colour in frying oils (Firestone, 2009). The Wesson and Lovibond are colorimetric methods that determine the colour of the oil by comparison with coloured glasses of known characteristics. In the spectrophotometric
method, the absorbance of the oil is measured at 460, 550, 620, and 670 nm, and the photometric colour index (PCI) is computed according to the equation: 

\[
\text{PCI} = 1.29(A_{460}) + 69.7(A_{550}) + 41.2(A_{620}) - 56.4(A_{670}).
\]

Where \(A_{460}, A_{550}, A_{620}, \) and \(A_{670}\), are absorbances at 460, 550, 620, and 670 nm, respectively.

In a study on the spectrophotometric method for the assessment of frying oils, Xu (2000) reported that the highest correlation was observed between the absorbance measured at 490 nm and the TPC value (\(r = 0.953\)). The results of a recent study by Bansal et al. (2010b), however, showed that any wavelength in the range of 400 – 500 nm could be utilized to provide a good correlation between TPC and the spectrophotometric absorbance. Irrespective of the methods used, results obtained on colour formation during frying must be interpreted with caution as the rate of colour development differs from oil to oil and also depends on the initial colour of the oil and the type of the food fried in it (Gertz 2000). Furthermore, oil components, such as tocotrienols and phenolics cause faster darkening of oil due to chemical changes in the molecules and by oligomerization.

2.5.6 Volatile carbonyls

A number of excellent reviews on the formation of volatile compounds has been published by Schieberle and Grosch (1981), Przybylski and Eskin (1995), Kiritsakis (1998), Frankel (2005), Valet et al. (2007). It is known that variety and different amounts of volatile compounds are generated during thermo-oxidative degradation of oil components and they relate to the nature and stability of the fatty acids involved, and to the frying conditions applied (Przybylski and Eskin, 1995; Malcolmson et al., 1996; Fullana et al., 2004; Katragadda et al., 2010). Thus, the amount and rate of formation of
volatile compounds during frying can be used as an indicator of an oil’s performance. Among the volatile compounds formed during thermo-oxidative degradation of oils, the carbonyls are the major component, and account for more than 60% of total volatiles depending on the conditions applied (Przybylski and Eskin, 1995).

Gas chromatography (GC) is the most common method for analyzing volatile compounds formed during thermal alteration of oils. The volatile samples are often introduced into the gas chromatograph by static headspace analysis, dynamic headspace analysis, or by direct injection. These methods are thoroughly reviewed by Przybylski and Eskin (1995), and Frankel (2005). In the direct injection method, an oil is injected directly into a GC injector special glass liner. The injector is maintained at a high temperature (>200°C) and a carrier gas purges the generated volatile compounds to the column. Static headspace analysis (SHS) consists of analyzing an aliquot of the vapours in equilibrium with the sample heated in a hermetically sealed vial. Although this method is relatively simple, the sensitivity is rather poor (Cer et al., 2000). However, Snyder and Mounts (1990) reported improved sensitivity and reproducibility by using a multiple headspace extraction technique (MHE). The headspace over oil heated at 90°C was sampled three times, and each sample was injected consecutively onto the gas chromatograph. Furthermore, solid-phase microextraction (SPME) also presents a more sensitive and convenient alternative to traditional static headspace analysis (Gromadzka and Wardencki, 2010). However, the SPME method has limitations, including difficulties in inter-fiber comparisons and diagnosis of fiber performance (Escuderos et al., 2007). Dynamic headspace analysis (DHS) has been the most used concentration technique for GC analysis and consists of sweeping the volatile compounds from the headspace sample.
with an inert gas; the volatile compounds are then trapped on a solid absorbent such as activated charcoal, tenax, poparak, chromosorb or amberlite (Cert et al., 2000; Escuderos et al., 2007).

The combination of GC and mass spectrometry (GC-MS) is also a versatile technique for analyzing volatile degradation products formed during frying. Snyder et al. (1986) evaluated the oxidative and thermal stability of soybean oil by measuring the amounts of volatile compounds with static headspace-gas chromatography-mass spectrometry (SHS-GC-MS). Carbonyl compounds generated during thermal treatment of canola and olive oils at 180 and 240°C for up to 15 h were adsorbed unto tenax and analyzed by GC-MS after thermal desorption (Fullana et al., 2004). In a similar study, Katragadda et al. (2010) evaluated the variety and the amounts of volatile compounds formed during heating of coconut, extra virgin olive, safflower, and canola oils at 180, 210, 240, and 270°C for 6 h by DHS-GC-MS. In a recent study, Uriarte and Gullén, (2010) evaluated the formation of volatile alkylbenzenes in extra virgin olive, sunflower, and virgin linseed oils heated at 190°C for 8 h by SPME-GC-MS. Jeleń et al. (2000) evaluated the efficiency of different types of fibers for their capacity to absorb the headspace volatile compounds generated during accelerated storage of canola oil at 60°C for 10 days. Divinylbenzene/carboxene/poly(dimethylsiloxane) (DVB/CAR/PDMS) offered the best performance compared to other tested fibers, namely, polyacrylate (PA), poly(dimethylsiloxane) (PDMS), and carbowax/divinylbenzene (CW/DVB).

As previously mentioned, carbonyls constitute the major group of volatile compounds formed during frying, and are the most important, qualitatively (Przybylski and Eskin, 1995). For instance, while the reported odour threshold value of pentane in oil
was 340 µg/g, the threshold value for pentanal, the corresponding aldehyde was 0.07 µg/g (Table 1). For the analysis of volatile carbonyl compounds, reversed phase HPLC is a viable alternative to GC analysis. The major advantage of HPLC compared to GC is that it operates at ambient temperatures; thus limiting the risk of artefact formation (Kolanowski et al., 2007). Typically, the volatile carbonyl compounds are trapped on a silica cartridge impregnated with 2,4-dinitrophenylhydrazine, after which the 2,4-dinitrophenylhydrazones of carbonyls are eluted and quantified by HPLC-UV at 360 nm (Possanzini and DiPalo, 1995; Katsuta et al., 2008). In a recent study, Bastos and Pereira (2010) utilized HPLC-MS for the quantification of the 2,4-dinitrophenylhydrazones obtained after derivatization of the volatile aldehydes generated during thermal treatment of canola oil at 180°C for 8 h.

2.5.7 Changes in antioxidants

Under thermo-oxidative conditions, endogenous or applied antioxidants are lost, either through direct antioxidant activity, evaporation or by thermal oxidation and polymerization of the antioxidants. It is well known that the observed decrease in antioxidant concentration increases with temperature and time (Gordon and Kourimska, 1995a; Normand et al., 2001; Warner and Moser, 2009). Accordingly, monitoring the level of antioxidants during frying can be used as an indicator of frying performance of oils. Analytical methods for assessing major lipid antioxidants have been reviewed by Cert et al. (2000), Abidi (2000), Ruperez et al. (2001), Carrasco-Pancorbo et al. (2005), Ladislav et al. (2005), Bendini et al. (2007), Jensen and Laurisen (2007), Liu et al. (2008), and Tarascou et al. (2010).
Tocochromanols are the most important natural antioxidants in vegetable oils, and the most widely used method for their quantification is the direct normal-phase HPLC analysis of the oil. A fluorescence detection, with an excitation wavelength set at 290 nm and emission wavelength at 330 nm is the most common approach, being the most sensitive for these compounds (Chase et al., 1994; Cert et al., 2000). Indeed, the AOCS official method requires that a UV detector be used only in the absence of fluorescence detector, and the UV detector should be set at 292 nm (Firestone, 2009). Although, analysis of tocochromanols by reversed-phase HPLC presents the advantage of shorter equilibrium and analysis time, and higher reproducibility than the normal-phase HPLC method, this method is limited by its inability to resolve β- and γ-isomers of both tocopherols and tocotrienols, and plastochromanol-8 (Cert et al., 2000; Ruperez et al., 2001). In addition, because normal-phase HPLC operates with organic solvents in which frying oils are easily soluble, higher loads of samples can be tolerated as they are easy to wash off the column by the nonpolar solvents (Ruperez et al., 2001).

There are discrepancies regarding the best column for the normal-phase HPLC analysis of tocochromanols. Kamal-Eldin et al. (2000) evaluated six silica, three amino, and one diol columns for the separation of tocochromanols in oat extracts and palm oil. Although the tested columns offered equally good separation depending on the mobile phase used, silica columns were reportedly more stable than diol column. Diol column, on the other hand, has been reported to offer more reproducible and consistent results than silica columns (Piironen et al., 1984; Rammel and Hoogenboom, 1985; Pocklington and Diefenbacker, 1988; Kramer et al., 1997). It was also mentioned by Abidi and Mounts (1996) that amino columns offer better selectivity than diol-bonded columns.
Unlike the diol columns that have no ionizable groups, however, the amino groups of 
amino-bonded columns ionize with the use of polar organic solvents, resulting in 
increased retention times and peak broadening (Kramer et al., 1997).

Other natural and synthetic polar phenolic antioxidants are usually analyzed by a 
reversed-phase HPLC, utilizing either isocratic or gradient elution methods with a UV-
Vis detector operated at 225, 240, or 280 nm (Cert et al., 2000; Carrasco-Pancordo et al., 
2005; Bendini et al., 2007; Harbaum-Piayda et al., 2010; Lafka et al., 2011). However, 
some phenolic compounds show several absorption maxima and the use of simultaneous 
multiple UV detection is recommended for quantification (Montedoro et al., 1992; Pirisi 
et al., 1997; Esti et al., 1998). Furthermore, use of a photodiode array detector allows the 
spectrum to be obtained at different wavelengths, and enables a peak identification and 
purity test (Ruperez et al., 2001). Due to its versatility, HPLC has been, and will remain a 
vital analytical technique for quantifying lipid antioxidants.

2.5.8 Formation of toxic components

As previously mentioned, a number of toxic compounds are produced under the 
conditions employed during deep frying. If the frying operation is not well controlled 
and/or the frying oil efficiently stabilized, the toxic compounds can be produced at a 
concentration that warrants health and safety concerns. Oxygenated α,β-unsaturated 
aldehydes are the most prominent toxic degradation products formed during frying, and 
include: 4-hydroxyhexenal (HHE); 4-hydroxyoctenal (HOE); 4-hydroxynonenal (HNE); 
4-hydroperoxynonenal (HPNE); 4-oxononenal (ONE); and 4,5-epoxynonenal (ENE), 
among others (Guillén and Goicoechea, 2008). While formation of other degradation
products, such as acrylamide, may depend on the type of food being fried, the formation of oxygenated α,β-unsaturated aldehydes is essentially oil-dependent, and may be used to assess the frying performance of an oil. With regard to the amounts and the degree of toxicity, 4-hydroxynonenal (HNE) is the most important, and remains the most studied of the oxygenated α,β-unsaturated aldehydes formed during frying (Niki, 2009). Indeed, the August-October 2003 issue (Volume 24, Issues 4-5) of Molecular Aspects of Medicine was entirely dedicated to reports on HNE. The chemistry and biochemistry of HNE has been extensively reviewed by Esterbauer et al. (1991). Guillén and Goicoechea, (2008) comprehensively reviewed the nature, reactivity, biological activity, formation pathways, and available detection and quantification methods for HNE and other oxygenated α,β-unsaturated aldehydes. More recent reviews of the chemistry, biochemistry and analytical methods for HNE and other oxygenated α,β-unsaturated aldehydes are provided by Gueraud et al., 2010; Huang et al. (2010), Negre-salvayre et al. (2010), Roede et al. (2010), and Spickett et al. (2010). Because of its great reactivity, HNE can modify proteins, nucleic acids and other biomolecules leading to several diseases and medical conditions. HNE has been shown to exhibit mutagenic, cytotoxic and genotoxic properties, which are related to pathogenesis of several human diseases such as Alzheimer’s and atherosclerosis. It inactivates various enzymes, inhibits proliferation of cells and acts as a chemotaxin (LoPachin et al., 2008; Niki, 2009; Gueraud et al., 2010; Spickett et al., 2010).

Most of the methods described for the determination of HNE in frying oils are derived from those originally developed for biological systems (Guillén and Goicoechea, 2008). The first method applied to oils was reported by Lang et al. (1985), and involves...
extraction of a sample with distilled water containing an antioxidant such as BHT; the water extract was then centrifuged for clarification; the supernatant was applied to an octadecyl silica gel column for clean up; and HNE was detected and quantified by HPLC-UV. Liu et al. (1996) evaluated the amounts of HNE in several samples of soybean and sesame oils by direct HPLC analysis of its derivatives with 4-(2-carbazolylpyrrolidin-1-yl)-7-nitro-2,1,3-benozadiazole (NBD-ProCZ). The resultant HNE-NBD-ProCZ hydrazone was detected using laser-induced fluorescence detector. Seppanen and Csallany (2006) evaluated the amount of HNE in thermooxidized soybean oil using the method described by Esterbauer et al. (1982) for biological samples which involves the following steps: (1) the direct derivatization with 2,4-dinitrophenylhydrazine (DNPH) in the oil; (2) extraction of the hydrazones with methanol/water mixture; (3) centrifugation and subsequent extraction of the supernatant with dichloromethane; (4) separation of extract on a silica gel TLC plate using dichloromethane; (5) removal of the bands corresponding to HNE; (6) elution of HNE by methanol; (7) centrifugation to remove residual silica gel; (8) concentration of the supernatant under nitrogen; and (9) quantification by HPLC-UV.

Gas chromatographic analysis of HNE formed in oxidized arachidonic and linoleic acids model systems was reported by Tamura and Shibamoto (1991). Hydroxynonenal was reacted with N-methylhydrazine, and the resultant 5(1’-hydroxyhexyl)-1-methyl-2-pyrazoline was analyzed on a fused silica capillary column using a nitrogen-phosphorus detector. In a more recent study by Surh and Kwon (2003), HNE was extracted from oil using distilled water containing 0.1% BHT; the aqueous phase was clarified by centrifugation, and the supernatant was subsequently extracted
with chloroform; the extracted HNE was reduced to the corresponding diols; and the ortho-esters, obtained by reacting the diols with trimethylorthoformate, were analyzed by GC-MS in a selected ion monitoring (SIM) mode.

The poor solubility of HNE in water, and its loss arising from the elaborate clean-up steps (derivatization, multiple extractions and purifications) involved in many of the methods described for HNE usually result in inefficient recovery of HNE. For instance, the recovery of HNE from sunflower oil spiked with 2.35 nM of HNE using the method by Lang et al. (1985) was 68%. Using a variation of the HNE-DNPH method, Uchida et al. (2002) reported HNE recovery rates of 88 and 76% from soybean oil spiked with 2.91 and 0.29 nM standard HNE, respectively. Thus, development of a simplified HNE extraction and clean-up protocol that eliminates lengthy, error-prone derivatization and multiple extraction and purification steps involved in the current methods is imperative.
Chapter 3 – Materials and methods

This chapter presents the materials and methods used in the study. They are presented in more detail in the relevant original publications and accepted manuscripts (I – X) which are an integral part of this thesis.

3.1 Materials

3.1.1 Oils and French fries

Commercially refined, bleached and deodorized regular canola oil (CO) was donated by Richardson Oilseed Processing (Lethbridge, Canada). High oryzanol rice bran oil (RBO) was supplied by Rito, Inc. (Stuttgart, Arkansas, USA) and crude palm oil was a gift from Golden Jomalina Food Industries (Kuala Langat, Malaysia). Frozen, par-fried French fries in an institutional pack, and sesame oil were obtained from a local store.

3.1.2 Chemicals

Standards of tocopherol were obtained from Calbiochem-Novabiochem (San Diego, CA). Standards of β-carotenes and γ-oryzanol were purchased from Sigma-Aldrich (St. Louis, USA) and Oryza Oil and Fat Chemical Co. Ltd. (Ichinomiya-City, Japan), respectively. Phytosterol standards were obtained from Steraloids (Newport, USA). Standards of fatty acid methyl esters were purchased from Nu-Chek-Prep (Elysian, USA).

Alumina (neutral Al₂O₃, 70 – 230 mesh), alumina 58Å (~150 mesh), silica gel 60 (70 – 230 mesh), celite 512 medium, hydrolyzed starch, and potato starch were obtained from Sigma-Aldrich (St. Louis, MO). Unless otherwise stated, all solvents and chemicals
of analytical grade used in the study were purchased from Sigma-Aldrich (St. Louis, USA).

Aldehydes for preparing standards for the analysis of volatile carbonyl compounds (VCC) were purchased from Bedoukian Research (Danbury, USA). The dinitrophenylhydrazone derivatives of the aldehydes were subsequently prepared according to method by Possanzini and DiPalo (1995). A saturated solution of dinitrophenylhydrazine (DNPH) in 2M HCl was reacted with the corresponding carbonyls, the solid dinitrophenylhydrazones were filtered, washed with 2M HCl and water, dried and stored in a capped glass vial.

3.1.3 Instruments

$^1$H and $^{13}$C NMR spectra were recorded on a 300 MHz Bruker Avance II spectrometer (Bruker Biospin Corporation, Billerica, MA, USA).

HPLC analyses were performed on Finnigan Surveyor LC system (Thermo Electron Corp., Waltham, MA, USA) equipped with a Finnigan Surveyor Autosampler Plus, FL Plus fluorescence detector, photo-diode-array detector, UV-Vis Plus detector, and Sedex 75 evaporative light scattering detector (Sedere, Alfortville, France).

GC analyses were carried out using a Trace GC Ultra (Thermo Electron Corporation, Rodano, Italy) or a Hewlett-Packard 6890 gas chromatograph (J & W Scientific, Folsom, CA, USA).

Spectrophotometric analyses were done on a Beckman DU-65 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA, USA) for UV-Vis data, a
Cary Eclipse fluorescence spectrophotometer (Varian, Palo Alto, CA, USA) for fluorescence.

Melting points were measured with an Electrothermal MEL-TEMP 3.0 (Barnstead, Dubuque, IA, USA).

High-resolution mass spectra were obtained with a QSTAR Elite mass spectrometer (AB SCIEX, Concord, ON, Canada).

Evaporation of solvents and concentration under vacuum was achieved using a BÜCHI rotary evaporator (BÜCHI Labortechnik AG, Flawil, Switzerland).

3.2 Methods

3.2.1 Frying procedures and oil samples

3.2.1.1 Standard frying conditions (I, III, IV)

Frying under standard conditions was performed in an 8-L capacity restaurant style stainless-steel fryer (General Electric Company, Niskayuna, USA). Canola oil (4 L) was heated at 185 or 215°C ± 5°C for 7 h daily for 7 days. A batch of frozen French fries was fried for 5 min for a total of eight batches daily. At the end of each frying day, the fryer was shut off and left to cool down overnight. Two 25 mL samples of oil and the last batch of French fries were collected daily and kept frozen at -20°C until analyzed. Before commencing frying each day, the oil was filtered to remove debris and replenished every second day with 500 mL of fresh oil.
3.2.1.2 Carbon dioxide blanketing (III, IV)

The frying procedure and sampling described above for standard frying condition (SFC) was used for frying under carbon dioxide blanketing (CDB). The carbon dioxide was delivered through stainless-steel tubing (2 mm i.d.) with 0.6-mm holes placed equally alongside of the fryer and at flow rate of 2.5 L/min. The tubing was attached to the upper edge of the fryer, and the gas outlets were 1 cm above the oil surface. Oil was purged with CO$_2$ for 10 min prior to commencing frying and at the end of each frying day; the flow of carbon dioxide was continued until the temperature of the oil dropped below 100°C.

3.2.1.3 Vacuum frying (III)

Vacuum frying was done using a BT-1 Industrial Vacuum Deep Fryer (Sakuma Corporation, Chiba, Japan). Canola oil (9.5 L) was placed in the 10 L capacity industrial vacuum fryer. A batch of 400 g of frozen French fries was fried under 9.7 kPa vacuum at 180 ± 5°C for 2.5 min. A total of eight batches of French fries were fried daily for 7 days. At the end of each day, the frying oil was allowed to cool overnight under vacuum. Two 25 mL samples of oil were taken daily and kept frozen at -20°C until analyzed. As in other frying protocols, oil was replenished every second day of frying with 500 mL of fresh oil.

3.2.2 Rapid frying test (V)

Silica gel, alumina and celite were placed in an oven preset at 160°C and heated for 24 h. The materials were then transferred into a desiccator and allowed to cool down.
An appropriate quantity of distilled water was added to adjust the water content to 10, 20 and 40%. The water-conditioned adsorbents were mixed and kept overnight. Formulated additives were added to 12.0 g of oil in an acid-washed glass vessel (Pyrex, USA; outer diameter 35 mm, capacity 30 mL). Clean octagonal stir bar (9.5 × 25 mm, Fischer Scientific, USA) was placed into the glass vessel, altering the final surface-to-volume ratio at 0.42. The oil was heated at 185 ± 5°C with stirring and the level of degradation assessed.

3.2.3 Isolation, processing and purification of minor components (VI, VII)

3.2.3.1 Column chromatography

A slurry of 1 kg of alumina (activated at 103°C for 16 h and 200°C for 8 h) in 2 L of hexane was loaded to a glass chromatography column (950 × 50 mm i.d.) and the hexane allowed to flow through the column until the solid phase was evenly packed. Oil (500 g) dissolved in 500 mL hexane was applied into the packed column. The first 800 mL of the eluting hexane was discarded. Purified triacylglycerols were eluted with 3.7 L of hexane (Scheme 1). The minor components were recovered from the column by eluting with 2.5 L of 10% methanol in methyl tert butyl ether. Both the column and collection vessels were wrapped in aluminum foil to prevent photo-oxidation (Scheme 1). The purified triacylglycerols were stored as hexane solutions at -16°C until used in the experiments.

The recovered minor components were further separated into two fractions by adsorption chromatography (Scheme 1). Conditioned silica gel was prepared by heating it for 24 h at 160°C, and the water content adjusted to 5% by addition of stoichiometric
amount of water. A slurry of 120 g of the conditioned silica gel in 150 mL of hexane was loaded into a glass chromatography column (600 × 45 mm i.d.) allowing constant flow of hexane until the packing was evenly packed. Minor components (20 g) dissolved in 30 mL hexane were introduced into the column. Tocopherol fraction was eluted with 750 mL of 5% methyl tert butyl ether (MTBE) in hexane while sterol fraction with the same volume of 50% MTBE in hexane. The purity of fractions was monitored by thin layer chromatography. Collected fractions were concentrated in vacuo at 35°C. Both the column and collection vessels were wrapped in aluminium foil to prevent photo-oxidation. The fractions were stored in hexane at -16°C until used in the experiments.

3.2.3.2 Preparative thin layer chromatography

The tocopherol and sterol fractions obtained by column chromatography were further purified by preparative thin layer chromatography (PTLC) on a 20 cm × 20 cm, 250 µm layer silica gel (Whatman, Piscatway, NJ, USA) (Scheme 1). The tocopherol fraction was developed using 20% MTBE in hexane. Bands corresponding to tocopherols were carefully scraped off the plate and subsequently eluted three times with 10 mL of MTBE. Combined extracts were concentrated in vacuo at 35°C, flushed with nitrogen and kept at -16°C until used in the experiments. The sterol fraction was similarly purified using 65% MTBE in hexane as developing solvent (Scheme 1).
Scheme 1. Schematic of isolation and purification of concentrated endogenous minor components from canola and rice bran oils. CC – Column chromatography; PTLC – preparative thin layer chromatography; MTBE – methyl-tert-butyl ether; CO – canola oil; RBO – rice bran oil; TAG – triacylglycerols; CMC, RBMC – minor components isolated from canola and rice bran oil by column chromatography, respectively; TCAN, TRBO – tocopherol fractions isolated from canola and rice bran oil by column chromatography, respectively; SCAN, SRBO – sterol fractions isolated from canola and rice bran oil by column chromatography, respectively.

3.2.3.3 Solvent extraction

Oil (200 g) was placed in a 500 mL capacity separatory funnel and 200 mL methanol was added followed by agitation for 5 min with periodic venting. The mixture
was kept at room temperature until layers separated, then the methanol portion was removed into round bottom flask. The extraction was repeated 12 times with the same volume of fresh methanol, and the combined extracts evaporated \textit{in vacuo} at 35°C. The concentrated fraction was flushed with nitrogen and stored at -16°C until used in the experiments.

3.2.4 Isolations

3.2.4.1 Lipids (II, IV)

Lipids were isolated according to the Folch et al. (1957) procedure. French fries (50 g) were chopped into small pieces and homogenized with 400 mL of chloroform:methanol (2:1, v/v). The solvent/lipid mixture was filtered through filter paper (Whatman #2, 24.0 cm) into a separatory funnel. Distilled water (100 mL) was added, and after mixing, the mixture was allowed to separate into two layers. The lower lipid-chloroform layer was collected in a round bottom flask, and the solvent removed under reduced pressure on a rotary evaporator at 35°C. The oil samples were transferred with iso-octane to appropriately labelled vials, flushed with nitrogen and stored at -16°C until analyzed.

3.2.4.2 Acrylamide (IV)

French fries (10 g) were ground using a Grindomax GM200 mill (Haan, Germany). To 4 g of ground sample 50 µL of deuterated acrylamide solution (150 µg/mL) was added as an internal standard and the mixture extracted with 50 mL of distilled water utilizing sonication at 60°C. The extract was centrifuged at 4500 g and the
supernatant defatted by extraction with petroleum ether and afterwards clarified with 5 mL of each Carrez I and II solution. For separation of the precipitate the solution was centrifuged and then acrylamide was salted out with sodium chloride. Thereafter, acrylamide was extracted three times from the aqueous phase with 30 mL ethyl acetate. The combined extracts was filtered using water-repellent filters (MN 616 wa ¼; Macherey-Nagel GmbH & Co. KG, Düren Germany), and evaporated under nitrogen to 0.5 mL.

3.2.4.3 4-hydroxy-2-nonenal (IV)

After removal of the nonpolar fraction (triacylglycerols) during polar components assessment, the polar fractions were recovered by eluting the column four times with 5 mL of methanol. The combined eluants were subsequently evaporated under stream of nitrogen to the final volume of 5 mL. The cloudy solution was then centrifuged at 1000 g for 5 min, and the clear supernatant transferred with a Pasteur pipette into a clean vial and analyzed by HPLC.

3.2.5 Volatile carbonyl compounds (VI – VII)

Volatile carbonyl compounds generated during the frying experiments were trapped on a Sep-Pak DNPH-Silica cartridge (Waters, Milford, MA) where they were converted to the stable dinitrophenylhydrazones. The cartridge was connected to a pump through a flow-meter. The wider end of the cartridge was suspended about 0.5 cm above the upper edge of the beaker used for frying test, and the samples of vapours were drawn through the cartridge at a flow rate of 350 mL/min for 2 h. The resulting
dinitrophenylhydrazones were eluted from cartridge with 5 mL of acetonitrile at a flow of 2 mL/min.

3.2.6 Synthesis of novel antioxidants (VIII – X)

3.2.6.1 Preparation of chromanol derivatives (VIII, IX)

Benzoic esters 5a, 5b (IX), and 3a – 3d (VIII) were prepared according to a procedure reported by Tranchimand et al. (2006). Potassium carbonate (77.2 mmol, 3.0 equiv) and benzyl bromide (77.2 mmol, 3.0 equiv) were added to a solution of the desired carboxylic acid (25.7 mmol) dissolved in 10 mL dry DMF under an argon blanket. The mixture was stirred for 15 h and transferred into 150 mL distilled water. The compound of interest was extracted three times with 100 mL diethyl ether. The combined extracts were washed with 100 mL distilled water, dried on magnesium sulfate and concentrated under vacuum using a rotary evaporator (Schemes 2 and 3).

The carboxylic acids 4a – 4d (VIII) and 6a – 6c (IX) were prepared according to the procedure described by Tranchimand et al. (2006). Potassium hydroxide (72.0 mmol, 5.0 equiv) was added to a solution of the corresponding benzoic ester (14.4 mmol) in a mixture of 53 mL distilled water and 210 mL ethanol. The mixture was refluxed for 2 h and the solvent evaporated under a vacuum using a rotary evaporator. The residue obtained was dissolved in 200 mL distilled water. The aqueous solution was washed twice with 50 mL diethyl ether and acidified with concentrated sulfuric acid until the formation of a white solid suspension. The suspension was then extracted thrice with 200 mL ethyl acetate. The combined extracts were washed with distilled water, dried on magnesium sulfate and finally concentrated under a vacuum using a rotary evaporator.
Scheme 2. Reactions, reagents, and conditions for the synthesis of benzoic acid derivatives. (i) BnBr, K₂CO₃, DMF; (ii) KOH, H₂O/MeOH, reflux. For details see the text.

Scheme 3. Reactions, reagents, and conditions for the synthesis of cinnamic acid derivatives. (i) BnBr, K₂CO₃, DMF; (ii) KOH, H₂O/MeOH, reflux. For details see the text.

For the preparation of the esters 8a – 8e (VIII) and 9a – 9c (IX), N,N'-dicyclohexylcarbodiimide (DCC; 3.06 mmol, 2.0 equiv) and 4-dimethylaminopyridine (DMAP; 0.23 mmol, 0.15 equiv) were added under an argon blanket to an alcoholic solution of (6'-benzyloxy-2',5',7',8'-tetramethylchroman-2'-yl)methanol (1.53 mmol) and the desired carboxylic acid (3.06 mmol, 2.0 equiv) in 40 mL dry dichloromethane (DCM) (Schemes 4 and 5). The mixture was stirred for 15 h at room temperature and 50 mL distilled water was added. The organic layer was removed and washed once more with 20
mL distilled water, dried over anhydrous magnesium sulfate and concentrated using a rotary evaporator. Finally, the crude product was purified by flash column chromatography on silica gel to give the desired ester.

Antioxidants 1a – 1e (VIII), 2a – 2c (IX), and 3 (IX) were prepared according to the following procedure (Schemes 4, 5 and 6): palladium on charcoal (10% wt) was added to a solution of the desired benzylated compound (0.7 mmol in 8 mL of dry THF). The mixture was stirred at room temperature under an atmosphere of hydrogen for 24 h, then filtrated on celite, and concentrated on a rotary evaporator under reduced pressure. The residue was purified by flash column chromatography on silica gel to give the desired antioxidant.

Scheme 4. Reagents and conditions used for the synthesis of antioxidants 1a – 1e.
(i) BnBr, K$_2$CO$_3$, DMF; (ii) LiAlH$_4$, THF, 0 °C; (iii) 4a-4e, DCC, DMAP, DCM; (iv) H$_2$, Pd/Charcoal 10%, THF. For details see the text.
**Scheme 5.** Reagents and conditions used for the synthesis of antioxidants 2a – 2c.
(i) BnBr, K$_2$CO$_3$, DMF; (ii) LiAlH$_4$, THF, 0 °C; (iii) 6a-6c, DCC, DMAP, DCM; (iv) H$_2$, Pd/Charcoal 10%, THF. For details see the text.

**Scheme 6.** Reagents and conditions used for the synthesis of antioxidant 3. (i) KOH, H$_2$O/EtOH, reflux; (ii) 8, DCC, DMAP, DCM; (iii) H$_2$, Pd/Charcoal 10%, THF. For details see the text.
3.2.6.2 Preparation of dihydrocaffeic acid derivatives (X)

General procedure for the synthesis of amines 2a – 2L (X): The aldehyde (10 mmol) and the aliphatic amine (15 mmol, 1.5 equiv) were dissolved in 10 mL methanol and the mixture was stirred for 3 h, then NaBH₄ (10 mmol, 1.0 equiv) was added in small portions (Scheme 7). The mixture again was stirred for 3 h followed by solvent removal under reduced pressure using a rotary evaporator. Then 10 mL distilled water was added and the solution extracted three times with 10 mL ethyl acetate. The organic layers were combined and dried on magnesium sulfate. Subsequent removal of solvent under reduced pressure using a rotary evaporator provided the desired crude product.

For the synthesis of the new dihydrocaffeic acid amides 3a – 3L (X), dihydrocaffeic acid (1.1 mmol) and triethylamine (1.1 mmol, 1.0 equiv) were added to 2 mL dimethylformamide and the mixture was stirred at 0°C for 30 min (Scheme 7). Then an amine (2.2 mmol, 2.0 equiv), dissolved in a minimum amount of DCM, and BOP (1.1 mmol, 1.0 equiv.), dissolved in 2 mL of DCM were added and stirred for 2 h at room temperature. The solution was concentrated under reduced pressure using a rotary evaporator. To the residue, 10 mL water was added and the solution extracted three times with 10 mL ethyl acetate. The organic layers were combined, dried on magnesium sulfate and the solvent removed under reduced pressure using a rotary evaporator. The crude product was further purified by flash column chromatography on silica gel.
Scheme 7. Reagents and conditions used for the synthesis of antioxidant 3a – 3L. (i) MeOH, NaBH₄; (ii) Dihydrocaffeic acid, Et₃N, DMF, BOP. For details see the text.

3.2.7 Radical scavenging activity (VIII – X)

3.2.7.1 DPPH assay

The DPPH assay was performed according to Nenadis and Tsimidou (2002). To 2960 µL of 0.1 mM ethanolic solution of DPPH 40 µL of synthesized antioxidant solution in ethanol was added at: 0.37, 0.74, 1.11, 1.85, 3.7, and 5.2 µM forming the ratios between the molar amounts of antioxidant to the molar amount of DPPH at 0.05, 0.10, 0.15, 0.25, 0.5 and 0.7, respectively. The decrease of absorbance at 516 nm was
measured at 25°C after 20 min of reaction where the blank solution contained the same amount of DPPH and 40 µL of ethanol. The results are expressed as the %DPPH inhibition calculated according to the following equation:

$$\text{%DPPH} = \left( \frac{\text{Ac} - \text{At}}{\text{Ac}} \right) \times 100$$

Where Ac and At are the absorbances of the control sample and the test sample, respectively. The IC$_{50}$ represents the concentration of antioxidant required to decrease the initial amount of DPPH by 50%.

3.2.7.2 ORAC assay

ORAC assays were performed according to Szydlowska-Czerniak et al. (2008). Fluorescein disodium salt and AAPH solutions were prepared in 75 mM phosphate buffer (pH 7.4). Antioxidant solutions consisting of 1 mM of each compound were dissolved in methanol and a specific volume of each dissolved in the buffer to provide the required amount of antioxidant within a range of 3.125 - 25.00 µM. Four different concentrations were tested for each antioxidant. A solution of fluorescein, 3.0 mL (0.0816 µM) was mixed with 0.5 mL of antioxidant solution directly in a quartz cuvette. The mixture was kept at 37°C for 10 min and 0.5 mL of the AAPH solution (153.0 µM) added. The fluorescence was measured at 37°C for 30 min at 30 s intervals. The emission and excitation were set at 525 nm and 485 nm, respectively. For a blank, phosphate buffer replaced the antioxidant solution. Each antioxidant solution was prepared in duplicate and three measurements were performed for each sample. A calibration curve was generated using trolox as the reference antioxidant.

The area under the fluorescence decay curve (AUC) was calculated as follows:
\[
AUC = \sum_{t=0.5}^{\infty} \left(f_t + f_{t-0.5}\right)/4
\]

Where \(f_t\) is the fluorescence at time \(t\) (min).

The net AUC corresponding to the sample was calculated using the following equation:

\[
AUC_{\text{net}} = AUC - AUC_{\text{blank}}
\]

For each antioxidant, a regression between \(AUC_{\text{net}}\) and the compound concentrations was calculated and the results are expressed in \(\mu\text{mol trolox equivalents per litre (TEq)}\).

3.2.8 Accelerated storage

To 1 g of pure canola triacylglycerols 350 \(\mu\text{g/g}\) of the tested antioxidant was added in a vial (National Scientific Target DP Vials; 2 mL, 12 × 32 mm). The samples were stored in the dark for up to seven days at 60\(^\circ\text{C}\), providing the surface area to volume ratio at 0.78. Samples were examined at 24 h intervals for peroxide value (PV).

3.2.9 Analysis by GC / GC-MS

3.2.9.1 Fatty acid composition (I – III)

Fatty acid methyl esters (FAME) were analyzed on Trace GC Ultra gas chromatograph using a Trace TR-FAME fused silica capillary column (100 m x 0.25 mm x 0.25 \(\mu\text{m}\)). Hydrogen was used as carrier gas with a flow rate of 1.5 mL/min. The column temperature was programmed from 70 to 160\(^\circ\text{C}\) at 25\(^\circ\text{C}/\text{min}\) and held for 30 min, and further programmed to 210\(^\circ\text{C}\) at 3\(^\circ\text{C}/\text{min}\). Starting and final temperatures were held for 5 and 30 min, respectively. Splitless injection was made using a PTV injector. Detector temperature was set at 250\(^\circ\text{C}\). FAME samples, 1 \(\mu\text{L}\) were injected with AS 3000
autosampler. Fatty acids were identified by comparison of retention time with authentic standards.

For the preparation of FAME, 25 mg of oil was weighed directly into a screw-capped tube and 0.5 mL of methyl heptadecenoate (internal standard) in isooctane (2 mg/mL) added. Then 5 mL of 0.5 M solution of sodium methoxide in methanol was added, vortexed and kept at 50°C in a heating block for 2 h. After the solution had cooled down to room temperature, 0.3 mL glacial acetic acid, 3 mL isooctane, and 3 mL distilled water were added and the solution was thoroughly mixed and centrifuged. The clear upper layer (FAME) was transferred into a dry chromatographic vial and submitted to GC analysis.

3.2.9.2 Phytosterols (VI, VII)

Compositions of phytosterols were analyzed using the procedure described by Rudzińska et al. (2003). Lipid samples were saponified with 1 M KOH in methanol at room temperature for 18 h, then water was added and unsaponifiables extracted with diethyl ether. Dry residues were silylated with BSTFA containing 1% TMCS. Derivatives of the sterols were separated on a Hewlett-Packard 6890 gas chromatograph with an HP-5 capillary column (30 m × 0.32 mm × 0.25 μm). Split injection with split ratio 1:25 was used. Separation was done isothermally at 290°C, with a helium flow rate of 1.6 mL/min. The injector and detector temperatures were set at 310°C. An internal standard, 5-α-cholestan, was used for quantification. Phytosterols were identified by comparison of retention data and by GC–MS using a Finnigan Trace 2000 gas chromatograph coupled to a Finnigan Polaris Q quadrupole ion-trap mass spectrometer after separation on a DB-5
capillary column (50 m × 0.2 mm × 0.32 μm). Helium was used as carrier gas at a flow rate of 0.6 mL/min. All mass spectra were recorded using electron-impact ionization mode at 70 eV and scanning mass in the range of 100–650 D.

3.2.9.3 Acrylamide (IV)

GC-MS analysis was carried out using the electron ionization mode (EI, 70 eV) on a Hewlett Packard instrument Model 5890 Series II/5989 A. For the determination of acrylamide the SIM-mode (selected ion monitoring) was used and the identification achieved by using characteristic ions with masses (m/z) 74, 71, 58 and 55. The quantification was carried out using ions with masses 71 and 74. The separation was achieved with a DB-23 capillary column (30 m x 0.25 mm, 0.25 μm; J&W Scientific Products GmbH, Köln, Germany). The carrier gas was helium at a flow rate of 1.0 mL/min. The column temperature was initially kept at 80°C for 2 min and then programmed to 220°C at 10°/min. The final temperature was held for 1 min.

3.2.10 Analysis by HPLC/HPLC-MS

3.2.10.1 Composition of polar material (I, III, V)

The composition of polar components was analyzed using high-performance size-exclusion chromatography (HPSEC) following the ISO Method 16931:2007. Components were separated on three size exclusion columns connected in series (Phenogel 500A, 100A and 50A; 5 μm, 300 × 4.6 mm; Phenomenex, Torrance, CA), with tetrahydrofuran (THF) as the mobile phase at a flow rate of 0.3 mL/min, and column
temperature of 30°C. A 10 µL sample was injected, and components were detected with an evaporative light scattering detector, operated at 35°C with air pressure of 2.5 bar.

3.2.10.2 Composition of minor component (VI, VII)

Isolated minor components were separated into lipid classes according to Silversand and Haux (1997). Analysis was performed on a Finnigan Surveyor LC system and components were separated on a normal phase Diol column (250 × 4.6 mm; MonoChrom, Varian, CA). The binary gradient was used consisting of: (A) hexane–acetic acid (99:1.0, v/v) and (B) hexane–isopropanol–acetic acid (84:15:1.0, v/v) solvents. The samples of 20 µL were injected and the gradient changed from 0% to 100% of the solvent B within 40 min. The final gradient was kept for 2 min and then returned to initial composition within 3 min followed by 5 min of equilibration. The flow rate was 0.6 mL/min and the column was kept at 45°C. Components were detected with an evaporative light scattering detector operated at 30°C with an air pressure of 1.5 bar. Triolein, diolein, monoolein, oleic acid, and stigmasterol linolenate were used as standards for external calibration to assess the amount of triacylglycerols, diacylglycerols, monoacylglycerols, free fatty acids, and sterol esters, respectively.

3.2.10.3 Tocopherols

Tocopherols were analyzed using high-performance liquid chromatography (HPLC) based on the AOCS Official Method Ce 8-89 (Firestone, 2009). Oil sample (50 mg) was weighed directly into a 1.5 mL vial and mixed with 1 mL hexane. Analysis was performed on a Finnigan Surveyor LC system. The detector was set for excitation at 292
nm and emission 325 nm. Separation of tocopherols was carried out on a normal phase Diol column (250 × 4.6 mm; MonoChrom). Injection volume was 10 µL. Mobile phase consisted of 7% methyl-tert-butyl-ether in hexane and with a flow of 0.6 mL/min. The amounts of tocopherols were quantified using external calibration for each isomer separately.

3.2.10.4 Synthetic antioxidants (VIII – X)

The new chromanol derivatives were analyzed similarly to tocopherols. However, the mobile phase contained 65% methyl-tert-butyl-ether in hexane and the fluorescence detector emission was set at 394 nm. For BHT, dihydrocaffeic acid and dihydrocaffeic acid amides, the mobile phase consisted of 50% methyl-tert-butyl-ether in hexane with a flow rate of 0.3 mL/min. Detection was at 281 nm using a photodiode-array detector (PDA).

3.2.10.5 γ-Oryzanol (VI)

A 20 µL sample was injected onto a C18 column (4 µm; 300 × 3.9 mm; Novapak, Waters, MA) held at 30°C (Przybylski et al., 2009). Separation was achieved by using acetonitrile-water (65:35, v/v). Detection was at 325 nm using a Finnigan Surveyor photodiode-array detector. Total amounts of γ-oryzanol are expressed as a group of esters and quantified by using the external calibration with γ-oryzanol standard.
3.2.10.6 Carotenoids (VI)

Carotenoids analyses were carried out according to the method of Khachik et al. (1992) with slight modifications. Analysis was carried out on Finnigan Surveyor Plus HPLC system. A 10 µL sample was injected onto a C18 column (4µm; 300 × 3.9 mm; Novapak) held at 25°C. Separation was achieved by the following gradient:

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Methanol</th>
<th>Acetonitrile</th>
<th>Dichloromethane</th>
<th>Hexane</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>15</td>
<td>75</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>75</td>
<td>5</td>
<td>5</td>
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<tr>
<td>20</td>
<td>17</td>
<td>60</td>
<td>11.5</td>
<td>11.5</td>
</tr>
<tr>
<td>35</td>
<td>15</td>
<td>40</td>
<td>22.5</td>
<td>22.5</td>
</tr>
<tr>
<td>40</td>
<td>15</td>
<td>75</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>45</td>
<td>15</td>
<td>75</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

The flow rate was 0.5 mL/min. Quantifications of carotenoids were carried out by using external calibration with a β-carotene standard.

3.2.10.7 Volatile carbonyl compounds (VI, VII)

Dinitrophenylhydrazones of volatile carbonyl compounds were detected and quantified by HPLC (Katsuka et al., 2008). A 20 µL sample was injected onto a C18 column (4 µm; 300 × 3.9 mm; Novapak) held at 30°C. Separation was achieved by the following gradient: (1) 40% acetonitrile and 60% water was held for 5 min; (2) followed by 100% acetonitrile within 40 min and maintained for an additional 5 min; (3) during a 5 min period, the mobile phase was returned to its initial composition followed by 5 min
equilibration. The flow rate was 0.5 mL/min. Detection was at 360 nm, and volatile carbonyl compounds were identified by comparison of retention data with standards and by HPLC-MS (Exactive Bench-Top; Thermo Fischer Scientific, West Palm Beach, FL). The HPLC conditions were similar to the above. The mass spectrometer was equipped with an atmospheric pressure chemical ionization (APCI) ion source operated in the negative mode. Spectra were collected using a mass scan range from 100 to 1000 Da.

3.2.10.8 Analysis of 4-hydroxynonenal (IV, VI, VII)

A 20 µL sample was injected onto a C18 column (4 µm; 300 × 3.9 mm; Novapak), and HNE was detected at 223 nm after elution with acetonitrile/water (30:70 v/v) at a constant flow of 0.75 mL/min (Lang et al., 1985). Identification of HNE was done by comparison of retention data and by co-elution of HNE standard with selected samples. To further verify the identity of HNE, identification was carried out on a QSTAR Elite mass spectrometer equipped with an APCI interface operated in a positive mode. Analyst QS 2.0 software was used for data acquisition and analysis. The conditions of mass spectrometric analysis were optimized for HNE as follows: The APCI source temperature was set at 450°C; the curtain gas at 25; the declustering potential at 45V; the focus potentials at 150V; and the ion source gas 1 and 2 at 20 and 60 psi, respectively. Quantification of HNE was carried out using external calibration.
3.2.11 Analysis by spectrophotometry

3.2.11.1 Anisidine value (I, III)

Anisidine values (AV) were determined according to ISO Method 6885 (2004). The oil was weighed directly into a 25 mL volumetric flask, dissolved, and made up to the mark with isooctane. The oil solution (5 mL) was pipetted to a clean screw-capped test tube, and 1 mL of anisidine reagent (0.25% solution of anisidine in glacial acetic acid) was added. Thereafter, the tube was closed, vortexed and kept in the dark at room temperature for 8 min. Within a further 2 min, the solution was transferred to a clean, dry spectrophotometer cell, and the absorbance was read at 350 nm. The experiment was repeated with an adjusted amount of oil whenever the measured absorbance was outside of the range 0.2 to 0.8. Unreacted test solution was similarly prepared but instead of anisidine reagent, glacial acetic acid (1 mL) was added. For the blank, the oil solution was replaced with isooctane (5 mL).

The anisidine value was calculated using the following formula:

\[ AV = \frac{25}{m} \left[ 1.2 \times (A_1 - A_2 - A_0) \right] \]

Where: \(A_0\) is the absorbance of the unreacted test solution, \(A_1\) is the absorbance of the reacted solution, \(A_2\) is the absorbance of the blank, and \(m\) is the mass of the oil in grams.

3.2.11.2 Colour analysis (I, III)

The colour of oils was assessed according to AOCS Official method Cc 13c-50 (Firestone, 2009). Oil (1 g) was weighed into a 2 mL volumetric flask. The oil was dissolved, made up to the mark with isooctane, and the absorbance at 490 nm was read against an isooctane blank.
3.2.11.3 Peroxide value (VIII – X)

The method originally published by Hornero-Mendez et al. (2001) and modified by Shantha and Decker (1994) was used. Oil (200 mg) was dissolved in 5 mL of hexane; to 200 µL of the sample solution 5 mL of methanol/chloroform/HCl solution (1:1:0.012 v/v), then 100 µL of NH$_4$SCN (30% w/w in water), and 100 µL of ferrous chloride (0.4% water solution) were added. After 5 min of incubation at room temperature, the absorbance at 480 nm was measured.

3.2.12 Data analysis

Data were analyzed by single factor analysis of variance (ANOVA) and regression analyses using Minitab 2000 statistical software (Minitab Inc., PA, ver. 13.2). Statistically significant differences between means were determined by Duncan’s multiple range tests. Statistically significant differences were determined at the P < 0.05 level.
Chapter 4 – Results

4.1 Influence of temperature on the frying stability of oils (I)

4.1.1 Total polar compounds (TPC)

TPC is one of the most reliable indicators of frying oil quality, providing information on the total amount of degradation compounds formed from triacylglycerols (Dobarganes et al., 2000b). The amount of total polar compounds increased consistently with time of frying at a rate dependent on temperature. At the end of the 7th day, the TPC was at 19.8 and 37.9% for frying at 185 and 215°C, respectively. The rate of polar compounds formation was 2.6 times faster during frying at 215°C compared to 185°C (Figure 17).

![Figure 17. Formation of total polar compounds during frying at different temperatures](image-url)
4.1.2 Composition of polar materials

In Figures 18 and 19, the relative contributions of the different groups of polar materials in oils used for frying at 185 and 215°C are presented. The contribution of polymers increased consistently with frying time at both frying temperatures, peaking at 8.0 and 15.6% for frying at 185 and 215°C, respectively. During frying at 185°C, the amount of dimers increased steadily from the initial 2% in the fresh oil to 33.6% at the end of the frying period (Figure 18). At 215°C, however, after the initial 16-fold increase at the end of the 1st day of frying, followed by a slight increase for the next two days, the amount of dimers consistently decreased for the rest of the frying time (Figure 19). The contribution of oxidized triacylglycerides steadily decreased from the initial 72.6 to 41.3% during frying at 185°C, and to 23.4% during frying at 215°C.
Figure 18. Changes in composition of polar materials during frying at 185°C. DG – diacylglycerols, OTG – oxidized triacylglycerols, dimers – dimers of triacylglycerols, polymers – polymers of diacylglycerols.

Figure 19. Changes in composition of polar materials during frying at 215°C. See Figure 18 for abbreviation.
4.1.3 Fatty acid composition

The fatty acid compositions of the fresh canola oil and the resultant changes during the 7-day of frying at 185 and 215°C in Table 9 are presented. A progressive decrease in the contributions of major polyunsaturated fatty acids was observed throughout the entire frying time. The contribution of linoleic acid (C18:2) decreased significantly from 18.9% in the fresh oil to 17.3% during frying at 185°C, and to 15.6% at 215°C. The deterioration of linolenic acid (C18:3) was more pronounced and showed a significant decrease of 24 and 57% at 185 and 215°C, respectively.

The amount of trans fatty acids formed during frying increased when temperature and time increased (Figures 20 and 21). During frying at 185°C, the contribution of total trans fatty acids significantly increased from the initial value of 2.4% to 3.3% at the 7th day of frying. Over the same period during frying at 215°C, the amount of trans isomers in oil increased 2.5 fold, from 2.4 to 5.9% (Figure 20). Regarding isomerisation of individual unsaturated fatty acids, the amount of trans 18:3 significantly increased from 1.6 to 2.0% during frying at 185°C, and to 2.8% at 215°C. Contribution from trans 18:2 increased from 0.1% in the initial oil to 0.4 and 1.0% at 185 and 215°C, respectively. The formation of trans 18:1 was also statistically significant, increasing from 0.7 to 0.9% at 185°C, and to 2.0% at 215°C over the 7 days of frying (Figure 21).

Analysis of the oxidized short-chain fatty acids as a group revealed a consistent increase in the contribution of oxidized fatty acids for the first 5 days of frying at 185°C, reaching a maximum at 1.8% (Figure 22). For the oil heated at 215°C, however, a considerable increase in the amount of oxidized fatty acids was observed in the first 3
days of frying with the maximum at 2.2%. Thereafter, the observed contribution decreased for the next 3 days of frying, lowering its contribution to 1.3% (Figure 22).

Table 9. Changes in contribution of canola oil fatty acid at different frying temperatures

<table>
<thead>
<tr>
<th>Frying time (h)</th>
<th>Contribution(^a) (relative percentage)</th>
<th>C(^{16:0})</th>
<th>C(^{18:0})</th>
<th>C(^{18:1})</th>
<th>C(^{18:2})</th>
<th>C(^{18:3\alpha})</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>185(^\circ)C</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td></td>
<td>4.00 ± 0.01</td>
<td>1.82 ± 0.02</td>
<td>60.03 ± 0.72</td>
<td>18.91 ± 0.16</td>
<td>8.40 ± 0.09</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>4.14 ± 0.03</td>
<td>1.91 ± 0.06</td>
<td>61.15 ± 0.61</td>
<td>18.10 ± 0.10</td>
<td>7.46 ± 0.05</td>
</tr>
<tr>
<td>14</td>
<td></td>
<td>4.22 ± 0.02</td>
<td>2.01 ± 0.03</td>
<td>61.35 ± 0.86</td>
<td>18.01 ± 0.16</td>
<td>7.16 ± 0.13</td>
</tr>
<tr>
<td>21</td>
<td></td>
<td>4.24 ± 0.07</td>
<td>2.01 ± 0.04</td>
<td>61.78 ± 0.64</td>
<td>17.90 ± 0.21</td>
<td>7.11 ± 0.11</td>
</tr>
<tr>
<td>28</td>
<td></td>
<td>4.25 ± 0.08</td>
<td>2.02 ± 0.08</td>
<td>61.96 ± 0.95</td>
<td>17.84 ± 0.21</td>
<td>6.85 ± 0.09</td>
</tr>
<tr>
<td>35</td>
<td></td>
<td>4.27 ± 0.06</td>
<td>2.02 ± 0.03</td>
<td>61.97 ± 0.91</td>
<td>17.85 ± 0.18</td>
<td>6.78 ± 0.08</td>
</tr>
<tr>
<td>42</td>
<td></td>
<td>4.30 ± 0.08</td>
<td>2.02 ± 0.04</td>
<td>61.98 ± 0.84</td>
<td>17.81 ± 0.22</td>
<td>6.56 ± 0.11</td>
</tr>
<tr>
<td>49</td>
<td></td>
<td>4.46 ± 0.05</td>
<td>2.03 ± 0.04</td>
<td>61.98 ± 0.68</td>
<td>17.27 ± 0.17</td>
<td>6.39 ± 0.10</td>
</tr>
<tr>
<td><strong>215(^\circ)C</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>7</td>
<td></td>
<td>4.19 ± 0.03</td>
<td>1.93 ± 0.04</td>
<td>61.20 ± 0.78</td>
<td>17.92 ± 0.19</td>
<td>6.79 ± 0.08</td>
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<tr>
<td>14</td>
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<td>4.27 ± 0.05</td>
<td>1.99 ± 0.05</td>
<td>61.77 ± 0.97</td>
<td>17.32 ± 0.17</td>
<td>5.68 ± 0.06</td>
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<td>4.34 ± 0.10</td>
<td>2.02 ± 0.03</td>
<td>62.37 ± 1.01</td>
<td>17.03 ± 0.18</td>
<td>5.13 ± 0.08</td>
</tr>
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<td>4.37 ± 0.09</td>
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<td>62.43 ± 0.77</td>
<td>16.43 ± 0.19</td>
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<td>2.06 ± 0.05</td>
<td>63.16 ± 0.63</td>
<td>16.25 ± 0.17</td>
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<td></td>
<td>4.61 ± 0.06</td>
<td>2.11 ± 0.06</td>
<td>63.46 ± 0.72</td>
<td>15.90 ± 0.18</td>
<td>3.84 ± 0.05</td>
</tr>
<tr>
<td>49</td>
<td></td>
<td>4.81 ± 0.09</td>
<td>2.18 ± 0.09</td>
<td>63.50 ± 0.98</td>
<td>15.54 ± 0.23</td>
<td>3.59 ± 0.04</td>
</tr>
</tbody>
</table>

\(^a\) All values are averages of triplicate analysis
Figure 20. Changes in total and linolenic acid trans isomers contributions during frying at different temperatures.

Figure 21. Changes in oleic and linoleic acid trans isomers contributions during frying at different temperatures.
4.1.4 Anisidine value

At the two frying temperatures, the anisidine value was not well-correlated with the frying time (Figure 23). The maximum value was achieved at the 3rd day of frying, increasing its value from 4.2 in the fresh oil to 129 at 185°C, and to 110 at 215°C. Thereafter, a consistent decrease until the end of frying time was observed, with the minimum value being at 110 and 79 for frying conducted at 185 and 215°C, respectively (Figure 23).
4.1.5 Colour

The amount and rate of formation of colour components in the oils increased with frying time and frying temperatures. At the end of the 7th day of frying, the absorbance of the oil increased from the initial value of 0.001 to 0.397 during frying at 185°C, and to 0.888 for frying at 215°C. The rate of formation of colour components was 2.2 times faster during frying at 215°C compared to frying conducted at 185°C (Figure 24).

Figure 23. Changes in anisidine values during frying at different temperatures
4.1.6 Residual tocopherols

The concentration of tocopherols in the frying oils decreased when frying temperature and time increased. At the end of the 7th day of frying, the total amounts of tocopherols decreased from an initial amount of 561 µg/g in the fresh oil to 175 µg/g during frying at 185°C. The entire tocopherols were, however, completely spent at the end of 6th day of frying at 215°C. At 185°C frying temperature, γ-tocopherol degraded faster than α-tocopherol whereas at 215°C, the order was reversed (Figure 25).
4.2 Influence of oxygen on the frying stability of oils and formation of toxic compounds (III)

4.2.1 Total polar compounds

To assess the effect of oxygen on frying stability of oils, French fries were fried using standard protocol (SFC) as control and utilizing carbon dioxide blanketing (CDB) and vacuum frying (VF). Irrespective of the frying protocols, the amount of total polar compounds consistently increased with frying time. At the end of the 7th day of frying, the amount of total polar compounds increased from an initial 3.3% to 22.2, 10.3, and 5.4% during standard frying, CDB and VF, respectively. The average rate of total polar compounds formation was 2.4, 1.2, and 0.3% per frying day during SFC, CDB, and VF,
respectively (Figure 26). At this rate, the oils will attain the 24% TPC discard level within 10, 21, and 83 days of frying under SFC, CDB, and VF, respectively.

![Graph showing the formation of TPC during frying under different conditions](image)

**Figure 26.** Formation of TPC during frying under different conditions

4.2.2 Residual tocopherols

The amounts of tocopherols in oils consistently decreased as frying time increased at a rate affected by frying protocol. At the end of frying period, 15, 70, and 90% of the initial amounts of tocopherols was detected in oils used for frying under SFC, CDB, and VF, respectively. The average rate of tocopherol degradation was 82, 17, and 7 µg/g per frying day for SFC, CDB, and VF, respectively (Figure 27).
Figure 27. Tocopherol degradation during frying under different conditions

4.2.3 Concentrations of HNE in French fries (IV)

Irrespective of the frying protocol, HNE concentrations in prepared fries increased consistently throughout the entire frying time (Figure 28). At the end of the frying period, 6.8 and 3.1 µg/g of HNE were found in fries prepared under SFC and CDB, respectively (Figure 28).
4.2.4 Concentrations of acrylamide in French fries (IV)

The acrylamide concentrations in French fries fried under SFC and CDB are presented in Figure 29. Under both frying protocols, acrylamide formation was not well correlated with the frying time. For the first day of frying, there was no significant difference between both frying conditions in the formation of acrylamide. At the 3rd day of frying, however, 125 and 38 µg/kg of acrylamide were found in fries prepared under SFC and CDB, respectively. Irrespective of the frying protocol, the lowest amount of acrylamide was found in fries prepared at the 4th day of frying (Figure 29).
4.3 Development of procedure for HNE quantification (IV)

The existing clean up procedure for HNE in frying oil is laborious, and the complexity of degradation products makes it difficult to obtain baseline separation of HNE peak from other components, hence, the need for a simple and reliable procedure for HNE analysis (IV). Preliminary study showed that methanol was a more efficient eluant than acetonitrile, and that modifying the methanol with formic acid did not increase its efficiency (Table 10). Furthermore, increasing the volume of methanol above 20 mL did not result in additional recovery of HNE (Table 10).

The developed method was compared with two commonly used methods to assess recovery of HNE recovery from canola oil. The results for four concentrations are
presented in Table 11. Solvent extraction with methanol-water resulted in 0, 51, 68 and 71% recovery when assessed for the following levels of the compound: 0.1, 1, 5 and 10 µg/g, respectively. The developed procedure offered recovery of 82, 103, 91 and 93%, respectively. The most commonly used procedure utilizing DNPH derivatives provided accordingly recovery at 0, 72, 80 and 85% for the same concentration of standard.

Table 10. Recovery of HNE using different eluants

<table>
<thead>
<tr>
<th>HNE added (µg/g)</th>
<th>Recovery (µg/g)</th>
<th>Recovery (µg/g)</th>
<th>Recovery (µg/g)</th>
<th>Recovery (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Methanol</td>
<td>Methanol + Formic acid (99.9:0.1, v/v)</td>
<td>Acetonitrile</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15 mL</td>
<td>20 mL</td>
<td>25 mL</td>
<td>20 mL</td>
</tr>
<tr>
<td>5</td>
<td>4.28 ± 0.21</td>
<td>4.54 ± 0.23</td>
<td>4.58 ± 0.14</td>
<td>4.63 ± 0.31</td>
</tr>
<tr>
<td>10</td>
<td>8.71 ± 0.35</td>
<td>9.31 ± 0.48</td>
<td>9.25 ± 0.23</td>
<td>9.40 ± 0.57</td>
</tr>
</tbody>
</table>
Table 11. Recovery of isolated HNE from spiked canola oil samples by different methods

<table>
<thead>
<tr>
<th>Method</th>
<th>HNE added (µg/g)</th>
<th>HNE found (µg/g)</th>
<th>HNE recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent Extractiona</td>
<td>0.1 0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1 0.51 ± 0.03</td>
<td>0.51 ± 0.03</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>5 3.47 ± 0.19</td>
<td>3.47 ± 0.19</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>10 7.09 ± 0.58</td>
<td>7.09 ± 0.58</td>
<td>71</td>
</tr>
<tr>
<td>HNE-DNPHb</td>
<td>0.1 0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1 0.72 ± 0.07</td>
<td>0.72 ± 0.07</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>5 3.98 ± 0.40</td>
<td>3.98 ± 0.40</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>10 8.50 ± 0.88</td>
<td>8.50 ± 0.88</td>
<td>85</td>
</tr>
<tr>
<td>Developed Method</td>
<td>0.1 0.08 ± 0.00</td>
<td>0.08 ± 0.00</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>1 1.03 ± 0.05</td>
<td>1.03 ± 0.05</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>5 4.54 ± 0.23</td>
<td>4.54 ± 0.23</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>10 9.31 ± 0.48</td>
<td>9.31 ± 0.48</td>
<td>93</td>
</tr>
</tbody>
</table>

a Method by Lang et al. (1985); b Method by Seppanen and Csallany (2006)

4.4 Development of a frying test (V)

The large number of frying experiments that was required to evaluate the effects of minor components on frying performance, and the limited amount of synthesized novel antioxidants demanded the development of a reliable and fast frying test to assess frying performance in small sized samples of oils (V). Preliminary results showed that there was no difference in the amount of total polar compounds formed at the end of 2 h of heating with silica gel, alumina and celite as additives (Table 12). Likewise, water content of the additives did not lead to an appreciable difference in the amount of TPC, although the contribution of diacylglycerides in polar materials increased when the water
content increased to 40\% (Table 13). Similarly, the amount of TPC increased when the surface-to-volume ratio and rate of oil stirring increased (Table 12).

Table 12. Effect of selected compounds and parameters on the formation of polar components during the frying test

<table>
<thead>
<tr>
<th>Additives</th>
<th>Water content (%)</th>
<th>S/V</th>
<th>Stirring at (rpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Silica gel</td>
<td>10%</td>
<td>0.75</td>
<td>14.1±0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.42</td>
<td>10.9±0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.27</td>
<td>7.5±0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.75</td>
<td>13.3±1.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.42</td>
<td>10.3±0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.27</td>
<td>7.5±0.5</td>
</tr>
<tr>
<td></td>
<td>20%</td>
<td>0.75</td>
<td>14.8±0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.42</td>
<td>11.0±0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.27</td>
<td>8.6±0.7</td>
</tr>
<tr>
<td></td>
<td>40%</td>
<td>0.75</td>
<td>15.7±1.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.42</td>
<td>10.9±0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.27</td>
<td>7.0±0.4</td>
</tr>
</tbody>
</table>

Samples were heated for 2 h at 185 ± 5°C; S/V – Ratio of oil surface area to volume
The effects of various additives on the thermo-oxidative degradation of oil under optimized conditions: heating time; stirring rate; surface-to-volume ratio; food-to-oil ratio; and water contents are reported in Table 13. At the end of the 2\textsuperscript{nd} h of heating, the amount of TPC increased from 3.8\% in the fresh oil to 24.5 and 26.7\% when CuSO\textsubscript{4} and FeSO\textsubscript{4} was used, respectively. Although, the TPC in oils heated with silica gel and a potato starch mixture were only marginally higher compared to the control, the composition of different groups of the polar materials was significantly different. Generally, oils heated with salts showed a significantly lower amount of residual tocopherols (5.1 – 7.3\%) compared to samples without salts. The optimal mimicking food formulation to reproduce standard frying was found to be a mixture of gelatinized potato starch, glucose and silica gel (4:1:1, w/w) (Table 13).
Table 13. Formation of polar components and degradation of tocopherols during frying and the frying test using canola oil and various formulations mimicking food (%)

<table>
<thead>
<tr>
<th>Component</th>
<th>TPC</th>
<th>Polymer</th>
<th>Dimers</th>
<th>OxTAG</th>
<th>DG</th>
<th>RTOC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canola oil</td>
<td>20.8±1.7</td>
<td>6.4±0.4</td>
<td>29.8±1.9</td>
<td>61.2±3.9</td>
<td>2.3±0.2</td>
<td>18.3±2.0</td>
</tr>
<tr>
<td>Silica (10% water)</td>
<td>22.2±2.4</td>
<td>8.8±0.3</td>
<td>33.2±2.4</td>
<td>54.7±3.1</td>
<td>2.4±0.1</td>
<td>14.8±1.2</td>
</tr>
<tr>
<td>Silica (20% water)</td>
<td>22.9±1.9</td>
<td>8.1±0.2</td>
<td>32.9±3.0</td>
<td>56.1±3.7</td>
<td>2.7±0.2</td>
<td>10.9±1.0</td>
</tr>
<tr>
<td>Silica (40% water)</td>
<td>21.8±2.0</td>
<td>7.9±0.4</td>
<td>35.3±2.1</td>
<td>52.2±4.0</td>
<td>3.9±0.2</td>
<td>15.1±1.3</td>
</tr>
<tr>
<td>CuSO₄ (50µg/g)</td>
<td>24.5±2.1</td>
<td>6.1±0.5</td>
<td>32.2±2.1</td>
<td>60.1±4.2</td>
<td>1.6±0.1</td>
<td>6.3±0.5</td>
</tr>
<tr>
<td>FeSO₄ (50µg/g)</td>
<td>26.7±2.5</td>
<td>6.9±0.2</td>
<td>37.8±3.0</td>
<td>52.5±2.8</td>
<td>1.9±0.1</td>
<td>5.1±0.5</td>
</tr>
<tr>
<td>Silica + CuSO₄ (50µg/g)</td>
<td>24.9±2.3</td>
<td>7.7±0.5</td>
<td>34.1±3.1</td>
<td>55.8±2.1</td>
<td>2.1±0.1</td>
<td>5.8±0.3</td>
</tr>
<tr>
<td>Silica + FeSO₄ (50µg/g)</td>
<td>25.6±2.0</td>
<td>8.1±0.5</td>
<td>38.4±2.5</td>
<td>51.8±3.0</td>
<td>1.7±0.1</td>
<td>7.3±0.4</td>
</tr>
<tr>
<td>Potato starch</td>
<td>22.1±1.9</td>
<td>7.6±0.3</td>
<td>32.1±2.9</td>
<td>57.5±3.3</td>
<td>2.5±0.1</td>
<td>14.1±1.4</td>
</tr>
<tr>
<td>Hydrolyzed starch</td>
<td>20.2±1.8</td>
<td>8.9±0.6</td>
<td>32.0±2.7</td>
<td>56.3±2.7</td>
<td>2.8±0.2</td>
<td>15.2±1.1</td>
</tr>
<tr>
<td>(Starch+glucose+silica)</td>
<td>23.7±1.5</td>
<td>13.3±0.9</td>
<td>36.4±2.1</td>
<td>44.7±3.0</td>
<td>4.5±0.2</td>
<td>12.0±1.1</td>
</tr>
<tr>
<td>Institutional frying</td>
<td>24.9±2.0</td>
<td>15.6±1.2</td>
<td>32.6±1.6</td>
<td>41.1±2.9</td>
<td>9.6±0.4</td>
<td>13.3±0.8</td>
</tr>
</tbody>
</table>

Testing conditions: temperature 185 ± 5°C; time 2h; the ratio of oil surface to volume at 0.42. a Apart from the salts, all components were added at 10% of the oil weight. b Canola oil heated at the frying test conditions. c Added silica gel containing 40% water. d A formulated food containing gelatinized starch, glucose, silica gel and water at 4:1:1:65 w/w. e Values from the 7th day of actual frying using canola oil.

4.5 Influence of minor components on frying stability (VI, VII)

4.5.1 Isolation, processing, and purification of minor components

The amount of respective minor components, and those of the corresponding fractions isolated from canola, rice bran, sesame, and palm oils are presented in Table 14.
With alumina as adsorbent, separation of 500 g of canola and rice bran oils provided 51.7 and 56.2 g of minor components, respectively. When 200 g of rice bran, sesame and palm oils were extracted with solvent, 9.4, 6.7 and 4.3 g of minor components were obtained, respectively (Table 14).

Using silica gel as an adsorbent for separation of minor components, 20 g of canola oil minor components provided 2.5 and 15.3 g of tocopherol and sterol fractions, respectively. The same amount of rice bran oil minor components yielded 1.8 and 14.1 g of tocopherol and sterol fractions, respectively. Further purification of the tocopherol fraction (1 g) by preparative thin layer chromatography (PTLC) provided 0.16 and 0.31 g of tocopherol fractions from canola and rice bran oils, respectively. Similarly, PTLC purification of the sterol fraction yielded 0.22 and 0.40 g of canola and rice bran sterol fractions, respectively.

Table 14. Amount of non-triacylglycerides isolated from different oils (g)

<table>
<thead>
<tr>
<th>Isolated method</th>
<th>Oil</th>
<th>Minor components</th>
<th>Tocopherol Fraction</th>
<th>Sterol Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromatography</td>
<td>Canola</td>
<td>51.7 ± 3.8</td>
<td>1.0 ± 0.1</td>
<td>8.7 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>Rice bran</td>
<td>56.2 ± 4.1</td>
<td>1.6 ± 0.1</td>
<td>15.9 ± 1.3</td>
</tr>
<tr>
<td>Solvent extraction</td>
<td>Rice bran</td>
<td>9.4 ± 0.8</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td></td>
<td>Sesame</td>
<td>6.7 ± 0.5</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td></td>
<td>Palm</td>
<td>4.3 ± 0.4</td>
<td>na</td>
<td>na</td>
</tr>
</tbody>
</table>

*Weight of oil used = 500 g; *Weight of oil used = 200 g; na = not applicable
4.5.2 Composition of isolated fractions

HPLC indicated that the canola tocopherol fraction (TCAN) was composed of 3.7% steryl esters, 4.8% triacylglycerols, 2.3% diacylglycerols, 82.2% tocopherols, and 7.0% other components (Table 15). The composition of the TCAN tocopherol homologous mixture was: 27.0, 50.5, and 4.7% α-, γ-, and δ-tocopherols, respectively (Figure 30, Table 15). GC analysis of the TCAN fatty acid composition showed that it contained 4, 2, 61, 21, and 10% palmitic, stearic, oleic, linoleic, and linolenic acids, respectively (Table 16). The tocopherol fraction from rice bran oil (TRBO) consisted of 7.6, 5.2, 3.7, 79.1, and 4.1% steryl esters, triacylglycerols, diacylglycerols, tocopherols, and other components, respectively. The HPLC and HPLC-MS analyses of the TRBO showed that it contained 13.9, 1.0, 17.9, 3.5, 8.4, 31.7, and 2.8% α-tocopherol, β-tocopherol, γ-tocopherol, δ-tocopherol, α-tocotrienol, γ-tocotrienol, and δ-tocotrienol, respectively (Figure 31, Table 15). The fatty acid composition of TRBO was typical of rice bran oil, being 11, 2, 50, 33, and 2% palmitic, stearic, oleic, linoleic, and linolenic acids, respectively (Table 16).

As analyzed by HPLC, minor components isolated from rice bran oil by solvent extraction (RBOS) were composed of 7.4% steryl esters, 12.5% triacylglycerols, 5.6% diacylglycerols, 1.4% free fatty acids, 13.9% tocopherols, 43.3% sterols, 10.5% γ-oryzanol, and 5.4% other components (Table 15). The major components of the γ-oryzanol in RBOS were identified as cycloartenyl ferulate, 24-methylenecholesterol ferulate, stigmasteryl ferulate, campesteryl ferulate, sitosteryl ferulate, and sitostanyl ferulate (Figure 32). GC analysis of RBOS fatty acid methyl esters (FAMEs) showed that it contained 7, 2, 44, 43, and 3% palmitic, stearic, oleic, linoleic, and linolenic acids,
respectively (Table 16). The minor components from palm oil, POS consisted of 3.8% steryl esters, 18.9% triacylglycerols, 9.5% diacylglycerols, 1.6% monoacylglycerols, 2.7% free fatty acids, 28.5% tocopherols, 20.9% sterols, 2.7% carotenoids, and 11.4% other components (Table 15). POS tocopherol homologous mixture consisted of 2.5, 4.9, 6.0, 9.7, and 5.4% α-tocopherol, δ-tocopherol, α-tocotrienol, γ-tocotrienol, and δ-tocotrienol, respectively. Campesterol and β-sitosterol were the major sterols detected in POS, accounting for 17.9 and 81.2% of the total sterols, respectively (Table 15). POS contained 33, 3, 44, 15, and 1% palmitic, stearic, oleic, linoleic, and linolenic acids, respectively, compared to 43, 4, 41, 10, and 0% respective amounts in the original palm oil (Table 16).
Figure 15. Composition of endogenous minor components isolated from canola, rice bran and palm oils

<table>
<thead>
<tr>
<th>Components</th>
<th>TCAN</th>
<th>TRBO</th>
<th>SCAN</th>
<th>SRBO</th>
<th>RBOS</th>
<th>POS</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-tocopherol</td>
<td>269.8±19</td>
<td>139.1±11</td>
<td>ND</td>
<td>ND</td>
<td>18.1±1.5</td>
<td>25.4±2.3</td>
</tr>
<tr>
<td>β-tocopherol</td>
<td>ND</td>
<td>9.8±0.5</td>
<td>ND</td>
<td>ND</td>
<td>0.4±0.1</td>
<td>ND</td>
</tr>
<tr>
<td>γ-tocopherol</td>
<td>505.1±39</td>
<td>178.9±11</td>
<td>ND</td>
<td>ND</td>
<td>37.2±1.4</td>
<td>ND</td>
</tr>
<tr>
<td>δ-tocopherol</td>
<td>47.3±3</td>
<td>34.5±2.5</td>
<td>ND</td>
<td>ND</td>
<td>10.7±0.4</td>
<td>48.6±1.9</td>
</tr>
<tr>
<td>α-tocotrienol</td>
<td>ND</td>
<td>83.5±4.4</td>
<td>ND</td>
<td>ND</td>
<td>17.5±1.6</td>
<td>59.8±4.3</td>
</tr>
<tr>
<td>β-tocotrienol</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>γ-tocotrienol</td>
<td>ND</td>
<td>317.4±24</td>
<td>ND</td>
<td>ND</td>
<td>51.8±5.0</td>
<td>97.4±5.4</td>
</tr>
<tr>
<td>δ-tocotrienol</td>
<td>ND</td>
<td>27.9±2.1</td>
<td>ND</td>
<td>ND</td>
<td>3.6±0.2</td>
<td>53.8±3.7</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>822.2±57</td>
<td>791.1±40</td>
<td></td>
<td></td>
<td>139.3±7.2</td>
<td>285.0±15</td>
</tr>
<tr>
<td>Brassicasterol</td>
<td>4.9±0.3</td>
<td>ND</td>
<td>115.0±9.3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Campesterol</td>
<td>11.9±1.0</td>
<td>7.9±0.4</td>
<td>264.1±16</td>
<td>143.5±10</td>
<td>63.8±4.1</td>
<td>44.7±2.3</td>
</tr>
<tr>
<td>Stigmasterol</td>
<td>1.8±0.1</td>
<td>6.0±0.4</td>
<td>14.5±1.2</td>
<td>157.2±11</td>
<td>78.1±4.9</td>
<td>1.5±0.1</td>
</tr>
<tr>
<td>β-Sitosterol</td>
<td>16.0±1.2</td>
<td>29.1±1.3</td>
<td>488.5±38</td>
<td>476.2±24</td>
<td>262.3±18</td>
<td>202.8±12</td>
</tr>
<tr>
<td>Δ5-Avenasterol</td>
<td>1.9±0.1</td>
<td>3.3±0.2</td>
<td>10.3±0.7</td>
<td>10.8±0.6</td>
<td>13.5±0.9</td>
<td>0.6±0.1</td>
</tr>
<tr>
<td>Δ7-Avenasterol</td>
<td>ND</td>
<td>3.5±0.2</td>
<td>5.1±0.6</td>
<td>17.5±1.4</td>
<td>16.0±1.3</td>
<td>ND</td>
</tr>
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<td>ND</td>
<td>ND</td>
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<td>27.2±1.9</td>
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<td>81.2±4.7</td>
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<td>53.5±4.8</td>
<td>114.0±11</td>
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* Methylenecycloartanol; ND – not detected. See text for abbreviation.
Table 16. Fatty acid composition of isolated minor components used in the study

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<th>Minor components</th>
<th>Fatty acids [mg / 100 mg]</th>
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<tr>
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<tr>
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<td>RBOS</td>
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<td>POS</td>
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<tr>
<td>Canola oil</td>
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<tr>
<td>Rice bran oil</td>
<td>13.78 ± 0.48</td>
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<tr>
<td>Palm oil</td>
<td>42.52 ± 1.77</td>
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</table>
Figure 30. Chromatogram of tocopherols fraction isolated from canola oil (TCAN).
See text for HPLC conditions. a – α-tocopherol; b – γ-tocopherol; c – plastochromanol; d – δ-tocopherol

Figure 31. Chromatogram of tocopherols fraction isolated from rice bran oil (TRBO).
See text for HPLC conditions. a – α-tocopherol; b – α-tocotrienol; c – β-tocopherol; d – γ-tocopherol; e – γ-tocotrienol; f – δ-tocopherol; g – δ-tocotrienol
4.5.3 Formation of polar components, volatile carbonyl compounds and hydroxynonenal (VI, VII)

In Figures 33 – 35, the effects of endogenous minor components on the TAG degradation as described by amount of total polar components (TPC) are presented. At the end of the frying test, the amount of TPC was at 24.3% in pure TAG, compared to 17.5, 18.0, 14.2, 13.8, 14.2, 14.5, 18.4, 9.9, and 10.5% in TAG containing TCAN, TRBO, SCAN, SRBO, RBOS, SOS, POS, PC and PE, respectively. Both SCAN and SRBO resulted in a significant reduction in the amount of TPC when applied at 3000 µg/g,
whereas only SRBO was effective when applied at 500 µg/g (Figure 33). At the end of the frying test, the amount of TPC was 18.4, 18.0, and 17.4% in TAG when supplemented with the following tocopherol homologous mixture: 450α + 450γ + 100δ; 450α + 100γ + 450δ; and 100α + 450γ + 450δ µg/g, respectively (Figure 36). Irrespective of the composition of the homologous mixture, increasing the concentration of tocopherols above 1000 µg/g did not result in further reduction in the amount of TPC at the end of the frying test (Figure 36). Similarly, the amount of TPC was at 15.9, 16.8, 15.4, 17.0, 17.8, and 18.8% when TAG were fortified with ethyl ferulate (EF), caffeic acid (CA), dihydrocaffeic acid (HCA), ferulic acid (FA), gallic acid (GA), and vanillic acid (VA), respectively (Figure 37).
Figure 33.  Formation of polar components during test frying of antioxidant-free canola triacylglycerols containing different amounts of tocopherol and sterol fractions isolated from canola and rice bran oils.

See text for abbreviations.
Figure 34. Formation of polar components during test frying of antioxidant-free canola triacylglycerols containing 5000 µg/g of endogenous minor components isolated from different oils. See text for abbreviations.
Figure 35. Formation of polar components during test frying of antioxidant-free canola triacylglycerols containing different amounts of phosphatidylcholine and phosphatidylethanolamine. See text for abbreviations.
Figure 36. Formation of polar components during test frying of antioxidant-free canola triacylglycerols containing different combinations and amounts of tocopherol isomers. Tocopherol concentrations are in µg/g.

See text for abbreviations.
Figure 37. Formation of polar components during test frying of antioxidant-free canola triacylglycerols containing different phenolic acids and minor components isolated from canola oil. Phenolic acid concentrations - 500 µg/g; SCAN – 3000 µg/g; TCAN – 1000 µg/g.
See text for abbreviations
In Table 17, the rate of emission of volatile carbonyl compounds (VCC) formed during frying in TAGs with and without endogenous minor components are presented. At the end of the frying test, the rates of emission of volatile carbonyl compounds from pure TAG and TAG fortified with TCAN, TRBO, SRBO, SCAN, POS, RBOS, and SOS were 426, 190, 210, 178, 190, 226, 159, and 180 µg/g/h, respectively. Within the same period, the rate of VCC emission was 122 and 138 µg/g/h for TAG supplemented with phosphatidylethanolamine (PE) and phosphatidylcholine (PC), respectively (Table 17). Compared to pure TAG, the rate of emission of VCC was reduced by 42, 58, 54, 52, 57, and 56% when the CTG was supplemented with vanillic acid (VA), ethyl ferulate (EF), gallic acid (GA), ferulic acid (FA), caffeic acid (CA), and dihydrocaffeic acid (HCA), respectively (Table 17). The VCC profile was independent of the composition of tocopherol isomers, sterols and the type of phenolic acids present in the mixture; in all cases, propanal was the most abundant volatile carbonyl compound, followed by 2-propenal and pentanal (Table 17).
Table 17. Rate of volatile carbonyl compounds emission (µg/g/h) during test frying with antioxidant-free canola triacylglycerols fortified with various minor components

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<th>45.0+45.0</th>
<th>65.0+65.0</th>
<th>85.0+85.0</th>
<th>105.0+105.0</th>
<th>125.0+125.0</th>
<th>145.0+145.0</th>
<th>165.0+165.0</th>
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<td>15.1</td>
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Table 17 cont’d

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<th>POS (0.5%)</th>
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<th>SROBO (3000 µg/g)</th>
<th>TRBO (1000 µg/g)</th>
<th>TCAN (1000 µg/g)</th>
<th>PC (2000 µg/g)</th>
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<td>189.5c</td>
<td>137.6i</td>
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Table 17 cont’d

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<th>EF</th>
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<td>0.5d</td>
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<td>0.1b</td>
<td>0.1b</td>
<td>0.1b</td>
<td>0.1b</td>
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<tr>
<td>Total</td>
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<td>178.8cfe</td>
<td>197.2e</td>
<td>203.2ce</td>
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<td>178.0cfe</td>
<td>189.7ce</td>
<td>189.5ce</td>
</tr>
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</table>

Values with the same superscript in the same row are not significantly different at p < 0.05. See text for abbreviations.
All the phenolic acids significantly reduced the amount of HNE formed during the frying test. At the end of the frying test the following amounts of HNE were found: 11.2 µg/g for pure TAG; 5.9, 7.1, 6.0, 5.7, 6.0, and 6.3 µg/g while TAG were supplemented with EF, VA, CA, HCA, FA, and GA, respectively (Figure 38). Thus, the amount of HNE found in TAG fortified with VA was significantly higher than those detected in TAG containing any of the other phenolic acids. However, effects of FA, CA, EF, HCA and GA were not significantly different. TAG containing tocopherol homologous mixtures contained significantly lower amount of HNE compared to pure TAG (Figure 38). At the end of the frying test, the amount of HNE accumulated in TAG without tocopherols was at 11.7 µg/g, compared to a maximum of 6.2 µg/g in TAG fortified with combination of tocopherol isomers. However, different tocopherol homologous mixtures affected HNE formation in the same manner (Figure 38). Compared to pure TAG, the rate of HNE formation was reduced by 64, 48, 57, 57, and 51% in TAG supplemented with RBOS, POS, SRBO, SCAN, and TCAN, respectively (Figure 39).
Figure 38. Formation of hydroxynonenal during test frying of antioxidant-free canola triacylglycerols containing different phenolic acids and minor components isolated from canola oil. Phenolic acid concentrations - 500 µg/g; SCAN – 3000 µg/g; TCAN – 1000 µg/g. See text for abbreviations.
4.6 Novel antioxidants (VIII – X)

4.6.1 Synthesis

In Table 18, the physical descriptions, melting point and experimental yields of the synthesized antioxidants are presented. The calculated molecular mass agreed very well with the mass established by mass spectroscopy (Table 18). The $^1$H and $^{13}$C NMR
data for the novel antioxidants and their precursors are presented in the corresponding published/accepted manuscripts (VIII – X).

<table>
<thead>
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<th>Antioxidant</th>
<th>Description</th>
<th>Mp (°C)</th>
<th>Yield (%)</th>
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<th>[M + H]⁺ Found</th>
<th>Rotamers ratio</th>
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</tbody>
</table>

NA = Not Applicable; Mp = Melting point
4.6.2 Antioxidant evaluation under storage conditions (VIII – IX)

The ability of the novel antioxidants to protect polyunsaturated oil from oxidation was determined utilizing Schaal oven test. All the novel antioxidants significantly inhibited TAG oxidative degradation under accelerated storage conditions (Figure 40 – 42). After 5 days of storage, peroxide value of TAG was at 111 meq/kg, whereas for TAG fortified with antioxidants 1a – 3L were between 5.7 – 44.4 meq/kg. No significant difference (P ≥ 0.12) was observed in the protective capacities of novel antioxidants 1a (44.4 meq/kg), 1b (41.0 meq/kg), 1c (40.1 meq/kg), 1d (39.4 meq/kg), 2a (39.9 meq/kg), 2b (39.3 meq/kg) and α-tocopherol (45.6 meq/kg), whereas antioxidants 1e (9.4 meq/kg), 2c (15.9 meq/kg), 3 (14.0 meq/kg), and 3a – 3L (5.3 – 8.9 meq/kg) were significantly more efficient (P ≤ 0.001) than α-tocopherol (Figure 40 – 42). Compared to BHT (8.9 meq/kg), the following antioxidants: 3g (6.8 meq/kg), 3h (6.8 meq/kg), 3i (7.0 meq/kg), 3j (5.3 meq/kg), 3k (5.6 meq/kg), and 3L (5.7 meq/kg) were significantly more effective at protecting TAG under accelerated storage conditions. The dihydrocaffeic acid amides, 3a – 3L (X) were significantly more efficient than the trolox derivatives 1a – 1e, 2a – 2c, and 3 (VIII, IX).
Figure 40. Changes in peroxide formation during the storage of canola triacylglycerols with antioxidants 1a – 1e added at 300 µg/g.

Figure 41. Changes in peroxide formation during the storage of canola triacylglycerols with antioxidants 2a – 2c, 3 added at 300 µg/g.
Figure 42. Changes in peroxide formation during the storage of canola triacylglycerols with antioxidants 3a – 3L added at 300 µg/g. DCA – dihydrocaffeic acid; BHT – butylated hydroxytoluene; α-T – α-tocopherols. See text for abbreviations.
4.6.3 Antioxidant evaluation under frying conditions

The effectiveness of the novel antioxidants to protect TAG from thermo-oxidative degradation was evaluated under frying conditions using the frying test developed in this study. All the novel antioxidants significantly protected TAG against thermo-oxidative degradation (Figure 43 – 45). At the end of the frying test, the amount of total polar compounds accumulated in pure TAG was 25.7%, while the amounts accumulated in TAG fortified with the new antioxidants ranged from 14.6% to 18.6%. Furthermore, the novel antioxidants were significantly better (P ≤ 0.004) at protecting TAG during frying than α-tocopherol and BHT, the natural and synthetic reference antioxidants, respectively. No significant difference was observed, however, in the protective capacities of the following novel antioxidants: 1d (17.3%), 2b (17.8%), 3d (17.0%), 3e (17.0%), 3f (16.8%), 3g (17.9%), 3h (17.8%), 3i (16.9%), 3j (17.1%), 3k (16.9%), and 3L (17.5%). Among the novel antioxidants, compounds 1e (16.0%), 2c (15.8%), 3a (15.0%), 3b (14.7%), and 3c (14.6%) were the most effective.
Figure 43. Formation of polar components during test frying of canola triacylglycerols containing antioxidants 1a – 1e added at 500 µg/g.

Figure 44. Formation of polar components during test frying of canola triacylglycerols containing antioxidants 2a – 2c and 3 added at 500 µg/g. See text for abbreviations.
Figure 45. Formation of polar components during test frying of canola triacylglycerols containing antioxidants 3a – 3L added at 500 µg/g. DCA – dihydrocaffeic acid; BHT – butylated hydroxytoluene; α-T – α-tocopherols. See text for abbreviations.
4.6.4 Antioxidant stability

The amounts of antioxidant retained in the oil at the end of the accelerated storage and frying tests were analyzed as a measure of their stability. All the novel antioxidants exhibited significantly higher thermal stability (P ≤ 0.001) than α-tocopherol and BHT (Figures 46 – 51). At the end of the 5th day of storage, 35 and 49% of tocopherol and BHT, respectively remained in the TAG, while the amounts of novel antioxidants retained ranged from 60% to 75%. BHT and α-tocopherol were completely depleted at the end of the frying period, whereas up to 28% of the novel antioxidants still remained.

![Graph showing residual antioxidant percentages over storage time](image)

**Figure 46.** Percentage of remaining antioxidant during the accelerated storage of canola triacylglycerols containing α-tocopherols and antioxidants 1a – 1e added at 300 µg/g. See text for abbreviations.
Figure 47. Percentage of remaining antioxidant during test frying of canola triacylglycerols containing α-tocopherols and antioxidants 1a – 1e added at 500 µg/g. See text for abbreviations.

Figure 48. Percentage of remaining antioxidant during the accelerated storage of canola triacylglycerols containing α-tocopherols, antioxidants 2a – 2c and 3 added at 300 µg/g. See text for abbreviations.
Figure 49. Percentage of remaining antioxidant during test frying of canola triacylglycerols containing α-tocopherols, antioxidants 2a – 2c and 3 added at 500 µg/g. See text for abbreviations.
Figure 50. Percentage of remaining antioxidant during the accelerated storage of canola triacylglycerols containing antioxidants 3a – 3L added at 300 µg/g. See text for abbreviations.
Figure 51. Percentage of remaining antioxidant during test frying of canola triacylglycerols containing antioxidants 3a – 3L added at 500 µg/g. See text for abbreviations.
4.7 Formulation of antioxidants to enhance frying stability

To design frying oil with improved frying stability, combinations of the best performing natural endogenous minor components (VI: PC and RBOS) and synthetic antioxidants (VIII – X: Compounds 1e, 3a, 3) were formulated. The formulated antioxidants were added to refined, bleached and deodorized canola oil, as opposed to purified triacylglycerols (TAG) which was used during the preliminary investigations (VI – X). The amount of TPC formed at the end of the 2 h test frying in canola oil, and the fortified canola oil in Figure 52 is presented. Canola oil containing the synthetic dihydrocaffeic acid amide antioxidant (X: 3a) showed the lowest amount of TPC among the tested synthetic antioxidants, although the result was not significantly different from the sample containing the di-chromanol antioxidant (IX: 3). On the other hand, the synthetic gallic acid derivative (VIII: 1e) offered no additional stability compared to canola oil without added antioxidants. Furthermore, several combinations of the synthetic antioxidants offered no synergistic advantage over individual antioxidants 3a and 3 (Figure 52). Compared to canola oil alone, the amount of TPC was reduced by 2, 14, 16, 11, 15, 7, and 13\% when synthetic antioxidants 1e, 3, 3a, 3+1e, 3+3a, 3a+1e, and 3+3a+1e were applied, respectively.

At the end of the frying test, canola oil fortified with solvent-extracted minor components from rice bran oil (VI: RBOS) and phosphatidylcholine (VI: PC) showed no significant difference in TPC values at 15.7 and 16.1\%, respectively; however, the TPC in the sample containing a mixture of PC and RBOS was significantly lower (P \leq 0.04). Canola oil fortified with the quaternary mixture containing PC, RBOS, 3, and 3a, or the ternary mixture consisting of PC, RBOS, and 3a offered the lowest level of TPC at
13.7% among all the tested combinations. No significant difference (P > 0.05) was observed, however, between these antioxidant mixtures and the binary systems consisting of PC and RBOS (14.0%) and PC and 3a (14.3%). Generally, antioxidant combinations containing compound 1e showed higher TPC values compared to combination of antioxidants containing 3 or 3a. At the end of the frying test, the amount of TPC in canola sample was 19.1%, compared to 16.3, 15.5, 15.6, 15.3, 14.3, and 15.7% in canola oil fortified with RBOS+1e, RBOS+3, RBOS+3a, PC+3, PC+3a, and PC+1e, respectively (Figure 52)
Figure 52. Formation of polar components during test frying in canola oil containing different combination of antioxidants: PC at 1000 µg/g; RBOS at 5000 µg/g; 1e, 3, 3a, at 350 µg/g. See text for abbreviations.
4.8 Effects of formulated antioxidants during actual frying

4.8.1 Total polar components

As shown in Figure 52, antioxidant mixtures: (1) PC+RBOS+3+3a; (2) PC+RBOS+3a; (3) PC+RBOS; and (4) PC+3a, were the most effective among the possible other combinations of the best performing individual natural and synthetic antioxidants, namely, 1e, 3, 3a, PC, and RBOS. Although the developed frying test offered a reliable prediction of frying performance (V), actual frying still remains the most reliable method for assessing frying stability of oils and accordingly, the effectiveness of one of the most promising formulation of antioxidants (FA) was tested. In Figure 53 is presented the total amount of polar compounds formed during a 6-day frying in control canola oil and oil containing the formulated antioxidant PC+3a. At the end of the frying period the TPC formed in canola oil was 34.1% compared to 20.2% found in fortified canola oil. Compared to fortified canola oil, the rate of total polar compounds formation was 1.7 times higher in control oil (Figure 53).
4.8.2 Anisidine value

Similar to TPC results, anisidine values for control oil were consistently higher compared to oil containing the formulated antioxidant (FA) throughout the entire frying time (Figure 54). At the end of the frying, the anisidine value for canola oil was 2.3 times higher than in fortified canola oil.
4.8.3 Colour formation

The rate of colour components formation was 2.4 times higher in fortified canola oil than control oil (Figure 55). At the end of the frying, the absorbances of control oil and fortified oil were 0.312 and 0.710, respectively.
4.8.4 Changes in fatty acids composition

*Table 19* shows the changes in the composition of major fatty acids during frying in canola oil and the fortified canola oil. In unfortified canola oil, the amount of linoleic acid decreased from 18.3 mg/100 mg in the fresh oil to 14.6 mg/100 mg at the end of the 6-day frying. Within the same period, the amount of linolenic acid decreased from 9.0 to 4.9 mg/100 mg. The decrease in linoleic and linolenic acids in fortified canola oil was significantly less pronounced: At the end of the frying, the amount of linoleic and linolenic acids decreased from 18.4 and 9.1 mg/100 mg in the fresh oil to 16.2 and 6.4 mg/100 mg, respectively. At the end of the 6th day of frying, the ratio of linoleic acid to palmitic acid (C18:2/C16:0) decreased from 4.7 to 3.7 and 4.2 in canola and fortified canola oil.
oils, respectively (Figure 56). The decrease in the ratio of linolenic acid to palmitic acid (C\textsubscript{18:3}/C\textsubscript{16:0}) was more pronounced, and was 1.4 times greater during frying in canola oil as compared to fortified canola oil.

Table 19  Change in fatty acids composition during frying in canola oil and canola oil fortified with formulated antioxidant, PC+3a.

<table>
<thead>
<tr>
<th>Frying time [days]</th>
<th>Fatty acids [mg / 100 mg]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C16:0</td>
</tr>
<tr>
<td>Canola oil</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>3.90 ± 0.10</td>
</tr>
<tr>
<td>1</td>
<td>3.82 ± 0.08</td>
</tr>
<tr>
<td>2</td>
<td>3.81 ± 0.10</td>
</tr>
<tr>
<td>3</td>
<td>3.74 ± 0.04</td>
</tr>
<tr>
<td>4</td>
<td>3.77 ± 0.05</td>
</tr>
<tr>
<td>5</td>
<td>3.98 ± 0.09</td>
</tr>
<tr>
<td>6</td>
<td>3.96 ± 0.07</td>
</tr>
<tr>
<td>Fortified canola oil</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>3.91 ± 0.10</td>
</tr>
<tr>
<td>1</td>
<td>3.84 ± 0.09</td>
</tr>
<tr>
<td>2</td>
<td>3.78 ± 0.06</td>
</tr>
<tr>
<td>3</td>
<td>3.75 ± 0.10</td>
</tr>
<tr>
<td>4</td>
<td>3.79 ± 0.09</td>
</tr>
<tr>
<td>5</td>
<td>3.82 ± 0.13</td>
</tr>
<tr>
<td>6</td>
<td>3.85 ± 0.07</td>
</tr>
</tbody>
</table>
Figure 56. Changes in the ratios of linoleic:palmitic and linolenic:palmitic acids during frying in canola oil fortified with formulated antioxidant, **PC+3a** ([PC, 1000 µg/g; 3a, 350 µg/g]). FA - formulated antioxidant. See text for abbreviations.
4.8.5 Residual Antioxidants

In Table 20 the decrease in the amount of the following antioxidants: tocopherols, dihydrocaffeic acid amide, and phosphatidylcholine, during frying in canola and fortified canola oil is presented. The amount of total tocopherols decreased from 604 µg/g in the initial oil to 27 and 204 µg/g in unfortified and fortified canola oils, respectively. The rate of tocopherol degradation was 91.8 µg/g per frying day for unfortified canola oil while 67.6 µg/g per frying day was observed for canola oil containing the formulated antioxidant (Figure 57). At the end of the 6th day of frying, 51 and 20% of the respective initial amount of dihydrocaffeic acid amide and phosphatidylcholine remained in the oil.
Table 20. Changes in the amounts of antioxidants during frying in canola and fortified with formulated antioxidant, PC+3a canola oils. See text for abbreviations.

<table>
<thead>
<tr>
<th>Frying time [days]</th>
<th>Canola oil (µg/g)</th>
<th>Fortified canola oil (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α-Toc</td>
<td>γ-Toc</td>
</tr>
<tr>
<td>0</td>
<td>195±10</td>
<td>409±21</td>
</tr>
<tr>
<td>1</td>
<td>127±9</td>
<td>319±11</td>
</tr>
<tr>
<td>2</td>
<td>102±8</td>
<td>262±14</td>
</tr>
<tr>
<td>3</td>
<td>63±5</td>
<td>196±10</td>
</tr>
<tr>
<td>4</td>
<td>45±3</td>
<td>130±10</td>
</tr>
<tr>
<td>5</td>
<td>18±1</td>
<td>78±4</td>
</tr>
<tr>
<td>6</td>
<td>0±0</td>
<td>27±2</td>
</tr>
</tbody>
</table>
4.8.6 Formation of 4-hydroxynonenal

The amount of 4-hydroxynonenal (HNE) formed during frying in canola and fortified canola oils is presented in Figure 58. No detectable amount of HNE was observed in the fresh oil. However, at the end of the frying period, the amount of HNE detected in canola oil and canola oil with the formulated antioxidant was at 5.7 and 2.5 µg/g, respectively.
Figure 58. Formation of 4-hydroxynonenal during frying in canola oil fortified with formulated antioxidant, **PC+3a** (PC, 1000 µg/g; 3a, 350 µg/g). FA - formulated antioxidant. See text for abbreviations.
Chapter 5 – Discussion

5.1 Effect of external frying factors (I – IV)

Thermo-oxidative degradation of vegetable oils is more pronounced during frying than when other cooking methods such as baking and stir-frying are employed (II). Thus, protecting oils against thermo-oxidative degradation during frying is of paramount importance. As previously mentioned, extending the fry-life of polyunsaturated (PUFA) oils involves conscious optimization of both the external and internal factors affecting frying performance of oils.

In the present study, the investigated indices describing frying performance showed that the fry-life of PUFA oils can be significantly prolonged by frying at temperature lower than 190°C (I), and under reduced oxygen atmosphere (III). Under the frying protocol employed in the present study, the oil used for frying at 185°C would require 13 days of frying before attaining the 24% TPC level, which is used as oil discarding level in EU countries (Gertz, 2000). On the other hand, frying at 215°C shortened utilization of oil to 5 days using the same TPC discarding level (Figure 17). Thus, the increase in the frying temperature accelerated hydrolytic, oxidative, and oligomerization reactions, leading to increase in the amount and rate of polar compounds formation. As frying progressed, the oxidized triacylglycerols formed at the onset of oxidation are degraded or converted to higher molecular weight oligomers, hence the consistent decrease in the contribution of oxidized triacylglycerols to total polar compounds (Figures 18 and 19). Arroyo et al. (1992) also reported a consistent decrease in the contribution of oxidized triacylglycerols to the total polar compounds during frying in sunflower oil. Similar explanation can be applied for the observed decrease in the
contribution of dimers to TPC at the later stage of frying at the higher temperature (Dobarganes and Marquez-Ruis, 2003). The results from our study are in agreement with those of Houhoula et al. (2003) who reported an increase in TPC as a function of frying temperature during the frying of potato chips in cottonseed oil in the temperature range 155 – 195°C.

According to Blumenthal (1991), the higher the amount of polar compounds in the oil, the higher the surfactants content, and the higher fat absorption by the fried food. Thus, apart from shortening the fry-life of oils, frying at elevated temperature will result in fried products with high fat contents which contain more degradation products. In most countries, diseases associated with high fat consumption are a major health burden, hence the recent global campaign against high fat intake (Mehta and Swinburn, 2001).

The health benefits attributed to polyunsaturated fatty acids (PUFA) such as linoleic and linolenic make the consumption of polyunsaturated oils very desirable. It is important, however, that these fatty acids are protected during food processing. This is particularly important in institutional frying operation where the oil is repeatedly used, with high turnover and minimal control of frying conditions (Mehta and Swinburn, 2001). The observed loss in the fatty acids during frying is due to oxidation, oligomerization, scission, cyclization, isomerization, and other side reactions, which were accelerated at the higher frying temperature; hence the significantly higher amount of short-chain glycerol-bound oxidized fatty acids observed at the higher temperature (Figure 22). The consistent decrease in the amount of these oxidized fatty acids after the 3rd day of frying at 215°C can be attributed to thermal degradation and involvement in oligomerization reaction (Dobarganes and Marquez-Ruis, 2003). Velasco et al. (2004)
observed that the amount of polar fatty acids decreased drastically as compared to the amount of total polar compounds in used frying fats. Short-chain glycerol-bound oxidized fatty acids are of particular chemical and nutritional interest since they remain in the frying oil, and are absorbed and subsequently ingested.

According to Tsuzuki (2011), heat induced cis/trans isomerisation of unsaturated fatty acids occurs mainly via the formation of radical species. Christy et al. (2009), on the other hand, proposed a heat induced double bond migration accompanied by hydrogen transfer. Because these reactions are energy-dependent, isomerisation rate increases with temperature; hence the observed significant increase in trans isomers content of the oil heated at the higher temperature. Increasing amounts of trans isomers during frying at a higher temperature can have practical implications related to nutritional zero trans content claims. When the amount of these isomers increased 2.5-fold during frying at the higher temperature, then the amount of trans isomers in fried products will exceed the specified by definition limit, making the claim for zero trans fat invalid. Our results agree with those of Tyagi and Vasishtha (1996) and Tsuzuki et al. (2010) regarding the effect of frying temperature on trans formation in soybean and canola oils, respectively.

Anisidine value (AV) measures the amount of nonvolatile aldehydes, principally 2-alkenal and 2,4-dienal formed during thermo-oxidative degradation of oils, and has been shown to be negatively correlated with the flavour scores of the frying oils (Frankel, 2005). Regardless of the general knowledge that the decomposition of hydroperoxides and subsequent formation of carbonyl compounds increases with increasing temperatures (Houhoula et al., 2003), AV observed in the present study followed an opposite trend.
This trend can be explained by the high chemical reactivity, involvement in the formation of other compounds, and thermal decomposition of the carbonyl compounds formed during the frying, effects which were more pronounced at the higher frying temperatures. A similar explanation can be offered for the observed decrease in AV after the 3rd day of frying at both frying temperatures.

Apart from the immediate effects on the frying oils, frying above the optimum temperature can also affect the nutritional quality, storage stability and thermo-oxidative degradation products of the prepared food. Tocopherols are the major antioxidants present in oils and offer protection against thermo-oxidative degradation principally by donating hydrogen atoms to lipid peroxy radicals thereby interfering with either chain propagation or initiation (Frankel, 2005; Seppanen et al., 2010). The faster rate of tocopherol depletion at the higher temperature can be attributed to the increase in the rate of oxidative initiation and propagation, producing higher concentrations of lipid peroxy radicals, which in turn placed an increasing demand on the number of tocopherol molecules participating in the antioxidant activity. According to Verleyen et al. (2002), the degradation of tocopherols could also be due to the non-selective oxidation of unsaturated fatty acids and tocopherols by the highly reactive alkoxyl and hydroxyl radicals generated by decomposition of hydroperoxides. The rate of hydroperoxide decomposition increases with temperature (Frankel, 2005). The higher temperature can further accelerate the rate of self oligomerization of the tocopheroxy radicals arising from the chain breaking mechanism, causing more tocopherol loss (Kamal-Eldin and Appelqvist, 1996). A similar observation was reported by Verleyen et al. (2001) during heating of α-tocopherol in a triolein model system in the temperature range of 180 to
260°C. Furthermore, at the higher temperature, the rate of tocopherol removal by evaporation/distillation would also increase (Marmesat et al., 2010).

According to Sebedio et al. (1990) and Dobarganes et al. (2000a), and also observed in our study, there was lack of a significant differences in the compositions between fried products and the frying oils, indicating extensive exchange of lipids during frying. Thus, with complete depletion of tocopherols at the higher temperature (Figure 25), food processed at the optimum temperature will show better storage stability.

The results from this study clearly emphasize the importance of controlling frying temperature. When frying is carried out at a temperature above the optimum, either due to a faulty temperature controller or deliberately, to increase turnover at the peak hours, the rate of oil degradation will significantly increase, shortening the fry-life of the oil (Mehta and Swinburn, 2001). For instance, a 1999 national survey of fast food outlets in New Zealand reported a wide range of frying temperatures used in institutional frying operations, ranging from 112 to 233°C, with 26% of the surveyed outlets operating above 190°C (Mehta and Swinburn, 2001). According to Collin (1993), the actual oil temperature could be up to 30°C higher than the temperature controller setting, and unfortunately, only a small percentage of institutional frying operators are consciously making effort to calibrate and ascertain the correctness of their fryers’ temperature controllers. For instance, a survey in Australia showed that only 56% of the fast food outlets had correctly calibrated temperature controllers (Mehta and Swinburn, 2001).

The main degradation process during frying is related to oxidation. Thus, apart from moderating the frying temperature, frying performance of PUFA oils can also be enhanced by controlling availability of oxygen during frying (publication III). In this
study, the amount and availability of oxygen during frying was reduced either by frying under carbon dioxide blanketing (CDB), or by frying under vacuum (VF), using canola oil as the frying medium. A superior frying performance of oil was observed when a reduced amount of oxygen was used, evident from the amount of TPC formed. The propagation steps of thermo-oxidative degradation involve a rapid reaction between alkyl radicals and molecular oxygen forming alkylperoxy radicals, followed by a rate determining hydrogen transfer reaction between the alkylperoxy radicals and unsaturated lipids to form hydroperoxides (Frankel, 2005). By limiting the amount and availability of oxygen through CDB and VF, the propagation steps of thermo-oxidation, and the subsequent formation of lipid hydroperoxides was effectively hindered. Lipid hydroperoxides are the precursors of the thermo-oxidative degradation products measured by TPC, AV, and other stability indices, hence the observed higher frying stability of the oils used for frying under CDB and VF, compared to SFC.

In addition, by hindering the propagation, the rate determining steps, thermo-oxidative degradation of polyunsaturated fatty acids such as linoleic and linolenic was inhibited during CDB and VF, hence the higher amounts of these fatty acids remaining in the oils under these conditions, compared to SFC. In the same manner, the reduction in the amounts of molecular oxygen, lipid peroxy and alkyl radicals during CDB and VF significantly reduced the amount of tocopherols lost to oxidative degradation. Verleyen et al. (2001) observed that degradation of $\alpha$-tocopherol in a triolein model heating was markedly inhibited even at $240^\circ$C under constant nitrogen flow, and concluded that the loss of tocopherols under frying conditions could be both thermal and oxidative. Accordingly, the average rates of tocopherol degradation were 82, 17, and 7 $\mu$g/g per
frying day during frying under SFC, CDB, and VF, respectively (Figure 27). At this rate, it would take 8, 39, and 96 days to completely consume the tocopherols in the oils used for frying under SFC, CDB, and VF, respectively. Thus, apart from extending the fry-life of the oil, food produced under the developed frying protocol would have a much higher nutritional value.

Many toxic degradation products have been identified in frying fats and fried foods, and the list keeps growing. Among them, acrylamide and 4-hydroxy-2-nonenal (HNE) have attracted much attention not only because of their unusually high toxicity, but also because they could be formed at concentrations that pose health concerns (Taubert et al., 2004; Niki, 2009). According to Mucci and Wilson (2008), more than one-third of the calories consumed by U.S. and European populations contain acrylamide. Because of their reactivity, acrylamide and HNE are known to modify proteins, nucleic acids and other biomolecules leading to several diseases and medical conditions (LoPachin et al., 2008; Niki, 2009). In the present study, the amount of HNE and acrylamide accumulated in the French fries were significantly reduced when the frying was conducted under the novel CDB instead of the traditional frying (Figures 28 and 29). The interaction between asparagine and reducing sugar is the most widely recognized acrylamide formation mechanism in foods (Zhang et al., 2009); however, the significant reduction in the amounts of HNE and acrylamide consequent to decrease in the oxygen amount observed in the present study evidently showed that oxidation is involved in the reaction mechanism for the formation of these compounds. Thus, results from this study support observations from model studies by Zamora and Hidalgo (2008).
and Hidalgo et al. (2009) that oxidation of fatty acids is producing precursors required for acrylamide formation.

Methods suggested in the literature for reducing acrylamide formation during frying include: (1) frying at lower temperature; for instance, regulations in Germany require that frying operations be carried out at a temperature not higher than 165°C to limit formation of acrylamide (Gupta, 2004); (2) Decreasing the amounts of reducing sugar, asparagine, and other acrylamide precursors in the raw potatoes either by breeding or through appropriate treatment of the potatoes before frying (Matthäus, 2009); and (3) frying under vacuum (Granda and Moreira, 2005). Breeding new varieties of potatoes low in acrylamide precursors is costly, time consuming, and success cannot be guaranteed (Matthaus, 2009). Furthermore, application of vacuum system in institutional frying is costly and operationally labour intensive and probably impossible at the current state of technology. Conversely, results from this study showed that CDB is an efficient method for impeding the formation of acrylamide during frying, offering a cost-effective alternative to vacuum frying and other proposed method to reduce acrylamide content of fried foods.

5.2 Effect of internal factor \((V - X)\)

Generally, PUFA oils are inherently unstable due to their high susceptibility to oxidative degradation (Parker et al., 2003; Martin-Polvillo et al., 2004). In agreement with published reports by Lampi and Kamal-Eldin (1998) and Warner (2005), the observed higher frying stability of regular canola oil over the minor components stripped canola oil (Figure 34) indicated that minor components exert significant influence on
frying performance of oils. Consequently, manipulation of the composition and concentration of minor components should enhance the frying performance of PUFA oils.

To use the typical actual frying protocol (section 3.2.1.1) for a detailed study of the effects of individual groups of minor components on frying performance would be uneconomical both in materials and time. Consequently, an effective, economical and fast frying test was developed for this purpose (V). The developed frying test was specifically designed to effectively mimic an actual frying protocol used in the present study and institutional frying. With this frying test, the effects of different concentrations and combination of the various groups of minor components isolated from canola (TCAN, SCAN), rice bran (TRBO, SRBO, RBOS), sesame (SOS), and palm oils (POS) on the frying performance of typical PUFA oil were assessed (VI). In this part of the study, pure triacylglycerols (TAG) isolated from canola oil were utilized as a test frying medium. Utilization of TAG allowed us to assess how individual groups of endogenous minor components affect frying performance. Further, using this model, we were able to eliminate and assess interaction of different components.

As expected, tocopherols, irrespective of source (pure or isolated as a fraction from oils) offered significant protection to TAG under frying conditions. As measured by the amount of total polar compounds formed during the frying test, tocopherol fractions isolated from canola, rice bran and palm oils, despite the diversity in isomer composition, offered a similar level of protection (Figures 33 and 34). Rice bran and palm oils are rich sources of tocotrienols, however, these tocochromanols did not add to the frying performance. Indeed, Romero et al. (2007) did not find an increase antioxidant activity when a mixture of α-tocopherol and α-tocotrienol was added to stripped canola oil as
compared to when added individually. The similar antioxidant mechanism of tocopherol and tocotrienol protection may account for this observation (Seppanen et al., 2010). Tocochromanols (tocopherols and tocotrienols) are chain-breaking antioxidants, interfering with either chain propagation or initiation by readily donating hydrogen and inactivating lipid peroxyl radicals. According to Frankel (2005), significant synergism is generally observed when the individual antioxidants operate by different mechanisms.

Using pure tocopherols, to remove any interference from other minor components in the oils, three combinations of isomers were set up as described in the table below:

<table>
<thead>
<tr>
<th>High α, High γ, Low δ (µg/g)</th>
<th>High α, Low γ, High δ (µg/g)</th>
<th>Low α, High γ, High δ (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>225α + 225γ + 50δ</td>
<td>225α + 50γ + 225δ</td>
<td>50α + 225γ + 225δ</td>
</tr>
<tr>
<td>450α + 450γ + 100δ</td>
<td>450α + 100γ + 450δ</td>
<td>100α + 450γ + 450δ</td>
</tr>
<tr>
<td>900α + 900γ + 200δ</td>
<td>900α + 200γ + 900δ</td>
<td>200α + 900γ + 900δ</td>
</tr>
</tbody>
</table>

All of the indices for stability measured in this part of the study indicated that the differences in protection observed among the isomeric systems were not of practical importance (VI). Results by Warner and Moser (2009) also showed that varying the ratio of tocopherol homologues has no significant impact on their antioxidant activity when the α-, γ-, δ- homologues were present in an oil. Furthermore, results from our study showed that increasing tocopherol concentration above 1000 µg/g did not result in concomitant improvement in frying stability of TAG, despite the increase in residual tocopherols. The
inefficiency of tocopherols at higher concentrations could be explained as follows: (1) Above the optimum concentration, tocopheroxyl radicals became prooxidant and chain carriers, regenerating alkyl and peroxy radicals through hydrogen abstraction from fatty acids and lipid hydroperoxides, respectively (Frankel, 2005); (2) Higher concentration of tocopheroxyl radicals resulted in the formation of higher amounts of oxidized and oligomerized tocopherols possessing prooxidant activity (Rietjens et al., 2002; Chapman et al., 2009); (3) A number of tocopherol oligomers, such as the α-tocopherol ethane dimer and the γ-tocopherol diphenol dimer still possess antioxidant activity, and could have moderated the prooxidant effect as stated above in (1) and (2), resulting in the reduced efficiency, rather than the prooxidant effect observed for tocopherols at concentrations higher than the optimum (Kamal-Eldin and Appelqvist, 1996). According to Burton et al. (1985) and Mukai et al. (1993), however, the rates of tocopheroxyl radicals bimolecular couplings were $10^4$ – $10^6$ times higher than those of hydrogen abstractions from fatty acids or lipid hydroperoxides by tocopheroxyl radicals. Thus, the observed inefficiency of tocopherols at higher concentrations might as well be due to a balance between the amounts of prooxidant and antioxidant oxidized and oligomerized tocopherols.

Interestingly, a significant protection of TAG was observed when the a sterol fraction from either canola (SCAN) or rice bran oil (SRBO) was applied as the principal minor component (Figure 33). This result is consistent with a published report by Gertz et al. (2000), and may be due to the ability to donate hydrogen to alkyl or peroxy radicals by those sterols with ethylidene side chain, thereby inhibiting the propagation step of thermo-oxidation (Sims et al., 1972; Gordon and Magos, 1983; White and Armstrong,
1986). The higher amount of such sterols in SRBO may explain the effectiveness of this fraction even at the lower concentration (500 µg/g) used, compared to SCAN. Unfortunately, the protection offered by sterols was lost whenever tocopherols were present in the mixture, indicating a lack of a synergistic interaction between these components. Although the antioxidant mechanism of sterols is not well understood, these results indicated that they are less efficient than tocopherols as a chain-breaking antioxidant, and are not the primary antioxidant in a mixture with tocopherols. Indeed, the thermo-oxidative loss of sterols has been reported to be significantly lower in the presence of tocopherols by Rudzinska et al. (2004) and Tabee et al. (2008). Thus, in the real system, the antioxidant activity of sterols during frying might not be realized since they coexist with tocopherols in the oil.

When minor components isolated from palm oil, POS, were applied to canola oil triacylglycerols as the exclusive minor components, frying stability was not significantly better than that of regular canola oil (Figure 34). This result indicates that characteristic minor components from palm oil, carotenoids and tocotrienols, may not contribute to the frying performance of PUFA oil already containing tocopherols. The carotenoids to tocochromanols ratio in TAG containing the POS was 1:10 (Table 15), thus, the much higher concentration, coupled with the better hydrogen donating potency of tocochromanols could have made them the primary antioxidants in the mixture. Incidentally, in their study on the oxidative stability of phosphatidylcholine liposomes at 120ºC in a Rancimat, a carotenoids to tocochromanols ratio of 1:10 was suggested by Schroeder et al. (2006) as the optimum ratio for synergistic interactions between these classes of antioxidants. The widely different thermo-oxidative conditions and lipid
substrates used in this study, however, make comparison with our results impossible. On the contrary, and in agreement with the present study, Romero et al. (2007) found no significant difference in the amount of TPC formed during the heating of canola oil and canola TAG containing a mixture of carotenoids and tocopherols for up to 18 h.

Addition of solvent-extracted minor components from rice bran (RBOS) or sesame oil (SOS) significantly enhanced the frying performance of polyunsaturated TAG (Figure 34). The significant antioxidant activity of RBOS is presumably due to the high concentration of γ-oryzanol in addition to tocochromanols or potential synergistic effect between these components (Table 15). This deduction became plausible considering the fact that RBMC, the minor components isolated from rice bran oil by chromatography (Scheme 1) did not show similar effectiveness as RBOS, the major difference in the compositions of RBMC and RBOS being the large amount of γ-oryzanol in RBOS, a component not detected in RBMC (Table 15). According to Gertz et al. (2000) and Kochhar and Gertz (2004), the addition of γ-oryzanol to canola and sunflower oils significantly increased their thermo-oxidative stability. Although Nystrom et al. (2007) did not observed any synergy between α-tocopherol and sitostanyl ferulate, a component of γ-oryzanol, during the heating of high oleic sunflower oil triacylglycerols, the thermo-oxidative conditions and the compositions of the components examined were very different from those used in our study, making comparison impossible.

The significant protection offered by SOS was likely due to the activity of the sesame lignans such as sesamol, sesaminol, sesamin, and sesamolin. Farhoosh and Kenari (2009), Alizera et al. (2010), and Serjouie et al. (2010) attributed the improved frying stability of blends of sesame and canola oils over the canola oil alone to the sesame oil
lignans. In a related study, the improved stability of a blend of soybean and sesame oils, and the food fried in it over soybean alone was assigned to the sesame oil lignans (Chung et al., 2004; Chung et al., 2006).

Among assessed minor components, the best protection was offered by phospholipids (Figure 35). The fry-life of TAG containing 0.2% phosphatidylcholine was nearly twice that of regular canola oil as measured by the amount of polar materials formed during the frying test. These results are consistent with previous reports by Chu (1991), Kourimska et al. (1994), and Gordon and Kourimska (1995b) regarding the antioxidant activity of phospholipids during frying. The observed activity of the tested phospholipids, namely PC and PE may be attributed to the formation of non enzymatic browning reaction products between these phospholipids and the sugar molecules or with some oxidation products produced during frying (Husain et al., 1984; Hidago et al., 2005; 2006; 2007; Zamora et al., 2011). It may also be due to the ability of the phospholipids to form an oxygen barrier between the oil and air interface, limiting the amount of oxygen penetrating the frying medium (Porter, 1980; Calvo et al., 1994).

The present study (VI) showed that phospholipids at a concentration up to 0.1% would significantly enhance the frying performance of PUFA oils. Unfortunately, most of the phospholipids are specially removed from oils during processing, leaving the protection of refined oils against thermo-oxidative degradation exclusively at the mercy of tocopherols (Shahidi, 2003; Gunstone, 2004). However, the inefficiency of tocopherols to protect polyunsaturated oils during frying is clearly demonstrated in the present study (VI), presumably due to their poor thermal stability, thermal deactivation, and tendency for prooxidant activity at concentrations higher than the optimum (Frankel, 2005;
As shown in Figures 34 and 36, none of the tocopherol isomer combinations resulted in additional protection for antioxidant stripped canola oil over the regular canola oil, suggesting that the tocopherol concentration in this oil is close to the optimum. Evidently, to enhance the frying performance of PUFA oils at a practical level, there is the need to look beyond tocopherols.

Looking beyond tocopherols, syntheses of several phenolic antioxidants were undertaken and their abilities to protect TAG during storage and frying were evaluated (VIII – X). All the synthesized antioxidants (nine chromanol derivatives and twelve dihydrocaffeic acid amides) provided significant protection for TAG both under storage and frying conditions, with the gallic acid derivative of trolox (VIII: 1e), the dichromanol (IX: 3), and the group of dihydrocaffeic acid amides in which the benzyl amine moiety was not hydroxylated (X: 3a – 3c) being the most active. The high thermal stabilities of the new compounds definitely enhance their potential as antioxidants for high temperature applications of PUFA oils. The radical scavenging mechanism of phenolic antioxidants is well established: they break the free radical chain forming a stable phenoxy radical, which is effectively stabilized by delocalization of unpaired electrons on the aromatic ring (Scott, 1963; Frankeel, 2005). Compared to tocopherol and BHT, the higher radical scavenging activity of the novel antioxidants, and the observed superior antioxidant activity during storage was likely due to the increase in the number of hydroxyl functional groups and the presence of electron donating groups in ortho position to the hydroxyl group (Scott, 1963; Saito and Kawabata, 2005). Conversely, the activity of the antioxidants under frying conditions is not that simple, and presumably involves radical scavenging, net effect of the degradation products of the original
antioxidants, interactions with the food components, and removal of secondary thermo-
oxidative products such as aldehydes through condensation reaction, among others
(Frankel, 2005; Hidalgo et al., 2008).

It is well known that antioxidant compounds can reinforce each other by cooperative effects known as synergism. This is particularly significant when radical scavenging antioxidants such as phenolic compounds are used together with metal chelators such as phospholipids (Frankel, 2005). To this end, all possible combinations of the best performing natural endogenous minor components (PC and RBOS) and synthetic antioxidants (Compounds 1e, 3a, 3) were formulated to maximize synergistic relationships among them. Furthermore, to understand the possibility of interactions between added antioxidants and endogenous minor components, regular canola oil was used as the frying medium instead of the antioxidant stripped canola oil used in the preliminary studies (V–X).

The fact that antioxidant 1e was effective during the frying test in canola TAG (VIII), whereas it is ineffective in regular canola oil suggested the possibility of a destructive interaction between the synthetic antioxidant and some canola oil endogenous minor components. The interaction between lipid hydroperoxides and added antioxidants has been reported to decrease antioxidant efficiency (Kamal-Eldin et al., 2002). Kortenska et al. (1991) reported that the antioxidant efficiency of p-methoxy phenol was reduced 2.3 fold in the presence of 1-palmitoylglycerol during oxidation of sunflower oil. The strong reducing power of gallic acid has also been used to explain the prooxidant activity of gallic acid and its derivatives (Murakami et al., 2000; Yen et al., 2002). The lack of synergy observed for all the possible combinations of the synthetic antioxidants,
1e, 3, and 3a (Figure 52) may be due to a similar mechanism of antioxidant activity, radical scavenging mechanism (Frankel, 2005). Conversely, the observed synergy between phosphatidylcholine (PC) and solvent extracted rice bran minor components (RBOS) may be related to an interaction between PC and γ-oryzanol or other phenolic compounds present in RBOS. Ramadan (2008) observed an increase in the antioxidant activity of soy lecithin when quercetin was present. This synergistic interaction between a metal chelator and radical scavengers may also be applied to explain the effectiveness of the following antioxidant mixtures: PC+RBOS+3+3a and PC+RBOS+3a (Figure 52).

Although the developed frying test offered a reliable level of prediction of the frying performance of oils (V), actual frying still remains the most reliable method for assessing frying performance of oils. Consequently, the effectiveness of one of the most promising formulated antioxidant mixtures (Figure 52) was tested in a standard frying condition during a 6-day actual frying of French fries in canola oil. For simplicity, and to remove the additional variable that might be introduced from an increased amount of tocopherols due to the presence of RBOS in the mixture, the binary antioxidant, PC with 3a, was used for the study. All the indices of frying performance indicated that canola oil containing the added formulated antioxidant (FA) was significantly more stable than the unfortified regular canola oil. Thus, the components of the applied formulated antioxidant, PC and 3a, working synergistically and were able to inhibit the formation of polar components, protecting the PUFA frying oil against thermo-oxidative degradation.

Aldehydes are major products formed during thermo-oxidative degradation of oil. Although some of the aldehydes produced are lost by evaporation during frying, a significant amount of nonvolatile compounds remains in an oil and is assessed by
anisidine value (AV). The higher the AV, the more prevalent the degradative reactions occurring in the frying oil. The significant antioxidant activity of the formulated antioxidant was evident from the significantly lower AV of the canola oil fortified with it, compared to unfortified canola oil (Figure 54). Aldehydes are secondary oxidation products formed by the decomposition of hydroperoxides. Hydroperoxides formation during frying proceeds via a free radical mechanism, thus the low AV value in the fortified canola oil could be due to the ability of the phenolic antioxidant, 3a, to donate hydrogen atoms to lipid peroxy radicals thereby interfering with either chain propagation or initiation (Frankel, 2005). The high radical scavenging activity of 3a has been established in a part of this study, being significantly better than α-tocopherol and BHT (X). The antioxidant 3a could also be removing aldehydes from the oil through a condensation reaction between amine and carbonyl group (Hidalgo et al., 2008). The synergy between PC and the phenolic antioxidant, 3a further enhanced the activity of the formulated antioxidant (Khan and Shahidi, 2000; Judde et al., 2003; Ramadan, 2008).

PUFA, notably linoleic and linolenic acids are the primary targets of thermo-oxidative degradation. As previously mentioned, the extent of degradation of these fatty acids could be a reliable indicator of frying performance of the oil (Dijkstra et al., 2007). Because of its higher radical scavenging activity, compound 3a might have inhibited the propagation step of thermo-oxidative degradation by donating hydrogen to lipid peroxy radicals more effectively than the endogenous tocopherols, protecting the fatty acids from being attacked by these radicals. The metal ion mediated decomposition of hydroperoxides to alkoxy and hydroxyl radicals might have been prevented by the PC component, further reducing the number of radicals available to initiate and propagate
fatty acid degradation. These two components working synergistically evidently ensured that the essential fatty acids, linoleic and linolenic acids were better protected in canola oil containing the formulated antioxidant.

The presence of phosphatidylcholine in the antioxidant mixture is responsible for the increased colour formation observed in canola oil fortified with the formulated antioxidant, and this is due to its well documented non enzymatic browning reactions (Pokorny, 1981; Hidalgo et al., 1990). It is well known that the presence of surfactant materials such as phospholipids enhances foaming during frying (Blumenthal, 1991; Dobarganes et al., 2000a). Contrary to expectations, however, no significant foaming was observed in the canola oil containing the formulated antioxidant throughout the entire frying period. In contrary, canola oil without FA showed excessive foaming at the beginning of the 4th day of frying. Thus, this observation suggested that the effect of degradation products on foaming was more effective than the emulsifying effect of phosphatidylcholine. Indeed a number of degradation products such as monoglycerides, diglycerides, and polar polymers are surface active compounds and can contribute to foaming, according to Blumenthal’s surfactant theory of frying (Blumenthal, 1991). Kourimska et al. (1994) found no significant increase in foaming of olive oil and no noticeable effect on the sensory quality of prepared French fries when lecithin was applied at 0.1%.

Tocopherols are the principal endogenous antioxidants in frying oils. The rate of tocopherol degradation has been related to the stability of the frying oil (Normand et al., 2001; Normand et al., 2003; Normand et al., 2006). The slower rate of tocopherol degradation in fortified canola oil (Figure 57) indicated that tocopherols were protected
by the formulated antioxidant used in the study. The observed protection of tocopherols could be due to the fact that the phenolic compound 3a was a more effective chain breaking antioxidant than α-tocopherol, thus sparing the latter (X). It could also be due to synergism between tocopherols and the components of the formulated antioxidant containing PC and 3a (Khan and Shahidi, 2000; Judde et al., 2003; Pazos et al., 2007). Some phenolic acids and phospholipids have been reported to regenerate α-tocopherol from the tocopheroxyl radical and oxidized tocopherols (Weng and Gordon, 1993; Facino et al., 1998; Jia et al., 1998; Pazos et al., 2007). Indeed, supplementing tocopherols with phenolic antioxidants has been recommended as a way to delay tocopherol degradation during frying (Sánchez-Muniz and Bastida, 2006). The significantly higher residual amount of tocopherols and the applied antioxidants in the fortified canola oil means that food processed in this oil will possess higher nutritional quality and a better storage stability compared to normal canola oil (Sebedio et al., 1990; Dobarganes et al., 2000a).

Hydroxynonenal (HNE) is one of the most toxic α,β-unsaturated aldehydes formed during lipid oxidation (Esterbauer et al., 1988; Seppanen and Csallany, 2006). Thus, the marked inhibition of HNE by the formulated antioxidant is of significant importance to the safety of prepared French fries considering the extensive mass exchange occurring between the food and the frying oil (Dobarganes et al., 2000a). Like most products of thermo-oxidative degradation, HNE formation from linoleic acid is by free radical reactions and proceeds via initial formation of 13-hydroperoxides (Pryor and Porter, 1990; Schneider et al., 2001). The higher radical scavenging potency of compound 3a in the formulated antioxidants was probably responsible for the lower formation of HNE in the fortified canola oil. The formulated antioxidant could also have
inhibited HNE formation by preventing or influencing the mechanism of decomposition of the 13-hydroperoxide precursors. Pan et al. (2010) observed that choline, ethanolamine, and soybean phospholipids decompose linoleic and linolenic acids hydroperoxides to the corresponding hydroxides at a higher rate than observed for α-tocopherol and BHT.
Chapter 6 – Conclusions and future research perspectives

Polyunsaturated (PUFA) oils are generally considered unsuitable for institutional frying operations because they are prone to thermo-oxidative degradation reactions occurring during frying. The major factors that determine frying performance of polyunsaturated oils can be broadly grouped into two – internal and external factors. The present study clearly demonstrated that by a careful control of some of these factors, the fry-life of PUFA oils can be significantly extended.

Results from the present study demonstrated that the effects of conducting a frying operation above 195°C are far reaching: (1) The fry-life of the PUFA oil will be dramatically shortened; (2) the food prepared under such conditions will not achieve optimal nutritional quality and storage stability; and (3) the food may accumulate large amounts of components with potential detrimental health effects. Moreover, the threshold level described by the “zero trans” definition found on a food label may be annulled by frying above the optimum temperature. Therefore, to improve the frying performance of PUFA oils it is of utmost importance that the frying operation be consciously carried out at a temperature below 195°C.

Furthermore, the present study demonstrated that the frying performance of PUFA oils will be significantly enhanced by frying under an atmosphere of reduced oxygen concentration. The study showed that frying under carbon dioxide blanketing can double the fry-life of PUFA oils and deliver healthier fried food possessing better nutritive quality and storage stability by impeding the formation of toxic compounds such as HNE and acrylamide, and protecting essential fatty acids and endogenous antioxidants.
An effective, economical and fast frying test was developed, making it possible to rapidly assess the frying performance in small samples of oils, covering a large number of tests in one working day. The influence of minor components on the frying performance of PUFA oils was extensively evaluated utilizing the developed frying test. The results from this study clearly revealed the limitation of tocopherols, the primary antioxidant in oils, to offer protection under frying conditions. The frying performance of PUFA oils, however, can be significantly improved by the addition of minor components from rice bran oil or the right amounts of phosphatidylethanolamine or phosphatidylcholine.

Looking beyond tocopherols, twenty one novel phenolic antioxidants were synthesized and evaluated as potential antioxidants for storage and frying applications. The results from the study evidently showed that the fry-life of PUFA oils and the storage stability of the fried food will be significantly enhanced in the presence of the new antioxidants.

Exploiting antioxidant synergism, formulations containing effective natural and synthetic antioxidants were developed. The results from this study clearly showed that the application of the formulated antioxidant containing phosphatidylcholine and N-propyl-N-benzyl-3-(3,4-dihydroxyphenyl)propanamide can double the frying performance of PUFA oils. Furthermore, the application of this formulated antioxidant will significantly inhibit the formation and subsequent accumulation in foods, of toxic thermo-oxidative degradation products such as 4-hydroxy-2-trans-nonenal (HNE), offering healthier fried products. Although, several other promising antioxidant mixtures established in the present study were not evaluated in an actual frying protocol, however,
as predicted by the developed frying test a similar level of effectiveness is expected from any of these antioxidant mixtures.

In this study, a simple, reliable and efficient novel procedure for HNE analysis was developed. The developed method eliminates derivatization and multiple extractions and purification steps in available methods in the literature. Furthermore, the novel method makes it possible to prepare in a single run, multiple samples within one working day for testing the amount and composition of polar components, and accurate quantification of HNE.

Overall, the present study showed that the frying performance of PUFA oils can be significantly improved by controlling important external factors such as frying temperature and oxygen concentration, and by enhancing the antioxidant potency of the endogenous minor components.

The stringent conditions employed during frying exert excessive pressure on both endogenous and applied antioxidants. Consequently, a majority of conventional antioxidants fail to perform under frying conditions. In the present study, antioxidants for frying applications were sought for within edible oils minor components, and by synthesis. However, much more attention needs to be given to other vegetal sources, particularly those possessing medicinal qualities.

The present study established the effectiveness of the novel antioxidants in a bulk oil system; however, their antioxidant activities in multiphase systems such as oil-in-water, water-in-oil, micelles and liposomes are subjects for further investigations. Toxicological studies on these antioxidants will also need to be undertaken before any practical applications in foods can be guaranteed. In addition, further studies should be
carried out to better understand the antioxidant mechanisms of the new compounds. Further studies on possible modifications of natural antioxidants such as tocopherol isomers should still continue to be explored as a way to improve their efficiencies and stabilities under frying conditions.
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Nutritional Claims for Fried Foods may be Annulled by Frying at Elevated Temperatures

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Abstract

The changes in regular canola oil as affected by frying temperature were studied. In seven consecutive days regular canola oil was heated at 185 ± 5 °C and at 215 ± 5 °C for seven hours daily with French fries fried intermittently. The thermo-oxidative alterations were measured by total polar compounds (TPC), anisidine value (AV), color components formation and changes in fatty acid composition. Results showed that TPC, AV, color and trans fatty acid content increased significantly (p < 0.05) as a function of frying temperature and time. The contribution of polyunsaturated fatty acids (PUFA) decreased in direct proportion to frying temperature and time. After 7 days of frying, the amount of PUFA was reduced by half during frying at 215 °C. Of the parameters assessed, total polar component and color showed the best correlation with correlation coefficients of 0.9650 and 0.9302 for frying at 215 °C and 185 °C, respectively. TPC formation correlated inversely with the reduction of tocopherols.

Keywords: Canola oil, Frying performance, Total polar component, Anisidine value, Color, Frying temperature, Tocopherols, French fries, Fatty acids.
Introduction

Deep-fat frying is probably one of the most dynamic processes in all of the food processing. Essentially, the process involves immersing a food item in a large quantity of heated oil or fat, which is normally topped up and reused several times before being disposed. Deep-fat frying produces a product with desired sensory characteristics including fried food flavor, golden brown color, and a crisp texture [1].

Most frying operations are conducted at temperature of 175–195 °C, nevertheless German regulation allows maximal frying temperature to 175 °C, to limit formation of acrylamides [2]. Extruded products and pellets are typically fried at 190 to 215 °C [3]. This high temperature requirement and the presence of air and moisture, from the food, initiate several chemical and physical changes affecting oxidative degradation of oil used. Published studies described chemical reactions involved and various volatile and non-volatile oxidation products were identified [4–6]. The chemical changes in the frying fats also affect physical characteristics of oil and fried product [7]. For instance, the color of frying oil was reported to darken as a result of oxidation and the formation of browning pigments when potato chips were fried [8, 9].

A number of studies have been undertaken to assess various chemical reactions and extent of oxidative deterioration as affected by frying temperature, but many of the published data were obtained using heating an oil and not during actual frying [10–12]. Meanwhile, it has been observed that the chemical reactions that take place during deep-fat frying are different from those during continuous heating [13, 14]. Besides, different oils have been found to behave differently regarding the rate of formation of polar components and secondary oxidation products. Guillen and Cabo [15] reported that secondary products were formed immediately after hydroperoxide formation in olive and rapeseed oils, whereas in sunflower and safflower oils, secondary products were formed when the concentration of hydroperoxides reached level of 180 and 270 meq/kg, respectively. Consequently, the need to study the frying performance of individual oil as a function of frying temperature during actual frying of food becomes imperative.
Oxidized short-chain fatty acids are secondary oxidation products formed through thermal degradation of lipids hydroperoxides. Recently, much concern has been on the biological effects of oxidized lipids, and there is increasing evidence that they may be detrimental to health, especially in connection with the development of atherosclerosis, liver damage, and promotion of intestinal tumor [16].

The objective of this study is to evaluate the effect of frying temperature on degradation of canola oil by monitoring the accumulation of total polar components, oxidized short-chain fatty acids, polymers formation, p-anisidine value, color components formation, changes in fatty acid composition, and change in tocopherol contents. According to our observation of some institutional operation, temperatures applied in this study are realistic in frying processes. We selected canola oil for this study due to the presence of more prone to oxidative degradation unsaturated fatty acids, such as oleic, linoleic and linolenic.

Materials and Methods

Oil and French fries

Commercially refined regular canola oil was obtained from Canbra Foods (Lethbridge, Canada). Frozen par-fried French fries in institutional pack were obtained from a local food store.

Frying procedure and oil sampling

The frying was simultaneously conducted in two 8 L capacity restaurant style stainless steel deep fryers (General Electric Company, New York, USA). Regular canola oil (3.75 L) was heated at 185 ± 5 °C and 215 ± 5 °C for 7 h daily for 7 days. A batch of 200 g of frozen French fries was fried for 5 minutes for a total of eight batches per frying day. At the end of each frying day, fryers were shut off and left to cool overnight. Two 25 mL samples of oil from each of the fryers were taken daily and kept frozen at -16 °C until analyzed. Before commencing frying each day, oils were filtered to remove solid debris. Oil was replenished every second day of frying with 500 mL of fresh oil.
Fatty acid analysis

Fatty acids were methylated following the AOCS Official Method Ce 1-62 [17]. The resulting fatty acid methyl esters (FAME) were analyzed on Trace GC Ultra gas chromatograph (Thermo Electron Corporation, Rodano, Italy) using a Trace TR-FAME fused silica capillary column (100 m × 0.25 mm × 0.25 µm; ThermoFisher Scientific, Waltham, MA, USA). Hydrogen was used as carrier gas with flow rate of 1.5 mL min\(^{-1}\). Column temperature was programmed from 70 °C to 160 °C at 25 °C min\(^{-1}\) and held for 30 minutes, and further programmed to 210 °C at 3 °C min\(^{-1}\). Starting and final temperatures were held for 5 and 30 minutes, respectively. Splitless injection was used utilizing PTV injector. Detector temperature was set at 250 °C. FAME samples, 1 µL, were injected with AS 3000 autosampler (Thermo Electron Corporation, Rodano, Italy). Fatty acids were identified by comparison of retention time with authentic standards (Nu-Chek-Prep, Elysian, MN).

Oxidized short-chain fatty acids methyl esters (OFAME) were identified by comparison with standards and quantified as a group [18]

Total polar compounds (TPC)
TPC were determined by gravimetric method after column chromatography separation of non-polar fraction following AOAC Method 982.27 [19]. Polar components were eluted from the column with diisopropyl ether and further analyzed for composition by size exclusion chromatography.

Anisidine values (AV)
AV, a measure of secondary oxidation products, was determined according to ISO Method 6885:2004 [20].

Tocopherols
Tocopherols were analyzed by the AOCS Official Method Ce 8-89 [17]. Briefly, oil samples (75 mg) were weighed directly into vial and dissolved in 1.5 mL hexane. Analysis was performed on a Finnigan Surveyor LC (Thermo Electron Corporation, Rodano, Italy) with a Finnigan Surveyor Autosampler Plus and Finnigan Surveyor FL Plus fluorescence detector,
set for excitation at 292 nm and emission 394 nm. The column was a normal-phase Microsorb 100-5 Si column (3 µm; 250 × 4.60 mm; Varian, CA). Of each sample, 10 µL was injected. Mobile phase consisted of 7% methyl-tert-butyl-ether in hexane with a flow rate of 0.6 mL/min. The amounts of tocopherols were quantified using calibration curves for each isomer separately.

Size exclusion chromatography
The composition of polar components was analyzed using high performance size exclusion chromatography (HPSEC) according to ISO Method 16931-2007 [21]. Separation was performed on a Finnigan Surveyor liquid chromatograph (Thermo Electron Corporation, Rodano, Italy). Components were separated on three size exclusion columns in series (Phenogel 500A, 100A and 50A, 5µ, 300 x 4.60 mm; Phenomenex, Torrance, CA), with tetrahydrofuran (THF) as the mobile phase at a flow rate of 0.3 mL/min, and column temperature of 30 °C. A 10 µL sample was injected, and components were detected with a Sedex 75 evaporative light scattering detector (Sedere, Alfortville, France), operated at 40 °C with air pressure of 2.5 bar.

Color analysis
Color of the frying oils was determined according to AOCS Official method Cc 13c-50 [17] using a DU®-65 spectrophotometer (Beckman, Fullerton, CA).

Statistical analysis
Data were evaluated by analysis of variance (ANOVA, single factor). Statistical significance is expressed at the p < 0.05 level unless otherwise indicated.

Results and Discussion

The fresh oil has 0.06% of free fatty acids (FFA), 1.0 meq/kg of peroxide value (PV), 4.2% of polar components and anisidine value at the level of 4.2, indicating good quality oil [22].
Total polar compounds

The determination of TPC in frying oil provides the most reliable measure of the extent of oxidative degradation [14, 23]. In this study, the contents of TPC increased almost linearly with the frying time at a rate affected by frying temperature (Fig. 1). Total polar content of the oil heated at 185 °C at the end of frying time was 19.8% which was still below 25% oil discarding level set up in many European countries [24]. However, when frying was done at 215 °C the total amount of polar components reached discarding level after 4 days of frying with polars amount close to 40% at the end of frying. The extent of deterioration as measured by TPC for the oil heated at 185 °C at the end of the 7 days frying period was only comparable to the third day of frying at 215 °C. These results are not unexpected; rate of oxidation is temperature dependent and roughly increasing by factor of two with each 10 degree increase in temperature [25]. Activity of tocopherols as antioxidant has been reported to decrease with the increase of frying temperature above 110 °C [26, 27].

Composition of polar components

The composition of polar compounds formed during frying was analyzed using HPSEC and components separated on diglycerides (DG), oxidized triglycerides (OTG), dimers and polymers, and their contribution calculated on the basis of peak areas. The contribution of polymers in total polar material increased consistently with frying time at both frying temperatures achieving maximum at 8% for frying at 185 °C (Fig. 2), and 15.6% at 215 °C (Fig. 3). The amount of polymers generated at 185 °C at the end of the 7 days frying period was comparable to third day of frying at 215 °C, similar trend was observed for the amount of total polar compounds (Fig. 1). Comparable increase in the amount of dimers for oil fried at 185 °C was observed throughout the frying period (Fig. 2). However, at 215 °C, after the 16-fold increase at the end of first day of frying, and slight increase for the next 2 days of frying, the contribution of dimers decreased until the end of the frying period (Fig. 3). This is probably due to the conversion of the dimers to polymers by reaction with degradation products of hydroperoxides [16]. Marquez-Ruiz et al. [28] reported a considerable contribution of dimeric linkages to the structures of polymeric components. As expected, the
contribution of OTG decreased consistently over the frying period at both tested temperatures, as a consequence of thermal degradation [25]. However, a more pronounced decrease in the amount of OTG was observed at the higher frying temperature used 215 °C (Fig. 3).

Anisidine values
Aldehydes formed during oxidative degradation are secondary decomposition products, and non-volatile portion of carbonyls remains in the frying oil [4, 13]. At the two testing temperatures, AV was not well correlated with frying time (Fig. 4). The maximum was reached at the third day of frying for both frying temperatures and then decreased consistently until the end of frying time, apparently oil replenishment play some role in the changes in carbonyls content but elevated temperatures was the main cause that affected amount of these labile and reactive components [29, 30]. An average of 20% decrease in AV for the 30 °C increase in temperature was observed. This result could be explained by the thermal degradation of the aldehydes formed at higher temperature which results in a lower accumulation in the oil at the higher frying temperature, 215 °C. These compounds may also be involved in polymers formation due to their high reactivity [25]. Houhoula et al. [30] reported a significant increase in AV as a function of temperature during frying of potato chips in cottonseed oil.

Fatty acid composition
The fatty acid compositions of the fresh canola oil and the resulted changes during the 7 days of frying at 185 °C and 215 °C in Table 1 are presented. The result indicates a progressive decrease in both linoleic and linolenic acids contributions throughout the frying period. These changes are comparable with previous reports [23, 31]. A significant decrease ($p < 0.05$) was observed for the oil used for frying at 215 °C. The contribution of linoleic acid decreased by 8.5% and 17.5% during frying at 185 °C, and 215 °C, respectively. The deterioration of linolenic acid was more pronounced and showed a decrease by 24.0% and 57.3% during frying at 185 °C and 215 °C, respectively. White et al. [23] reported decrease by 7–11.5% in linoleic acid and 27–46% in linolenic acid when soybean oils were heated at 180 °C for 40 hrs.
The amount of *trans* fatty acids formed during frying increased when temperature and time increased (Figs. 5 and 6). At standard frying temperature the amount of *trans* isomers increased from 2.4% to 3.3%. This is relatively small increase for the 7 days of frying, slightly above analytical error of measurement. The increase in frying temperature to 215 °C caused very extensive *trans* isomerization of fatty acids (Figs. 5 and 6). The total contribution of *trans* isomers in oil increased 2.5 fold, from 2.4% to 5.9% (Fig. 5). This indicates how important temperature is to the formation of *trans* isomers during frying, and explains the amount of *trans* isomers observed in the initial oil (Fig. 5). Deodorization step of canola oil processing is usually performed at temperature above 200 °C under vacuum, where the main amount of *trans* isomers is formed [32]. Regarding individual fatty acid *trans* isomers formation, the amount of these isomers formed from fatty acids decreased in the following order: linolenic > linoleic > oleic (Figs. 5 and 6). The quantity of *trans* isomers formed at elevated temperature indicates that specific amount of energy is required to transfer double bond from *cis* to *trans* configuration. Data from this work are supported by published results, confirming that activation energy for isomerization decreases when the numbers of *cis* double bonds increases. [33].

Increase in the amount of *trans* isomers during frying at higher temperature, can have practical implications related to nutritional claim about “zero *trans* content in serving portion of fried products”. When the amount of these isomers is increasing 2.5 fold during frying at the higher temperature, then the amount of *trans* isomers in fried products will increase by the same amount and easily exceed specified by definition limit, making claim for the product annulled. These data clearly indicates importance of the frying temperature control and keeping it below 190°C.

The ratio of linoleic acid to palmitic acid (C_{18:2}/C_{16:0}) has been suggested as a valid indicator of the level of PUFA deterioration [34]. Our result showed the decrease in this ratio from 4.74 to 3.87 and 4.74 to 3.23 during frying at 185 °C and 215 °C, respectively. This implies that the decrease in this ratio was 1.2 times greater in oil heated at 215 °C as compared to 185 °C. Ornal and Ergin [35] fried a batch of potato per day in canola oil for 10 days at 190 °C and reported a decrease in the ratio from 4.04 to 3.49 at the end of frying time. Houhoula *et al.* [30] reported a reduction of the ratio from 2.39 to 2.03 for cottonseed oil.
heated at 185 °C for 12 h. The decrease in the ratio of linolenic acid to palmitic acid \((C_{18.3}/C_{16:0})\) was more pronounced, reducing it 1.9 times faster in oil heated at 215 °C compared to 185 °C.

Short-chain glycerol-bound aldehydes, acids, ketones and alcohols are non-volatile secondary oxidation products formed during oxidative degradation of lipids [18]. They are of particular chemical and nutritional interest since they remain in the frying oil, and are absorbed and subsequently ingested. Analysis of the oxidized short-chain fatty acid methyl esters (OFAME) as a group revealed a consistent increase in their contribution for the first 5 days of frying at 185 °C, reaching a maximum at 1.83% (Fig. 7). However, for the oil heated at 215 °C, a significant increase in the amount of oxidized fatty acids was observed in the first 3 days of frying with the maximum at 2.20%. Thereafter, a decrease for the next 3 days of frying was observed. This result suggests prevalence of degradation reactions such as dehydration and polymerization, which may not be unexpected at this frying temperature, especially considering the reactive nature of this group of polar compounds. Velasco et al. [36] observed that the amount of polar fatty acid methyl esters decreased drastically as compared to the amount of total polar compounds in used frying fats.

Peers and Swoboda [37] suggested the quantification of methyl octanoate as an oxidation index since octanoic acid is formed during the oxidation of linoleic acid and remains bound to the parent triacylglycerols. In this study, the accumulation of octanoic acid in the frying oil was specifically monitored. The amount increased significantly \((p < 0.05)\) as a function of frying temperature (Fig. 8). The increase in frying temperature from 185 °C to 215 °C resulted in 2-fold increment in the contribution of octanoic acid at the end of frying time. Slope of regression line shows that oxidative degradation of linoleic acid is happening 2.5 times faster at the higher frying temperature, compared to lower frying temperature assessed.

Color analysis
A significant \((p < 0.05)\) effect of frying temperature on formation of color components in the oil was observed. The 30 °C increase in frying temperature produced an over 100% increase in the optical density of frying oil (Fig. 9). The result indicated that the color at the seventh
day of frying at 185 °C was comparable to the color of the oil at the fourth day of frying at 215 °C. After 70 hrs of frying at 170, 180 and 190 °C in different oils, it was observed that color changes were influenced by frying temperature rather than frying medium [31]. Increase in frying temperature also stimulates formation of polymers by non-enzymatic browning which can be the main coloring agent in the oil [9, 38].

Tocopherols
Tocopherols are important minor constituents in oils, acting as natural antioxidants. The tocopherols profile of the fresh canola oil used in this study was found to be: 214 ± 10 ppm α-tocopherol, and 347 ± 18 ppm γ-tocopherol. The extent of tocopherols degradation increased significantly (p < 0.05) as a function of frying temperature. At the end of the seventh day of the frying period, approximately 31% of the total tocopherols present in the fresh oil still remained when the oil was heated at 185 °C (Fig. 10). For oil heated at 215 °C, however, the entire tocopherols were completely spent at the end of sixth day of frying. The calculated half-life of tocopherols for oil heated at 185 °C was 8 hrs while for frying at 215 °C 5.3 hrs. Consistent with previously published results [39], a strong inverse relationship was observed between TPC formation and the reduction of tocopherol at both frying temperatures. Thus, the different degradation rate of tocopherols can be partially accountable for the significant differences observed in oil deterioration at tested temperatures. In this study, γ-tocopherol degraded at the faster rate than α-tocopherol at the lower frying temperature, but the order was reversed during frying at 215 °C (Data not shown).

Correlation between assessment parameters
Although TPC remains the best assessment parameter for evaluating frying oil performance and oxidative stability, a faster and yet objective alternative is desirable. In that case, assessment methods that correlate well with TPC may provide the much needed alternative. In this study, poor correlation was found between AV and TPC, and AV and color at both frying temperatures (Table 2). However, a good correlation was observed between color and TPC at both frying temperatures (Table 2). Lopez-Varela et al. [40] reported a correlation coefficient of 0.885 between color and TPC for sunflower oil used in 75 successive frying of
potatoes. Stevenson et al. [41] also concluded that the color development parallels the development of polar component.

In general, the effect of temperature on frying performance of canola oil as measured by TPC, AV, fatty acid composition and color was significant. Furthermore, despite the general knowledge that the decomposition of hydroperoxides and consequent generation of secondary oxidation products increase with temperature [10, 30], a decrease in AV was observed as the frying temperature increased from 185 °C to 215 °C. Although the disappearance of non-volatile aldehydes as a consequence of their reactive nature at higher frying temperature could have been responsible for lowering of AV. This study showed that increasing frying temperature above 200 °C can cause intensive isomerization of PUFA and the amount of these isomers can increase above threshold level described by “zero trans definition” annulling the nutritional claim for fried product.
References

Fig. 1 Changes in polar components during frying at different temperatures
Fig. 2 Changes in composition of polar components during frying at 185 °C
Fig. 3 Changes in composition of polar materials during frying at 215 °C.
Fig. 4 Changes in anisidine values during frying at different temperatures.
Fig. 5 Changes in total and linolenic acid *trans* isomers amounts during frying at different temperatures.
Fig. 6 Changes in oleic and linoleic acids trans isomers contribution during frying at different temperatures
Fig. 7 Changes in oxidized fatty acids content during frying at different temperatures.
Fig. 8 Changes in octanoic acid content during frying at different temperatures.
**Fig. 9** Changes in oil color during frying at different temperatures.
Fig. 10 Total tocopherols remaining over frying time at different temperatures.
Table 1 Changes in contribution of canola oil fatty acid at different frying temperatures

<table>
<thead>
<tr>
<th>Frying time [h]</th>
<th>Temperature [°C]</th>
<th>Contribution (^a) (Relative Percentage)</th>
<th>C(_{16:0})</th>
<th>C(_{18:0})</th>
<th>C(_{18:1})</th>
<th>C(_{18:2})</th>
<th>C(_{18:3}) (\alpha)</th>
<th>C(<em>{18:2}/C</em>{16:0})</th>
<th>C(<em>{18:3}/C</em>{16:0})</th>
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<tbody>
<tr>
<td>0</td>
<td>185</td>
<td></td>
<td>4.00±0.01</td>
<td>1.82±0.02</td>
<td>60.03±0.72</td>
<td>18.91±0.16</td>
<td>8.40±0.09</td>
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<td>4.24±0.07</td>
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<td>17.90±0.21</td>
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<td>1.68±0.02</td>
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<td>2.02±0.08</td>
<td>61.96±0.95</td>
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<td>1.59±0.02</td>
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<tr>
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<td>4.19±0.03</td>
<td>1.93±0.04</td>
<td>61.20±0.78</td>
<td>17.92±0.19</td>
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<td>0.98±0.01</td>
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<td>15.90±0.18</td>
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\(^a\)All values are averages of triplicate analyses
### Table 2 Correlation coefficient for some assessment parameters at frying temperatures

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<tr>
<th></th>
<th>TPC 185°C</th>
<th>AV 185°C</th>
<th>Polymers 185°C</th>
<th>C&lt;sub&gt;18:2&lt;/sub&gt; 185°C</th>
<th>C&lt;sub&gt;18:3&lt;/sub&gt; 185°C</th>
<th>TPC 215°C</th>
<th>AV 215°C</th>
<th>Polymers 215°C</th>
<th>C&lt;sub&gt;18:2&lt;/sub&gt; 215°C</th>
<th>C&lt;sub&gt;18:3&lt;/sub&gt; 215°C</th>
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<td><strong>Color</strong></td>
<td>0.9302</td>
<td>0.6281</td>
<td>0.9688</td>
<td></td>
<td></td>
<td>0.9650</td>
<td>0.8350</td>
<td>0.9818</td>
<td></td>
<td></td>
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<tr>
<td><strong>AV</strong></td>
<td>0.8609</td>
<td>0.7226</td>
<td></td>
<td>0.5554</td>
<td>0.5719</td>
<td></td>
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<td></td>
</tr>
<tr>
<td><strong>Tocopherol reduction</strong></td>
<td>0.9540</td>
<td>0.8728</td>
<td>0.9313</td>
<td>0.9600</td>
<td></td>
<td></td>
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</tr>
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<td><strong>C₈</strong></td>
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<td>0.9388</td>
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<td>0.9987</td>
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</tbody>
</table>
Formation of $trans$ fats during food preparation

ROMAN PRZYBYLSKI, Ph.D; FELIX A. ALALEDUNYE, M.Sc, Department of Chemistry and Biochemistry, University of Lethbridge, Lethbridge, AB, T1K 3M4 Canada;
ABSTRACT

**Purpose:** The aim of this study was to investigate how typical cooking procedures such as baking, stir-frying used in food preparation affect trans fats formation.

**Methods:** Canola oil was used as the main fat ingredient. Zucchini cake and ginger cookies were baked at 180 and 200°C while stir-fry chicken was prepared at 200 and 275°C. The lipids from the food were extracted following the Folch procedure, and analyzed for trans fatty acids according to ISO Official Method 15304.

**Results:** A minimal changes in the amount of trans fats during baking were observed. Application of extreme temperatures during the baking which caused carbonization of the outer layer of products yielded insignificant increase in the amount of trans isomers. As in baking, stir-frying did not result in significant isomerization of the fatty acids, even when the oil was heated to 275°C and smoking heavily prior to placing the food in it. Irrespective of the cooking procedure, linolenic acid was the most prone to isomerization with the highest amount of trans isomers formed.

**Conclusion:** Baking and stir-frying at normal and/or extreme temperatures do not affect significantly the amounts of trans fats. Likewise, heating oil to the smoking point during stir-frying may decrease the amount of polyunsaturated fatty acids (PUFA) due to oxidative degradation.
INTRODUCTION
Naturally occurring unsaturated fatty acids mostly contain cis-configuration of double bonds, with the lesser amounts of trans conjugated isomers occurring mainly in ruminants. However, treatment of foods and food ingredients at high temperatures, such as deodorization of vegetable oils, are known to initiate isomerization of PUFA (1). It is well established that processing temperature above 190\(^{\circ}\)C are the main cause of PUFA isomerization, where time plays the major role (2,3).

Consistently, metabolic and epidemiological studies indicate that trans fats are more harmful than any other type of fat (4-6). Besides increased risk of developing cardiovascular diseases, the level of low density lipoprotein (LDL) is increasing, concurrently decreasing the high density lipoprotein (HDL). This type of fat was implicated with breast cancer, poor fetal and early infant development and affecting linoleic acid metabolism (7-9). Consequently, maximum limits for daily intake of trans fats have been set by World Health Organization and the instituted regulatory bodies in the most developed countries. Such interest has raised consumer’s fear and skepticism related to the potential formation of trans isomers in polyunsaturated oils when food is prepared using high temperature procedures (10,11).

Thermo-oxidative degradation of lipids during food preparation utilizing elevated temperatures have been studied extensively (12-15), however data on the effect of these cooking methods on the formation of trans fats are rather limited (15,16). Currently, baked goods are the main source of trans fats in our diet and reformulation of baking fats is directed into implementation of liquid oils containing higher amounts of PUFA and with it potential of trans fat formation during baking (15,17).

PURPOSE
In the current study, we investigated the effect of standard and extreme temperatures used in typical food cooking procedures such as baking, stir-frying on trans fat formation.
**METHODS**

**Materials:** Refined, bleached and deodorized canola oil without antioxidants was obtained from Richardson Oilseed Processing (Lethbridge, Canada). All reagents of analytical or HPLC purity were purchased from Sigma–Aldrich (St.Louis, MO, USA). Standards of tocopherols were obtained from Calbiochem-Novabiochem (San Diego, CA, USA) while standards of fatty acid methyl esters were purchased from Nu-Chek-Prep, Inc. (Elysian, MN, USA).

**Food preparation:** Zucchini cake, Betty’s gingersnaps cookies and stir-fried oriental chicken were prepared following the recipes available on the Canola Council webpage using ingredients specified in Table 1.

**Lipids Extraction:** Lipids were extracted from foods following the Folch procedure (18).

**Fatty acid composition:** Fatty acids were analyzed following the ISO method 15304 and as previously reported (3,19).

**Statistical analysis:** Results were analyzed by single factor analysis of variance (ANOVA) using Minitab 2000 statistical software (Minitab Inc, PA, ver. 13.2). Statistical differences between means were determined by Duncan’s test at p < 0.05.

**RESULTS**

**Canola oil**

Typical canola fatty acids composition was found in fats extracted from products (Table 2). The initial amounts of *trans* fat in canola oil was usual for processed oil and will not affect “zero *trans*” claim when the amount is calculated per serving portion of any product where the oil is used as ingredient (1).

**Baking**

The oil extracted from the dough exhibited the same profile of *trans* fatty acids as found in the fresh oil, indicating that ingredients used in the dough did not have *trans* isomers
Zucchini cake baked at 200°C had crust carbonized to about 2 cm in depth, while the cookies were black to about one third of their depth with only small portion having lighter colour. Baking at 200°C showed a slight but statistically insignificant increase in the amount of trans fat above the initial amount observed in the oil. The major changes occurred for linolenic acid, and its trans isomers contribution was at 70% of all trans fat (Figure 1A). Similar trends in trans fat formation were observed for gingersnap cookies (Figure 1B and Table 2).

**Stir-frying**

Statistically insignificant increase in the amount of trans isomers was observed during stir-frying at both temperatures, 200°C and 275°C (Figure 1C and Table 2). Application of temperature well above smoking point of oil did not promote isomerization, however caused oxidative degradation of PUFA (Data not included).

**DISCUSSION**

The data presented in this paper clearly validates that preparation of food by baking and stir-frying did not accelerate isomerization of PUFA, no additional amounts of trans fats were observed. Applying elevated temperatures accidentally or intentionally also did not produced additional amount of trans fat in the baked products. The main factors affecting trans isomers formation are temperature and time (2,3). During stir-frying the oil was heated at elevated temperature was short time. In baking and stir-frying the fat was protected by water present in a prepared food which lowers temperature to a maximum of 100°C. Maga et al (16) observed similar and insignificant changes in trans fat formation during potatoes baking. The activation energy for PUFA isomerization decreases when the number of cis double bonds increases (2,20), hence the linolenic acid trans isomers dominated among trans fat.

In conclusion, baking and stir-frying at normal or/and extreme temperatures did not cause formation of trans fats. However, during deep-fat frying, where oil is used for extended period of time and multiple batches of food are fried, as is happening in institutional frying, with passing time the amount of formed trans fats is increasing.
During this process extensive exchange between frying fat and fat in fried product occurs and all degradation products including trans fat are incorporated into fried foods (3).

**RELEVANCE TO PRACTICE**

The results from this study verify that the preparation of food by baking, stir-frying and deep-fat frying at standard conditions did not lead to the formation of trans fats (3). In addition, baking and stir-frying when carried out at abusive conditions did not alter the amount of trans fats. However, deep-fat frying where the same oil is used for long time, can be a source of trans fat during food preparation, particularly when operational frying temperature is above the optimal range of 180 -190°C (3). Thus proper temperature and time control during frying and baking is the most important parameter to minimize formation of the trans fatty acids.

**Acknowledgments**

The project was supported financially by Canola Council of Canada and Alberta Value Added Corporation.
**References**


11. Alexandra C, Jim M, Wayne S, Ashley D, Murray S, Christopher F. Effect on lipoprotein profile of replacing butter with margarine in a low fat diet: randomized


Table 1 Ingredients used for zucchini cake, gingersnap cookies and oriental stir-fry chicken preparation.

<table>
<thead>
<tr>
<th>South Pacific Zucchini Loaf</th>
<th>Gingersnaps cookies</th>
<th>Oriental Chicken Stir-fry</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 cups all purpose flour (750 mL)</td>
<td>Canola oil (125 mL)</td>
<td>Chicken breast strips (450 g)</td>
</tr>
<tr>
<td>2 tsp baking soda (10 mL)</td>
<td>1.25 cup granulated sugar (125 mL)</td>
<td>1 tsp canola oil (15 mL)</td>
</tr>
<tr>
<td>1.5 tsp baking powder (7 mL)</td>
<td>Egg (1 piece)</td>
<td>1 tsp minced gingerroot (5 mL)</td>
</tr>
<tr>
<td>1.5 tsp ground cinnamon (7 mL)</td>
<td>0.25 cup molasses (50 mL)</td>
<td>4 cups assorted oriental</td>
</tr>
<tr>
<td>1 tsp ground nutmeg (5 mL)</td>
<td>1.75 cups all purpose flour (425 mL)</td>
<td></td>
</tr>
<tr>
<td>1 tsp salt (5 mL)</td>
<td>2 tsp ginger (10 mL)</td>
<td>2 tsp soy sauce (30 mL)</td>
</tr>
<tr>
<td>Eggs (3 pieces)</td>
<td>1 tsp cinnamon (5 mL)</td>
<td>1 tsp vinegar (15 mL)</td>
</tr>
<tr>
<td>Canola oil (250 mL)</td>
<td>1 tsp baking powder (5 mL)</td>
<td>1 tsp cornstarch (15 mL)</td>
</tr>
<tr>
<td>1.5 cups granulated sugar (375 mL)</td>
<td>1 tsp baking soda (5 mL)</td>
<td>1 tsp honey (15 mL)</td>
</tr>
<tr>
<td>2 tsp vanilla (10 mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 cups shredded zucchini (500)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 can crushed pineapple (398)</td>
<td></td>
<td></td>
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<tr>
<td>1 cup chopped pecans (250 mL)</td>
<td></td>
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<tr>
<td>Yields 2 loaves (565 g each)</td>
<td>Yields 36 cookies (12 g each)</td>
<td>Yields 4 servings</td>
</tr>
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</table>
Table 2

Composition of fatty acids during different cooking methods

<table>
<thead>
<tr>
<th>Processing condition</th>
<th>Contribution(^a) (%)</th>
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<td>SAT</td>
<td>MUFA</td>
<td>PUFA</td>
<td>TRANS</td>
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<tr>
<td>Canola oil</td>
<td>5.82 ± 0.10(^a)</td>
<td>60.03 ± 0.72(^b)</td>
<td>27.31 ± 0.34(^c)</td>
<td>1.97 ± 0.02(^d)</td>
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<tr>
<td>Zucchini cake 180(^o)C</td>
<td>5.82 ± 0.09(^a)</td>
<td>60.00 ± 0.89(^b)</td>
<td>27.33 ± 0.43(^c)</td>
<td>1.98 ± 0.04(^d)</td>
</tr>
<tr>
<td>Zucchini cake 200(^o)C</td>
<td>5.83 ± 0.11(^a)</td>
<td>60.02 ± 0.96(^b)</td>
<td>27.29 ± 0.39(^c)</td>
<td>2.12 ± 0.07(^d)</td>
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<tr>
<td>Ginger cookies 180(^o)C</td>
<td>5.89 ± 0.07(^a)</td>
<td>59.98 ± 0.68(^b)</td>
<td>27.11 ± 0.51(^c)</td>
<td>2.07 ± 0.04(^d)</td>
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<tr>
<td>Ginger cookies 200(^o)C</td>
<td>5.85 ± 0.10(^a)</td>
<td>59.89 ± 0.91(^b)</td>
<td>27.01 ± 0.48(^c)</td>
<td>2.14 ± 0.08(^d)</td>
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<td>Stir frying 200(^o)C</td>
<td>5.91 ± 0.10(^a)</td>
<td>59.81 ± 0.88(^b)</td>
<td>27.00 ± 0.28(^c)</td>
<td>2.03 ± 0.05(^d)</td>
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<tr>
<td>Stir frying 275(^o)C</td>
<td>6.01 ± 0.09(^a)</td>
<td>60.07 ± 0.66(^b)</td>
<td>26.78 ± 0.31(^c)</td>
<td>2.22 ± 0.06(^d)</td>
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</table>

\(^a\) Data are presented as mean ± SD and all values are averages of triplicate analyses from three separate trials (n = 9). Means within a column marked with same superscripts do not differ significantly at p < 0.05. SAT: Saturated fatty acids, MUFA: Monounsaturated fatty acids, PUFA: Polyunsaturated fatty acids, TRANS: Trans fatty acids.
Figure 1 Formation of *trans* isomers during baking and stir-frying at different temperatures. Results represent total contribution of *trans* isomers and for specific fatty acids. (A) - Zucchini cake; (B) - Gingersnap cookie; (C) - Stir-fry.
Protecting Oil during Frying – A Comparative Study

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Abstract

The effect of carbon dioxide blanketing and vacuum frying on the frying performance of regular canola oil was evaluated. For seven hours daily and for seven days French fries were fried in regular canola oil at 185 ± 5 °C without and with carbon dioxide blanketing and in vacuum fryer. Extend of changes in oil were assessed by analysis of total polar compounds (TPC), anisidine value (AV), color components formation and changes in composition of fatty acids and tocopherols. Frying under CO₂ blanket (CDB) reduced the amount of total polar compounds by 54% while 76% reduction was observed during vacuum frying compared to standard frying conditions (SFC). Similarly lower oxidative degradation was observed when measured by anisidine value. At the end of frying period, the reduction in unsaturated fatty acids content was 3.8%, 1.9% and 12.7% when frying under CDB, vacuum and SFC, respectively. The rate of tocopherols degradation was three and twelve times slower in vacuum frying when compared to CDB and SFC, respectively.

Keywords: Canola oil, Carbon dioxide blanket, Vacuum frying, Frying performance, Total polar compounds, Anisidine value, Color, Tocopherols, French fries.
Introduction

Deep-fat frying is always performed with access of atmospheric oxygen, the main cause of oxidative degradation of frying oil. Fried foods offer a product with desired sensory characteristics including fried food flavor, golden brown color and a crisp texture [1]. However, applied high temperature, the presence of air and moisture initiate several chemical and physical changes in the oil which affect the sensory and nutritive qualities of both the oil and the food fried in it. During frying intensive mass exchange is happening between frying oil and fried food. As effect of it, degradation products present in oil are fast transferred into fried food [2]. Compounds formed during thermo-oxidative degradation of oil may have detrimental health effect whereas volatile components are part of pollutants affecting negatively frying facility [3, 4].

Lipid oxidation has been identified as single the most important factor affecting the performance of edible oil, and the presence of oxygen is suggested to be the main factor stimulating oxidative degradation of oil during frying [5, 6]. Therefore, approaches aimed to reduce the oxygen presence during frying have been reported as ways of protecting oil from oxidative degradation. These include flushing frying oil with nitrogen or carbon dioxide or recently, applying frying under vacuum [7–9]. It has been established that carbon dioxide offers greater protection than nitrogen for an oil during heating [8].

Shyu et al. [9] reported the superior performance of palm, soybean oils, and lard during vacuum frying of carrot chips over standard frying. Potato chips fried under vacuum absorbed more oil when frying temperature increased [10, 11]. Additionally, most of the reports on vacuum frying focused on its effect as drying process with an aim at quality of dried products, neglecting performance of the frying fats [11–14].

It is a general knowledge that both frying under carbon dioxide blanketing and vacuum frying offer protection to frying oil, although quantitative data on frying performance of oils under these conditions are very scanty and there is no data on the effectiveness of these protection methods.
The purpose of this study was a comparison of extent of canola oil degradation under carbon dioxide blanketing, vacuum frying, as compared to standard frying conditions.

Materials and Methods

Materials

Oil and French fries

Commercially refined, bleached and deodorized regular canola oil (peroxide value 2 meq/kg; FFA 0.01%) without antioxidant added was donated by Canbra Foods Ltd (Richardson Oil Processing, Lethbridge, Canada). Frozen par-fried French fries in institutional packs were obtained from a local food store.

Chemicals

All solvents and chemicals of analytical grade were used in this study and purchased from Sigma-Aldrich (St. Louis, MO). Standards of tocopherols were obtained from Calbiochem-Novabiochem (San Diego, CA). Standards of fatty acid methyl esters mixtures were purchased from Nu-Check-Prep (Elysian, MN). Carbon dioxide of anaerobic purity was purchased from Liquid Air (Calgary, Canada).

Frying procedure and oil sampling

Standard frying and under carbon dioxide blanketing

The standard frying (SFC) was conducted in 8 L capacity restaurant style stainless steel fryers (General Electric Company, Niskayuna, USA). Canola oil (4 L) was heated at 185 ± 5°C, 7 hours daily for 7 days. A batch of 400 g of frozen French fries was fried for 5 minutes for a total of eight batches per frying day. At the end of each frying day, fryers were shut off and left to cool down overnight. Two 25 mL samples of oil from each of the fryers were taken daily and kept frozen at -20°C until analyzed. Before commencing frying each day, oils were filtered to remove solid debris. Oil was replenished every
second day of frying with 500 mL of fresh oil. Standard frying conditions was used as control in this assessment.

The set up described above was used for frying under carbon dioxide blanketing (CDB). The carbon dioxide was delivered through stainless steel tubing (2 mm i.d.) with 0.6 mm holes placed equally alongside of fryer and at a flow rate of 2.5 L/min. The tubing was attached to the upper edge of the fryer and gas outlets were 1 cm above the oil surface. Oil was purged with CO$_2$ for 10 minutes prior commencing frying and at the end of each frying day, the flow of carbon dioxide was continued until the temperature of oil dropped below 100°C.

Vacuum frying
Vacuum frying (VF) was done using BT-1 Industrial Vacuum Deep Fryer (Sakuma Corporation, Chiba, Japan). Canola oil (9.5 L) was placed into a 10 L capacity industrial vacuum fryer. A batch of 400 g of frozen French fries was fried under 9.7 kPa vacuum at 180 ± 2°C for 2.5 minutes. A total of eight batches of French fries were fried daily for 7 days. At the end of each day, the frying oil was allowed to cool overnight under the vacuum. Two 25 mL samples were taken daily and kept frozen at -20°C until analyzed. Oil was replenished every second day of frying with 500 mL of fresh oil. The vacuum frying conditions produced fried French fries comparable in sensory properties to those obtained under SFC.

Fatty acid analysis
Fatty acids were methylated according to AOCS Official Method Ce 1-62 [15]. The resulting methyl esters (FAME) were analyzed on Trace GC Ultra gas chromatograph (Thermo Electron Corp., Rodano, Italy) using a Trace TR-FAME fused silica capillary column (100 m × 0.25 mm × 0.25 µm; Thermo, Waltham, MA, USA). Hydrogen was used as carrier gas with flow rate of 1.5 mL min$^{-1}$. Column temperature was programmed from 70°C to 160°C at 25°C min$^{-1}$ then held for 30 minutes, and further programmed to 210°C at 3°C min$^{-1}$. Initial and final temperatures were held for 5 and 30 minutes, respectively. Splitless injection was used utilizing PTV injector. Detector temperature was set at 250°C. FAME samples, 1 µL, were injected with AS 3000 autosampler
(Thermo Electron Corporation, Rodano, Italy). Fatty acids were identified by comparison of retention time with authentic standards (Standard mixture #617; Nu-Chek-Prep, Elysian, MN).

Tocopherols
Tocopherols were analyzed according to AOCS Official Method Ce 8-89 [15]. Briefly, oil samples (75 mg) were weighed directly into autosampler vials and dissolved in 1.5 mL hexane. Analyses were performed on a Finnigan Surveyor LC (Thermo Electron Corp., Waltham, MA, USA) with a Finnigan Surveyor Autosampler Plus and Finnigan Surveyor FL Plus fluorescence detector, the latter set for excitation at 292 nm and emission 394 nm. The column was a normal phase Diol column (5µm; 250 × 4.6 mm; Monochrom, Varian, CA). Of each sample, 10 µL was injected. Mobile phase consisted of 7% methyl-tert-butyl-ether in hexane with a flow rate of 0.6 mL/min. The amounts of tocopherols were quantified using external calibration method where each isomer was calibrated separately.

Total polar compounds (TPC)
TPC were determined by gravimetric method following AOAC Method 982.27, using column chromatography to separate non-polar fraction from polar one [16]. This procedure was modified according to Schulte [17]. Polar fractions were eluted with 50% MTBE in hexane and further analyzed for composition by size exclusion chromatography.

Size exclusion chromatography
The composition of polar components was assessed using high performance size exclusion chromatography (HPSEC) according to ISO Method 16931-2007 [18]. Separation was performed on a Finnigan Surveyor liquid chromatograph (Thermo Electron Corporation, Waltham, MA). Components were separated on three size exclusion Phenogel columns connected in series (500A, 100A and 50A; 5µ, 300x 4.6
mm; Phenomenex, Torrance, CA) kept at 30ºC. Tetrahydrofuran (THF) was used as a mobile phase at flow rate of 0.3 mL/min. Sample of 10 µL was injected, and eluting components detected with evaporative light scattering detector (Sedex 75; Sedere, Alfortville, France), operated at 30ºC with purified air at pressure of 2.5 bar.

Anisidine values (AV)
AV, a measure of secondary oxidation products, was determined according to ISO Method 6885:2004 [19].

Color
Color of the frying oils was assessed according to AOCS Official method Cc 13c-50 [15] using a DU®-65 spectrophotometer (Beckman, Fullerton, CA).

Statistical analysis
Samples from three repetitions of frying for each frying protocol were collected and were analyzed in triplicate. Data are presented as mean value ± SD. Data was analyzed by single factor analysis of variance (ANOVA) and regression analyses using Minitab 2000 statistical software (Minitab Inc, PA, ver. 13.2). Statistically significant differences between means were determined by Duncan’s multiple range tests. Statistically significant differences were determined at $P \leq 0.05$.

Results and Discussion
Frying
Frying was performed under typical parameters used on the American continent and it is in contrast to Europe where frying temperature is often regulated. During VF lower by 5ºC frying temperature was applied to obtain comparable quality of French fries as in the other methods. To explain further the differences in frying temperature, during SFC and CDB temperature drop to 165ºC for about 2 to 3 minutes was observed when frozen fries were loaded. After about 4 minutes temperature of the oil return to 185 ºC. During VF drop of temperature was only a few degrees, mainly due to lower boiling point of water and in reality average temperature was similar for all frying conditions.
During frying oil is top up due to the removal of oil by fried foods. In CDB and SFC 10%, while in VF 5% of frying oil was replaced every second day. Larger amount of replaced oil in the former frying conditions should have more efficient protecting effect than in vacuum frying; however presented data in this paper shows opposite. Added fresh oil has minimal effect on oxidative degradation of oil where frying conditions played the major role.

Total polar compounds
Polar materials, among them polymerized triacylglycerols, are formed as major secondary oxidation products during frying. Since they remain in the oil, their presence is the most reliable measure of the extent of oxidative degradation [20]. For the three frying protocols, TPC amount increased significantly (P ≤ 0.05) as the frying time progressed. Oil during SFC underwent the most rapid oxidation and accumulated the highest amount of polar components (Fig. 1). At the end of the frying time, the amount of TPC was 2.2 and 4.1 times higher in SFC compared to CDB and vacuum frying, respectively. During SFC the rate of polar components formation was 2 and 8 times faster compared to CDB and VF, respectively.

Anisidine values
Carbonyls are the most abundant secondary oxidation products formed from oxidative degradation of unsaturated fatty acids during frying [21]. CDB and vacuum frying resulted in 65% and 93% reduction in anisidine value compared to SFC, respectively (Fig. 2). During the first day of frying, 80% increase in AV was observed for all frying conditions. This can be related to the initial amounts of dissolved oxygen in the frying oil, and potential thermo-oxidative degradation stimulated by it presence. Przybylski and Eskin [8] reported that the amount of oxygen dissolved in oil is sufficient to provide peroxide value of 10 meq/kg. From the second day of frying, the effectiveness of CDB protection was comparable to vacuum frying where AV reached plateau with insignificant increase up to the end of frying time (Fig. 2). At SFC the value for AV increased significantly through all frying time (Fig 2).
Composition of polar components

The individual classes of polar compounds formed during the various frying conditions were separated by HPSEC as oxidized triacylglycerides (OTG), dimers and polymers. Formation and accumulation of dimers increased consistently over the entire frying period regardless of the frying conditions (Fig. 3). Excluding the first day of frying, 1.5 and 3 times lower rate of dimers formation were observed for VF when compared to CDB and SFC, respectively (Fig 3). Since the formation of hydroperoxide is a prerequisite for dimers production, the above result implied a greater ability of vacuum frying over carbon dioxide blanketing to defer oxidation [22, 23]. Similarly to AV, the amount of dimers formed during the first day of frying in SFC and CDB was the highest compared to VF, indicating better removal of dissolve oxygen by vacuum (Fig 3).

The amounts of formed polymers increased consistently with frying time for all frying conditions (Fig. 4). Since formation of oligomers is dependent on hydroperoxides presence, similar pattern of their development was observed, where first day offered the highest increase in contribution. The rate of polymers formation was three and seven times lower for frying under vacuum when compared to CDB and SFC, respectively (Fig 4). However, differences in polymers formation might be partially affected by a shorter frying time, 2.5 minutes per batch during vacuum frying as compared to a 5 minutes/batch for frying under CDB.

The contribution of oxidized triacylglycerides consistently decreased over the frying time regardless of the frying conditions (Fig. 5). These compounds are precursors to oligomers and other larger molecules. The decrease was most pronounced for SFC compared to both CDB and vacuum frying, indicative of higher rate of polymerization. During the first day of frying the largest decrease in contribution of oxidized triacylglycerides was observed for all frying conditions. After that, rate of these components disappearance was similar regardless of applied frying conditions.

Fatty acid composition

The fatty acid composition of fresh canola oil and the resulted changes during the frying under the three frying protocols in Table 1 is presented. A progressive decrease in
contributions of linoleic and linolenic acids throughout the frying period were observed, with the later one the most affected. The contribution of linolenic acid decreased by 24.5%, 7.1% and 5.3% under SFC, CDB and vacuum frying, respectively. However, for linoleic acid losses were smaller, and the contribution decreased by 0.5%, 2.4% and 7.6% during vacuum frying, CDB and SFC, respectively. Fujisaki et al. [6] reported that in environment with reduced amount of oxygen to 2%, the decrease of linoleic acid was negligible. Shyu et al. [9] found that contributions of linoleic and linolenic acids decrease by 12.7% and 19.6% during vacuum frying of carrot slices in soybean oil, respectively.

Tocopherols
Tocopherols are the single most important natural antioxidants in edible oils. The tocopherol profile of the fresh canola oil used in this study was: 317 µg/g of α-tocopherol, and 353 µg/g of γ-tocopherol. The degradation of tocopherols in canola oil during frying at different conditions in Figure 6 is shown. At the end of frying period, 15% of the total amounts of tocopherols remained in the oil during standard frying. Whereas, during CDB frying 70% of the initial amounts of tocopherol remained at the end of frying period. The lowest degradation of tocopherols was observed during vacuum frying with 90% left at the end of the frying. The average rates of tocopherol disappearance were 82 µg/g, 17 µg/g and 7 µg/g per frying day during SFC, CDB and vacuum frying, respectively. Slow degradation of tocopherols can improve storage stability of fried products and offer better nutritional value. Fujisaki et al. [6] reported insignificant changes in the tocopherols content during heating of high oleic safflower oil in environment with reduced amount of oxygen to 2%.

Regarding individual tocopherols, irrespective of the frying conditions, γ-tocopherol degraded faster than α-tocopherol (Fig. 7). The average rates of disappearance for α-tocopherol were 37 µg/g, 7 µg/g, and 2 µg/g per frying day for SFC, CDB and vacuum frying, respectively. Whereas, the corresponding rates for γ-tocopherol were 44 µg/g, 15 µg/g and 4 µg/g per frying day. These results are in agreement with the finding of Matthäus [24] who reported a faster rate of degradation of γ-tocopherol compared to α-tocopherol during frying in high-oleic rapeseed oil, high-oleic sunflower oil, partially hydrogenated rapeseed oil and palm olein.
Color

Frying conditions significantly ($P < 0.05$) affected a rate of pigments formation. Generally formation of pigments increased as a function of frying time regardless of the frying conditions (Fig. 8). Pigments formed 3 and 7 times faster during CDB and SFC compared to vacuum frying. This directly indicates that formation of pigments is affected by oxidation of fatty acids and thermal decomposition of oxidation products.

Conclusions

The present study evidently showed the superior ability of vacuum frying to protect frying oil from oxidative degradation, extending fry-life of it and improving nutritional quality of fried foods. However, the initial capital cost of equipment is very high and operation is complicated and cumbersome. Application of the vacuum system in institutional and industrial frying will be costly and operationally labour intensive and probably impossible to apply at current state of technology available. Important enhancement in performance of frying oil was also achieved when frying under carbon dioxide blanket. The later offer simple modification to the fryer and with it significant improvement in quality of fried foods by impeding oxidative degradation of oil and extending fry-life of the oil. Proposed protection method can have significant impact on the amounts and rate of formation of oxidative degradation products in frying oil and their transfer into fried foods.

Acknowledgements

We thank the Food Processing Development Centre, Leduc, Alberta for allowing us to use vacuum fryer and Marek Gierus for his knowledgeable help during vacuum frying.

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References


Table 1 Changes in contribution of fatty acids during frying in canola at different conditions

<table>
<thead>
<tr>
<th>Frying time[h]</th>
<th>Frying Conditions</th>
<th>Contribution a (Relative Percentage)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C16:0</td>
</tr>
<tr>
<td>0</td>
<td>Standard</td>
<td>4.04±0.04^b</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>4.04±0.04^b</td>
</tr>
<tr>
<td>14</td>
<td></td>
<td>4.24±0.06^c</td>
</tr>
<tr>
<td>21</td>
<td></td>
<td>4.21±0.12^c</td>
</tr>
<tr>
<td>28</td>
<td></td>
<td>4.25±0.09^c</td>
</tr>
<tr>
<td>35</td>
<td></td>
<td>4.20±0.12^c</td>
</tr>
<tr>
<td>42</td>
<td></td>
<td>4.29±0.05^c</td>
</tr>
<tr>
<td>49</td>
<td></td>
<td>4.47±0.05^d</td>
</tr>
<tr>
<td>0</td>
<td>With CO2</td>
<td>4.04±0.03^b</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>4.08±0.05^b</td>
</tr>
<tr>
<td>14</td>
<td></td>
<td>4.15±0.05^c</td>
</tr>
<tr>
<td>21</td>
<td></td>
<td>4.22±0.11^c,d</td>
</tr>
<tr>
<td>28</td>
<td></td>
<td>4.31±0.08^d</td>
</tr>
<tr>
<td>35</td>
<td></td>
<td>4.28±0.04^d</td>
</tr>
<tr>
<td>42</td>
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<tr>
<td>49</td>
<td></td>
<td>4.19±0.05^e</td>
</tr>
<tr>
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<td>Vacuum</td>
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<td>7</td>
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<td>4.07±0.03^b</td>
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<tr>
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<tr>
<td>21</td>
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<tr>
<td>28</td>
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<td>4.11±0.09^b,c</td>
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<td>35</td>
<td></td>
<td>4.20±0.11^e</td>
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<tr>
<td>42</td>
<td></td>
<td>4.18±0.06^e</td>
</tr>
<tr>
<td>49</td>
<td></td>
<td>4.17±0.09^e</td>
</tr>
</tbody>
</table>

a All values are averages of triplicate analyses from three repetitions. Means within a column of the same frying condition marked with the same superscripts do not differ significantly at P < 0.05.
Fig. 1 Changes in polar components during frying at different conditions.

Abbreviations: Control – Standard frying conditions; With CO₂ – Frying with carbon dioxide blanketing; Under vacuum – Vacuum frying.
Fig. 2 Changes in anisidine values during frying at different conditions.

For abbreviations see Fig 1.
Fig. 3 Changes in contribution of dimers during frying at different conditions.

For abbreviations see Fig 1.
Fig. 4 Changes in polymers during frying at different conditions. For abbreviations see Fig 1.
**Fig. 5** Changes in contribution of oxidized triglycerides during frying at different conditions. For abbreviations see Fig 1.
**Fig. 6** Total tocopherols changes during using different frying conditions.

For abbreviations see Fig 1.
Fig. 7 Changes in α and γ-tocopherol isomers during frying using different conditions.

For abbreviations see Fig 1.
Fig. 8 Changes in pigments formation during frying using different conditions.

For abbreviations see Fig 1.
Novel procedures for quantifying 4-hydroxynonenal and impeding formation including acrylamide during frying with carbon dioxide blanketing

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Running title: Inhibiting HNE and acrylamide formation during frying

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**Abstract**

Acrylamide and 4-hydroxynonenal (HNE) are among the most detrimental compounds formed during high temperature processing of food. The effect of carbon dioxide blanketing (CDB) on the formation and accumulation in food of these compounds during deep-fat frying was investigated. French fries were fried for 7 h daily and for 7 days in canola oil at 185 ± 5°C without and with CO₂ protection. The amount of acrylamide and HNE accumulated in the French fries were analyzed. Compared to standard frying conditions (SFC), frying under CDB reduced the amount of HNE by 62%. On the 3rd day of frying, the amount of acrylamide in fries fried under SFC was 3.3 times higher compared to frying with CO₂ protection. Frying with carbon dioxide protection is an effective and practical way to impede formation of toxic components during deep-fat frying. To assess formation of HNE a simple, sensitive and reliable procedure for HNE analysis in frying oils and fried products was developed and evaluated.

**Practical applications:** The toxicity of HNE and acrylamide, coupled with the increasing consumption of fried foods necessitates that measures be taken to reduce their formation and subsequent accumulation in fried foods. The frying method proposed in this study is very effective and requires only a simple modification to the fryer. Developed rapid and simple procedure for HNE analysis allows more accurate quantification.
1. Introduction

Polyunsaturated oils such as canola are susceptible to thermo-oxidative reactions during food processing. The extent of degradation, the amount and nature of degradation products depend, among other factors, on the temperature applied and accessibility of oxygen. During deep fat frying where elevated temperatures (160 – 190°C) are employed, these reactions rate is particularly high and usually leads to the formation of a variety of degradation products [1]. Because of the intensive mass exchange that occurs between the frying oil and the fried food, most of the degradation products are transferred into the fried food [2].

Many toxic degradation products have been identified in the deep-fat fried foods, and the list keeps growing. However, acrylamide and 4-hydroxynonenal (HNE) have attracted much attention not only because of their unusually high toxicity, but also because they could be formed at concentrations that may pose health concerns [3, 4]. Indeed, their detection in food has been of both national and international concerns. It has been reported that more than one-third of the calories consumed by U.S and European populations contain acrylamide [5]. Because of their great reactivity, acrylamide and HNE are known to modify proteins, nucleic acids and other biomolecules leading to several diseases and medical conditions [4, 6]. Acrylamide is also regarded as mutagenic, cytotoxic and carcinogenic as demonstrated by both in vitro and in vivo studies [8]. As a matter of fact, the risk of acrylamide estimated by the Scientific Committee on Toxicity, Ecotoxicity and the Environment (CSTEE) in the European Union demands that the exposure to this compound should be minimized to the lowest possible level [9].

HNE is a secondary oxidation product of omega-6 polyunsaturated fatty acids. It is reasonable therefore to postulate that any measure that reduces the availability of oxygen or discourages oxidative degradation will inhibit the formation of HNE and acrylamide. HNE has been shown to exhibit mutagenic, cytotoxic and genotoxic properties, which are related to pathogenesis of several human diseases such as Alzheimer’s and atherosclerosis [4, 7]. It inactivates various enzymes, inhibits the proliferation of cells and acts as a chemotaxin [7].
The toxicity of these compounds, coupled with the increasing consumption of fried foods necessitates that measures have to be taken to reduce their formation and accumulation in foods. Studies have shown that frying under vacuum inhibits the formation of acrylamide because this process allows using lower frying temperature (< 140°C) and accessibility to oxygen is limited [10, 11]. German regulations require that frying operations be carried out at a temperature not higher than 165°C to limit formation of acrylamide [12]. The interaction between asparagine and reducing sugar is the most widely recognized acrylamide formation mechanism in foods [13]. Recent studies have shown the significant contributions of lipid oxidation products to acrylamide formation in model systems [14 – 16].

Recently, we reported the efficiency of CDB to protect oil from thermo-oxidative degradation during frying [17]. In the present study, we continued to investigate the effectiveness of CDB to reduce the formation and accumulation of HNE and acrylamide during frying.

Most of the methods applied for the sample preparation and analysis of 4-hydroxynonenal from oils and fried products are derived from procedures developed initially for biological systems utilizing its derivatization to 2,4-dinitrophenylhydrazones [18 – 22]. However, the complexity of the degradation products formed during frying, where the main compounds are carbonyls, demands an efficient and rapid procedure for HNE analysis. Purification of the complex mixture of carbonyl hydrazones is multi step and labourious, and potential source of errors. Complete separation of purified samples on HPLC is impossible due to similar properties of carbonyl hydrazones and usually HNE peak is placed in the middle of the peaks cluster, affecting proper integration and quantification [19]. Furthermore, its labile nature, lengthy extraction and clean-up procedure may be a source of errors and artefacts formation. Taking into account all problems related to currently used procedures to quantify HNE content, we developed simple, rapid, reproducible and efficient method to analyse this compound in oil samples, which omit derivatization and purification.
2. Materials and methods

2.1 Materials

2.1.1 Oil and French fries

Commercially refined, bleached and deodorized regular canola oil without antioxidant was donated by Richardson Oilseed Limited (Lethbridge, Alberta). Frozen par-fried French fries in institutional packs were obtained from a local food store.

2.1.2 Chemicals

Deuterated acrylamide (98%) was supplied by Cambridge Isotope Laboratories (Andover, USA). Ethyl acetate, petroleum ether, and sodium chloride were purchased from Merck (Darmstadt, Germany). Acetonitrile, methanol, chloroform, hexane, methyl-tert-butylether, and 4-hydroxynonenal were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anaerobic carbon dioxide was purchased from Liquid Air (Calgary, Canada). All solvents and chemicals were of analytical grade.

2.2 Frying procedure and sample preparations

2.2.1 Standard frying (SFC) and carbon dioxide blanketing (CDB)

Frying under SFC was performed in 8 L capacity restaurant style stainless-steel fryer (General Electric Company, Niskayuna, USA). Canola oil (4 L) was heated at 185°C ± 5°C for 7 h daily for 7 days. A batch of 400 g of frozen French fries was fried for 5 min for a total of eight batches per frying day. At the end of each frying day, the fryer was shut off and left to cool down overnight. The last batches of fries from each frying day were kept frozen at -16°C until analyzed. Before commencing each frying day, the oil was filtered to remove solid debris. The oil was replenished every second day of frying with 500 mL fresh oil.

The setup described above was used for frying under CDB. The carbon dioxide was delivered through stainless-steel tubing (2 mm i.d.) with 0.6-mm holes equally distributed alongside of the fryer and at flow rate of 2.5 L/min. The tubing was attached to the upper edge of the fryer, and the gas outlets were 1 cm above the oil surface. Oil
was purged with CO₂ for 10 min prior to commencing frying. At the end of each frying day the flow of carbon dioxide was continued until the temperature of oil dropped below 100°C.

2.2.2 Extraction of lipids
Samples of French fries (50 g) from each day of frying were chopped into small pieces and homogenized with 400 mL of chloroform/methanol (2:1, v/v) following the Folch procedure [23]. The solvent/lipid mixture was filtered through paper filter (Whatman #2) into a separatory funnel. Distilled water (100 mL) was added, and after mixing, the mixture was allowed to separate into two layers. The lower layer was collected in a round bottom flask, and the solvent removed under vacuum on a rotary evaporator (BÜCHI Labortechnik AG, Flawil, Switzerland) at 35°C. The oil samples were transferred with isooctane to appropriately labelled vials, flushed with nitrogen and stored at -16°C until analyzed. Extracted oils were used for analysis parameters described in this paper, including HNE and acrylamide.

2.2.3 Analysis of HNE

2.2.3.1 Novel procedure
Sample of 200 mg of oil in isooctane was loaded into column with conditioned to 5% water silica gel. Non-polar fraction was eluted with 10 mL of 15% diisopropyl ether in hexane following AOAC Method 982.27 with Schulte modification [24, 25]. Polar fraction was eluted with four 5 mL portions of methanol. Combined polar fractions were subsequently concentrated under gentle stream of nitrogen to the volume of 5 mL. The cloudy solution was then centrifuged at 2300 rpm for 5 min, and the supernatant analyzed for HNE by HPLC. Whole polar fraction without prior separation was used directly for HNE analysis.

2.2.3.2 DNPH method
Synthesis and isolation of DNPH derivative of HNE was carried out following the Seppanen and Csallany procedure [19]. Briefly, sample of fresh canola oil (1 g) containing known amount of added HNE was mixed with 2 mL of freshly prepared
DNPH reagent and incubated overnight at room temperature in the dark [19]. Then, the oil was extracted three times with 5 mL methanol/water (75:25, v/v) and separated by centrifugation at 2500 rpm for 10 min. The clear methanol solution was then extracted three times with dichloromethane (5 mL each). After centrifugation, the dichloromethane extract was evaporated under gentle stream of nitrogen until the sample size was reduced to about 1 mL. The sample was separated on a silica gel TLC plate (20 cm × 20 cm, 0.2 mm thickness; Whatman) using dichloromethane as the developing solvent. The band corresponding to HNE-DNPH was extracted three times with methanol (10 mL each). The combined methanol extract was centrifuged at 2500 rpm for 10 min to eliminate residual silica. The supernatant was concentrated under nitrogen to 2.0 mL and analyzed by HPLC [19].

2.2.3.3 Solvent extraction of HNE
Fresh canola oil sample (200 mg) containing known amount of added HNE was dissolved in isooctane (2 mL) and homogenized with 5 mL of methanol/water (8:2, v/v) following the procedure by Lang et al. [20]. Extraction was repeated three times and the combined extract was centrifuged at 2500 rpm for 5 min and the methanol-water layer transferred to a clean vial using a Pasteur pipette and volume of sample reduced under nitrogen to 5 mL. The resulted cloudy solution was centrifuged and clear supernatant transferred to a clean vial for HPLC analysis.

2.2.4 Acrylamide
Acrylamide was determined by a GC-MS method after extraction of it from the fries. Deuterated acrylamide was used as internal standard for quantification. Briefly, 10 g of the sample was grinded in a laboratory mill (Grindomax GM200, Retsch, Haan, Germany). To 4g of ground sample 50 µL of internal standard solution (150 µg/mL) was added and extracted with 50 mL distilled water utilizing sonication at 60°C. The extract was centrifuged at 4500 g and the supernatant defatted by extraction with petroleum ether and further clarified with 5 mL of each Carrez I and II solution. For separation of the precipitate the solution was centrifuged and then acrylamide was salted out with sodium chloride. Acrylamide was extracted from the aqueous phase by threefold extraction with
30 mL ethyl acetate, each. The organic phase was filtrated by water-repellent filter MN 616 WA (Macherey-Nagel GmbH, Düren, Germany) to eliminate water and finally sample concentrated to about 0.5 mL before analysis on GC-MS.

2.3 Instrumentation

2.3.1 Gas chromatography-mass spectrometry

GC-MS analysis were carried out using the electron impact ionization mode (EI) at 70 eV on a Hewlett Packard system Model 5890 Series II GC with 5989 series MS A. For the determination of acrylamide and deuterated acrylamide the selected ion monitoring (SIM) mode was applied using the ions with the following m/z: 74, 71, 58 and 55. The quantification was done utilizing the ions with masses 71 and 74. The compounds were separated on DB-23 capillary column (30 m x 0.25 mm i.d., 0.25 µm; J&W Scientific Products GmbH, Köln, Germany) using helium as carrier gas at a flow rate of 1.0 mL/min. The column temperature was initially kept at 80°C for 2 min and then programmed from 80 to 220°C at 10°C/min. The final temperature was held for 1 min. Other operating temperatures were as follows: a splitless injector was held at 240°C; an interface at 250°C, and an ion source at 200°C.

2.3.2 High performance liquid chromatography (HPLC)

Analysis of HNE from the polar fraction was carried out on a Finnigan Surveyor Plus HPLC (Thermo Electron, Waltham, MA, USA) with a Finnigan Surveyor Autosampler Plus and a Finnigan Surveyor UV-Vis Plus detector. A 20 µL sample was injected onto a C18 Novapak column (300 × 3.9 mm, 4 µm; Waters, MA). HNE was detected at 223 nm. Acetonitrile/water (30:70 v/v) was used as mobile phase at a flow rate of 0.75 mL/min. HNE was identified by comparison of the retention time and co-elution with this standard. Confirmation of HNE peak was achieved by mass spectrometry. Quantification of HNE was carried out using external calibration.

2.3.3 HPLC-MS/MS

HPLC was carried out using an Agilent 1200 series HPLC system equipped with binary pump and autosampler (Agilent technologies, Palo Alto, CA, USA). Of each sample, 10
µL was injected. The sample was separated on the same column as described above using a mobile phase consisting of a solvent A (acetonitrile + 0.1% formic acid) and a solvent B (water + 0.1% formic acid) (30:70 v/v) at a flow rate of 0.75 mL/min. The UV detector was set at 223 nm. The column effluent was split at 73:27 ratio, UV detector 0.55 mL/min while MS 0.2 mL/min. The identification was carried on a QSTAR Elite mass spectrometer (AB SCIEX, Concord, ON, Canada) equipped with an APCI interface operated in a positive mode. Analyst QS 2.0 software was used for data acquisition and analysis. The mass spectrometric conditions were optimized for 4-hydroxynonenal as follows: the APCI source temperature was set at 450°C, the curtain gas at 25, the declustering potential at 45V, the focus potentials at 150V and the ion source gas 1 and 2 at 20 and 60 psi, respectively.

2.4 Statistical analysis
Samples from two repetitions of frying for each frying protocol were collected, and were analyzed in duplicate. Data are presented as mean value ± SD. Data were analyzed by single-factor analysis of variance (ANOVA) and regression analysis using Minitab 2000 statistical software (Minitab, PA, USA; ver. 13.2). Statistical differences between means were determined by Duncan’s multiple range tests. Statistically significant differences were determined at p ≤ 0.05.

3. Results and discussion
3.1 Analysis of HNE
A widely applied method for the isolation of HNE from oil matrix prior to HPLC analysis usually utilizes 2,4-dinitrophenylhydrazone derivatives of carbonyl compounds, followed by solvent extraction and subsequent clean up by thin layer chromatography [19]. The procedure is tedious, laborious and it is a possibility that some portion of materials is lost during extraction from the TLC plate which rarely is complete. Additionally, all carbonyl compounds present in analyzed matrix are derivatized forming complex mixture very difficult to separate on individual components. The method developed in the present study is simple, and allows assessment of HNE, total polar components (TPC) and their composition in a single preparation run. Additionally, the method is sensitive and precise,
observed coefficient of variation was below 6%. The new method was compared with two commonly used procedures to assess recovery from canola oil. The results for four concentrations in Table 1 are presented. Solvent extraction with methanol-water resulted in 0, 51, 68 and 71% recovery when assessed for the following levels of the compound: 0.1, 1, 5 and 10 ppm, respectively [20]. The developed procedure offered recovery at 82, 103, 91 and 93%, respectively. The most commonly used procedure utilizing the DNPH derivatives provided accordingly recovery at 0, 72, 80 and 85% for the same concentration of standard. Recovery data indicates that HNE can be measured quantitatively at all analyzed levels utilizing the novel procedure. Very low coefficient of variation indicates that the new methodology is rapid and potential sources of errors have been eliminated. Specific isolation of polar components allows baseline separation of HNE peak from other components and improves significantly quantification. Additionally, procedure has been simplified by eliminating derivatization and multiple extractions and purification steps. The developed procedure makes possible to prepare in the single run multiple samples within one working day for the assessment of the amount and composition of polar components, and accurate quantification of HNE.

3.2 Identification of HNE in polar fraction by HPLC-MS/MS

The HPLC chromatogram of HNE standard is presented in Fig. 1a. The peak at 12.7 min corresponded to HNE standard. The mass spectrum of the peak is presented in Fig. 1b. The base peak at m/z = 157 was consistent with the expected molecular ion [M + H]^+. The observed daughter ions at m/z 139, 121, 93 and 79 representing [MH – H$_2$O]^+, [MH – 2H$_2$O]^+, [MH – 2H$_2$O – C$_2$H$_4$]^+, [MH – 2H$_2$O – C$_3$H$_6$]^+, respectively and are in the agreement with data published by Gioacchini et al. [26]. The observed ions with m/z: 139 [MH – H$_2$O]^+, 97 [MH – H$_2$O – C$_3$H$_6$]^+, 83 [MH – H$_2$O – C$_4$H$_8$]^+, 69 [MH – H$_2$O – C$_5$H$_{10}$]^+, and 55 [MH – H$_2$O – C$_6$H$_{12}$]^+ observed for MS/MS fragmentation of the HNE molecule (Fig. 1c) are consistent with the expected fragmentation pattern for this compound [26, 27]. A typical chromatogram of polar components separation for frying oil samples in Fig. 2a is presented. The mass spectrum of the peak corresponding to HNE is shown in Fig. 2b. The identical retention time and fragmentation pattern with HNE
standard further confirms the presence of this compound in the analyzed samples, further validates the newly developed procedure.

3.3 Concentrations of HNE in French fries
4-Hydroxynonenal is one of the most abundant and most toxic 4-hydroxyalkenal formed during lipid oxidation. The same level of HNE was observed in both the frying oil and the fries fried in it, and it is in an agreement with previously reported data by Seppanen and Csallany [28]. This observation further confirms extensive exchange between frying oil and fried products. Irrespective of the frying protocol, HNE concentrations in fries increased consistently throughout the entire frying time. Results indicated that frying under CDB reduced the amount of HNE by 62% compared to standard frying condition (Fig. 3). HNE is a secondary product of lipid oxidation, and the presence of oxygen is the main factor stimulating oxidative degradation of oil during frying [12, 29]. Therefore, it is expected that any measure that limits the availability of oxygen will inhibit the formation of HNE. Carbon dioxide blanketing has been reported to reduce the incident of oxidation reaction during frying and current results further support this observation [17].

3.4 Concentration of acrylamide in French fries
Acrylamide is a toxic alkylamide formed under frying conditions. The acrylamide concentrations in French fries fried under SFC and CDB are presented in Fig. 4. For the first day of frying, there was no significant difference (p ≤ 0.05) between both frying conditions in the formation of acrylamide. This may be due to the fact that fresh oil in both fryers contained comparable amount of dissolved oxygen which is absorbed during manipulation and pre-frying purge with CO₂ was not effective to remove it. Frying under CDB resulted in an average 58% reduction in the amount of acrylamide formed for the remaining frying time when compared to SFC (Fig. 4). Apart from the application of carbon dioxide during CDB frying, other frying conditions such as frying temperature, frying time, surface-to-volume ratio, frying product load, were essentially identical for both frying procedures. Therefore, the observed difference in the amount of acrylamide can be attributed to the ability of CDB to reduce availability of atmospheric oxygen and impede lipid oxidation. Zamora and Hidalgo [14] reported that lipid oxidation products
contributed to acrylamide formation in a model system. The potential role of alkadienals and other carbonyl compounds as precursors interacting with asparagine which lead to formation of acrylamide have been reported [15, 16]. Results from this study support observation from model study by Hildago, verifying that oxidation of fatty acids is producing precursors required for acrylamide formation.

4. Conclusions
The present study showed that frying under CDB can considerably reduce the amount of toxic components in fries during deep fat frying. Furthermore, the study revealed a significant connection between lipid oxidation and acrylamide and HNE formation. Additionally, a simplified, quantitative and reliable procedure for analysis of HNE has been developed and verified its efficiency.

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Conflict of interest statement
The authors have declared no conflict of interest.
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and control of the risks of existing substances. Opinion expressed at the 22nd CSTEE plenary meeting, Brussels, 6/7 March 2001.


[28] C. M. Seppanen, A. S. Csallany: Incorporation of the toxic aldehyde 4-hydroxy-2-

Table 1. Recovery of isolated HNE from spiked canola oil samples by different methods.

<table>
<thead>
<tr>
<th>Method</th>
<th>HNE added (µg/g)</th>
<th>HNE found (µg/g)</th>
<th>HNE recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent Extraction</td>
<td>0.1 0.00±0.00 0</td>
<td>1 0.51±0.03 51</td>
<td>5 3.47±0.19 68</td>
</tr>
<tr>
<td>HNE-DNPH</td>
<td>0.1 0.00±0.00 0</td>
<td>1 0.72±0.07 72</td>
<td>5 3.98±0.40 80</td>
</tr>
<tr>
<td>Modified TPC</td>
<td>0.1 0.08±0.00 80</td>
<td>1 1.03±0.05 103</td>
<td>5 4.54±0.23 91</td>
</tr>
</tbody>
</table>

Solvent Extraction - procedure according to Lang et al [20]; HNE-DNPH derivatization procedure according to Seppanen and Csallany [19]; Modified TPC – novel procedure developed in this study, for details see text.
Figure 1a. HPLC chromatogram of HNE standard. (Refer to text for conditions)
Figure 1b. Mass spectrum of the HNE standard. Refer to text for conditions.
**Figure 1c.** MS/MS spectrum of the HNE with molecular ion at m/z 157.
Figure 2a. HPLC chromatogram of polar material isolated from frying oil with the novel procedure. HNE peak is marked. Refer to text for conditions.
Figure 2b. Mass spectrum of peak corresponding to HNE in extracted polar material from frying oil. For details see text. Peak at m/z = 157.1246 representing HNE.
Figure 3. Formation of HNE during frying under standard frying conditions (SFC) and carbon dioxide blanketing (CDB).
Figure 4. Formation of acrylamide during frying without (SFC) and with carbon dioxide blanketing (CDB).
Rapid assessment of frying performance using small size samples of oils/fats

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Abstract: A rapid, effective and mimicking actual frying test was developed to assess the frying performance of oils and fats using small size samples. To small volume of tested oil a formulated food consisting of gelatinized potato starch, glucose and silica gel, (4:1:1 w/w) were added and content heated at 185 ± 5°C with mixing for 2 h. Thermo-oxidative degradation of oil was assessed by the measurement of total amount of polar components and their composition, including degradation of tocopherols. The developed fast test is accurately mimicking actual frying done using institutional fryer as assessed by the accumulation and composition of total polar components and the amount of residual tocopherols. The validity of the test was assessed using the following oils: regular canola, high oleic low linolenic canola, and high oleic sunflower. Comparison of data between the fast frying test and institutional frying revealed lack of significant differences. The developed frying test is providing reliable quantitative and qualitative data describing performance of frying oil/fat. The rapid frying procedure allows assessment of the frying performance of oils at the early stages of a development where usually small amounts of sample is available and when large number of samples have to be tested assessing effects of oil additives.

Keywords: Frying performance, fast test, polar components, additives, tocopherols, oils
Introduction

During deep fat frying, oils are subjected to hydrolysis, oxidation and polymerization reactions. In institutional frying operation oils are used for several cycles, which accelerate degradation leading to a variety of products and impacting the physical properties, flavor and nutritional value of the frying oil and fried food. Thus, the heat stability of frying oils is a vital criterion in the selection of fats and oils for institutional and commercial frying [1].

Often, official and recommended methods such as the Rancimat and Schaal oven tests are employed to assess the oxidative stability of oils at elevated temperature [2]. Currently, none of the standard procedure is testing performance at the standard frying temperatures ranging from 165 to 190°C. It has been observed that the type of chemical reactions taking place during frying is different from those happening during heating without food and from ones occurring at ambient temperature [3]. Furthermore, the performance of some endogenous antioxidants is affected by temperature. For instance, sterols, ascorbyl palmitate, or sesamolin are nearly inactive at temperatures below 130°C, whereas all are powerful antioxidants in deep fat frying [4]. Additionally, various components of the fried food are known to participate in the reactions occurring at frying conditions [5].

In addition to the well known radical mechanism of lipid oxidation and polymerization, Brütting and Spitteller [6] proposed nonradical mechanism for the formation of dimers and cyclic triacylglycerides during frying. Recently, based on the information on the nonradical mechanism, Gertz et al [4] developed the oxidation stability at elevated temperature index (OSET). The principle of this method is based on the accelerated triacylglycerides dimerization stimulated by water conditioned silica gel and treatment at the frying temperature for 2h. Employment of this procedure in our laboratory, and subsequent HPLC analyses for composition of polar materials and the amount of residual tocopherols indicated that the results were similar to the actual frying for less than 6 h, or about 1 day of intermittent frying operation. Besides, the performance and behavior of oils during first day of frying does not represent its performance over prolonged time such as is utilized during typical institutional frying. Thus, the method is limited when it comes to acquiring quantitative and qualitative data describing frying performance of oils, or to assess performance of natural and/or synthetic antioxidants during prolonged frying. The need to assess performance of oil during prolonged frying when small size sample is available still remains imperative. Furthermore,
assessing effect of different factors, particularly endogenous minor components or antioxidants where large number of tests has to be performed requires rapid and reliable testing procedure.

Different factors interplay in the determination of types of chemical reactions and the nature of chemical products formed during deep fat frying includes: (1) the type of fryer used; (2) the ratio of oil surface area to volume; (3) the ratio of oil volume to mass of food fried and (4) availability of oxygen. Although at the elevated temperature during deep fat frying, oxygen supply is limited by blanketing with the steam coming from fried food and is also limited by the lower solubility at elevated temperature, even at these limiting conditions the influence of oxygen still remains important [7]. Oxygen is continually introduced into the frying medium when a new portion of food containing adsorbed and absorbed oxygen is placed in it, and by the subsequent agitation of the oil during frying mainly by escaping water vapours. In the reliable frying test these factors ought to work together to truly mimic actual institutional frying.

Actual deep fat frying in standard fryer remains the best method when assessing frying performance of oils. However, a rapid and cost-effective procedure generating reproducible and comparable results remains a very interesting task. This becomes particularly important when it is required to assess the frying performance of expensive, newly designed oils with limited quantity, or to evaluate novel antioxidants designed for frying applications. Previous attempts to simulate the prevailing conditions during actual frying for routine evaluation of thermo-oxidative degradation of frying oils include: Frying of moist cotton balls [8]; Spraying oil with water during heating at frying temperature [9]; Heating to a frying temperature in a Rancimat apparatus [10]; and recently, Heating to frying temperature in the presence of water conditioned silica gel [4]. Some of the shortcomings of the proposed procedures include: (1) The need for prolonged heating, in the excess of 8 h, to achieve sufficiently high amount of TPC formed; (2) Heating without food may not be representative of the reactions occurring during actual frying; and (3) Relatively large amount of sample required. In the present study, the development of the rapid frying test (FT) was directed to its optimization to mimic as close as possible the accumulation and composition of polar compounds, and the amount of residual tocopherols (RTOC) achieved when frying using standard fryer (SF). Furthermore, the heating period and the amount of sample were reduced for faster and more effective routine assessment of stability of the frying oil. Developed test is allowing utilization of the most effective analytical parameters directly describing degradation rate of frying oil and its
performance in standardized conditions. One of the main reasons why rapid test was developed is a comparison of oil/fat performance measured at well established conditions, allowing direct evaluation of stability.

**Materials and Methods**

Oils and French fries
Commercially refined oils were supplied by Richardson Oilseed Limited (Lethbridge Canada). Three oils were used in investigation: canola oil (CAN), high oleic low linolenic canola oil (HOLLCAN), and high oleic sunflower oil (HOSUN). Frozen par-fried French fries in institutional packs were obtained from a local food store.

Ingredients
Silica gel 60Å (70 – 230 mesh), alumina 58Å (150 mesh), Celite 512, hydrolyzed starch, potato starch, D-glucose, FeSO$_4$, and CuSO$_4$ used for formulation of replacement food in the rapid test were obtained from Sigma-Aldrich (St. Louis, MO). Water conditioned silica gel, alumina and Celite were prepared by heating the material for 24 h at 160°C, and adjusting the water content to 10, 20 or 40%.

Mixture to mimic fried food, abbreviated as formulated food (FF), was prepared by mixing 4 g of potato starch, 1 g of glucose and 1 g of silica gel with 5 mL of cold distilled water, and then 15 mL of boiling water was added. The mixture was transferred onto a hot plate preset at 110°C, and heated for 2 min with continuous mixing. The resulted gel was left uncovered to cool to room temperature. The moisture content of the gel was at 64.7 ± 2.1%

Fast frying test and oil sampling
Vegetable oil (12.0 g) was weighed into a clean glass beaker (30 mL, Pyrex, USA). Clean octagonal stir bars (9.5 × 25 mm, Fischer Scientific, USA) was placed into the glass vessel, altering the final ratio of oil surface area to volume at 0.42. The oil sample in glass beaker was heated at 185 ± 5°C for 10 min, and 1.2 g of FF was added. The heating was continued for another 20 min without mixing and then was stirred at 500 rpm. Heating and stirring were continued for the additional 90 min. About 0.5 g of oil sample was withdrawn at the 60th, 80th, 100th, and 120th min for the analysis.
Actual frying and oil sampling

Actual frying was conducted in an 8 L capacity restaurant style stainless steel deep fryer (General Electric Company, New York, USA). Vegetable oil (4 L) was heated at 185 ± 5°C, 7 h daily for 7 days. A batch of 400 g of frozen French fries was fried for 5 min for a total of eight batches per frying day. At the end of each frying day, fryers were shut off and left to cool overnight. Two 25 mL samples of oil from the fryer were taken daily and kept frozen at -16 °C until analyzed. Before commencing next frying day, oils were filtered to remove solid debris and were replenished every second day of frying with 500 mL of fresh oil.

Total Polar components (TPC)
The amounts of polar compounds were determined by gravimetric procedure following AOAC Method 982.27 with the Schulte modification [11, 12].

Composition of polar components
The composition of polar components was analyzed by high performance size exclusion chromatography (HPSEC) following the ISO Method 16931-2007 [13]. Separation was performed on a Finnigan Surveyor chromatograph (Thermo Electron Corporation, West Palm Beach, FL). Components were separated on three size exclusion columns connected in series (Phenogel 500A, 100A and 50A, 5µ, 300 x 4.6 mm; Phenomenex, Torrance, CA), with tetrahydrofuran (THF) as the mobile phase at a flow rate of 0.3 mL/min. Columns were held at temperature of 30°C. A 10 µL sample was injected and components detected with a Sedex 75 evaporative light scattering detector (Sedere, Alfortville, France) operated at 35°C with air pressure of 2.5 bar.

Tocopherols
Tocopherols were analyzed using high performance liquid chromatography (HPLC) based on the AOCS Official Method Ce 8-89 [2]. Analysis was performed on a Finnigan Surveyor LC (Thermo Electron Corporation, West Palm Beach, FL) with a Finnigan Surveyor Autosampler Plus and Finnigan Surveyor FL Plus fluorescence detector, set for the excitation at 292 nm and the emission at 325 nm. Separation of tocopherol isomers was carried out on a normal phase Diol column (250 × 4.6 mm; MonoChrom, Varian, CA). Of each sample, 10 µL was injected. Mobile phase contained...
7% methyl-tert-butyl-ether in hexane with a flow rate of 0.6 mL/min. The contents of tocopherols were quantified using calibration curves for each isomer separately.

Statistical analysis
Samples from three repetitions of each model frying experiment were analyzed in duplicate. For the actual frying experiments, samples from two repetitions of frying in each oil were collected and were analyzed in triplicate. Data are presented as mean ± SD. Data was analyzed by single factor analysis of variance (ANOVA) and regression analyses using Minitab 2000 statistical software (Minitab inc. PA, ver. 13.2). Statistically significant differences between means were determined by Duncan’s multiple range tests for P ≤ 0.05.

Results and Discussion
The type of reactions, the nature of products and the consequent performance of vegetable oil during deep fat frying depend, among other factors, on: the ratio of oil surface area to volume (S/V), availability of oxygen, and the presence of antioxidant/prooxidant in the frying medium. The effects of these parameters in a frying test were studied and accordingly adjusted to reproduce the amount and composition of polar components, and degradation of tocopherols as it happen in standard institutional frying.

Results showed that there was no significant difference in the amount of total polar compounds (TPC) formed at the end of 2 h of test frying when silica gel, alumina or celite were used to imitate frying foods (Table 1). A direct relationship was observed between the amount of TPC and S/V ratio (Table 1). Irrespective of the size of the heating vessel, systems with the S/V ratio of 0.75 provided the highest level of thermo-oxidative degradation. Lower S/V ratios, particularly at 0.27, exhibited the slowest rate of degradation (Table 1). Mezouari and Eichner [14] reported a significant increase in the rate of degradation of tocopherols with a concomitant increase in the accumulation of polymeric materials when oil was heated with stirring. This is probably due to the increased surface area of oil and better access to oxygen during stirring of the heated oil. A similar agitation of oil is happening when food, usually frozen, is introduced into the oil during frying. Rock and Roth [15] demonstrated that circulation of oil significantly increased the rate of fat deterioration.
Likewise, the water contents in the FF did not lead to the substantial differences in the amount of TPC, however, the contribution of diacylglycerides in the polar material significantly increased when the water content was increased to 40% (Table 2). This may substantiate effect of water content in frying food on hydrolysis of triacylglycerides.

The determination of TPC in frying oil is the most reliable measure of the extent of thermo-oxidative degradation [16, 17]. In order to achieve a rate of TPC formation similar to usually obtained during the 7 days of SF the effect of stirring at 400, 450, 500 and 600 rpm were studied. Within the 2 h heating period, a combination of 500 rpm stirring and S/V ratio of 0.42 was established as optimal. Utilizing developed conditions, the effect of several FF combinations on the amount and composition of polar components, and the degradation of tocopherols were studied (Table 2). The pro-oxidant effects of copper and iron ions are well established [7]. Gertz et al [4] suggested that hydrated silica gel catalyzed the non-radical dimerization of triacylglycerides, and utilized it in the OSET test. The rate of oil degradation during frying was increased when starchy product are fried [18]. The formulated food to mimic typical food product usually fried has to have similar water content to stimulate hydrolytic reaction. Pokorny [19] observed that frying gelatinized starch impregnated with glucose produced a sweet flavor, indicating an interaction between the fried food and the oil at frying temperature. Thus, the FF used in the developed frying test contained gelatinized starch mixed with 16% glucose and the same amount of silica gel. Selection of ingredients was directed by the major reactions involved in oil degradation during frying, including: (1) The water content was adjusted to the average amount among most fried food; (2) Starch and glucose are the most common ingredients present in the most foods, sometimes as an ingredient or is added to the food in different form (e.g. breading); (3) Silica gel is simulating the acid catalyzed non-radical dimerization of triacylglycerides; (4) The potato starch contains: 80% starch, 0.1% protein, and approximately 3 - 5 ppm of iron, providing ingredients which are usually present in the fried foods. (5) To further explain, low amount of proteins in FF is dictated by the type and role of it during the frying. Potato protein contains mainly low molecular proteins which are the most active in pigments formation. Additionally, proteins are not directly involved in lipids degradation, mostly involved in pigments formation utilizing lipids degradation precursors. Although the optimized FF used in this study does not entirely represents the variation of the food ingredients used in frying, the components were deliberately chosen to simulate reactions and processes taking place during SF. Utilization of this formulation in the frying test endow with the degradation rate observed during
institutional frying. The rate of TPC formation in heated oil in the absence of food was significantly different from the results of the FT when oil was heated with the presence of FF (Table 2). On the other hand, differences were observed in the distribution of polar components. In samples heated without FF, over 60% of the polar material was formed by oxidized triacylglycerides, indicating prevalence of oxidation over oxides degradation reactions, contrary to what is usually observed during institutional and prolonged deep fat frying [4]. Generally, addition of metal ions such as iron and copper, exhibited a significantly higher rate of TPC formation and significantly faster degradation of tocopherols (Table 2). Of the tested FF’s, when ingredients were added to gelatinized starch the amounts and the compositions of TPC formed was very close to the targeted values usually achieved during the 7 days of SF, suggesting similar mechanism of oxidative degradation (Table 2).

Three frying oils, namely canola, HOLL canola and HO sunflower oils were tested by both the frying tests and actual frying. The formation of TPC is presented in Figure 1. At the end of the frying test, the amount of formed TPC in CAN, HOLL CAN, and HOSUN were 23.7, 22.5, and 20.3%, respectively (Fig. 1). These values were between 93.1 – 96.6% of the values obtained for these oils at the 7th day of the actual frying (Fig. 1). Comparable results were also obtained in the distribution of polar components (Fig. 2 - 5). The respective contribution of polymers at the end of the frying test using CAN, HOLL CAN, and HOSUN oils were 9 to 14% lower than the amounts observed for the actual frying (Fig. 2). Oil samples from the frying test contained higher amounts of dimers and oxidized triglycerides than corresponding oils from the actual deep frying (Fig 3). The contribution of dimers was lower by 12% in samples from the actual frying, this difference can be within experimental error usually achieved between frying experiments. Unlike other groups of polar material, the amounts of diacylglycerides formed during the frying test using different oils was close to 50% lower when comparing to the actual frying (Fig. 5). The amounts of tocopherols remaining in the oils during the test frying were comparable to the values obtained during the actual frying (Fig 6). Controlled stirring at 500 rpm with the FF added resulted in the residual tocopherol amounts within 83.5 to 90.8% of the values observed for the actual frying at 1, 3, 5 and 7 day of frying (Fig. 6).

To extend the prediction of the frying test to earlier stages of frying, samples were collected at several sampling points, and were analyzed for degradation products. Results reflected that samples
withdrawn at the 60th min provided information on the frying performance of the oil during the first day of the actual frying. While the samples collected at 80th min for 2nd and 3rd day, at 100th min for 4th and 5th day, and at 120th min for 6th and 7th day, are comparable to performance of oil during the actual frying. Calculated slopes for polar components formation and degradation of tocopherols, which describe kinetics of degradation, were the same in all cases further proving that mechanism of changes was the same in actual and test frying (Data not included).

The consistency of the results obtained for the tested oils evidently showed that the developed frying test can offer a fast, reliable and reproducible prediction of frying performance of oils and fats during the actual frying. With the frying test the rate of tocopherols degradation can be predicted, which is a useful indicator of frying oil stability.

In conclusion we developed rapid procedure that offers fast and reliable assessment of frying performance of oil(s), oil additives and antioxidants. The procedure is designed to be utilized in assessment of the frying performance at the early stages of oil development, where usually small size samples are available. This procedure can be a practical test to be applied at the breeder level to improve selection of proper lines of new oils intended for frying operation. The procedure presented in the paper is specific to the standard frying condition, nevertheless, the following assessments can be done with it: (1) Establishing performance and fry-live of the frying oil; (2) Assessing effectiveness of antioxidants; (3) Assessing performance of oilseed lines at the breeder level; (4) Establishing activity of minor components during frying where usually large number of combination need to be evaluated.

Acknowledgement: This work was financed by the Alberta Value Added Corporation, the Agriculture Funding Consortium and by the Bioactive Oil Program.
References

Table 1: Effect of selected compounds, water content, mixing rate and oil surface to volume ratio on the formation of polar components during frying test.

<table>
<thead>
<tr>
<th>Additives</th>
<th>Water content</th>
<th>S/V</th>
<th>Stirring (rpm)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>400</td>
</tr>
<tr>
<td>Silica gel</td>
<td>10%</td>
<td>0.75</td>
<td>14.1±0.9</td>
<td>17.7±1.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.42</td>
<td>10.9±0.7</td>
<td>15.7±0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.27</td>
<td>7.5±0.5</td>
<td>8.8±0.4</td>
</tr>
<tr>
<td></td>
<td>20%</td>
<td>0.75</td>
<td>13.3±1.1</td>
<td>18.4±1.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.42</td>
<td>10.3±0.8</td>
<td>14.9±1.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.27</td>
<td>7.5±0.5</td>
<td>9.1±0.8</td>
</tr>
<tr>
<td></td>
<td>40%</td>
<td>0.75</td>
<td>15.3±1.2</td>
<td>18.2±1.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.42</td>
<td>10.7±0.8</td>
<td>15.2±1.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.27</td>
<td>7.3±0.6</td>
<td>8.9±0.7</td>
</tr>
<tr>
<td>Alumina</td>
<td>10%</td>
<td>0.75</td>
<td>13.9±1.3</td>
<td>17.9±1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.42</td>
<td>11.1±1.0</td>
<td>14.8±1.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.27</td>
<td>7.8±0.4</td>
<td>8.5±0.6</td>
</tr>
<tr>
<td></td>
<td>20%</td>
<td>0.75</td>
<td>14.8±0.9</td>
<td>17.0±1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.42</td>
<td>11.0±0.9</td>
<td>15.5±0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.27</td>
<td>8.6±0.7</td>
<td>9.9±0.7</td>
</tr>
<tr>
<td></td>
<td>40%</td>
<td>0.75</td>
<td>15.7±1.3</td>
<td>18.0±1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.42</td>
<td>10.9±0.8</td>
<td>14.9±0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.27</td>
<td>7.0±0.4</td>
<td>9.2±0.5</td>
</tr>
</tbody>
</table>

Samples were heated for 2 h at 185 ± 5°C with and without stirring. S/V – Ratio of oil surface area to volume.
Table 2: Formation of polar components and degradation of tocopherols during frying and frying test using canola oil and various formulations of mimicking food (%).

<table>
<thead>
<tr>
<th>Component(^a)</th>
<th>TPC</th>
<th>Polymer</th>
<th>Dimers</th>
<th>OxTAG</th>
<th>DG</th>
<th>RTOC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canola oil(^b)</td>
<td>20.8±1.7</td>
<td>6.4±0.4</td>
<td>29.8±1.9</td>
<td>61.2±3.9</td>
<td>2.3±0.2</td>
<td>18.3±2.0</td>
</tr>
<tr>
<td>Silica (10% water)</td>
<td>22.2±2.4</td>
<td>8.8±0.3</td>
<td>33.2±2.4</td>
<td>54.7±3.1</td>
<td>2.4±0.1</td>
<td>14.8±1.2</td>
</tr>
<tr>
<td>Silica (20% water)</td>
<td>22.9±1.9</td>
<td>8.1±0.2</td>
<td>32.9±3.0</td>
<td>56.1±3.7</td>
<td>2.7±0.2</td>
<td>10.9±1.0</td>
</tr>
<tr>
<td>Silica (40% water)</td>
<td>21.8±2.0</td>
<td>7.9±0.4</td>
<td>35.3±2.1</td>
<td>52.2±4.0</td>
<td>3.9±0.2</td>
<td>15.1±1.3</td>
</tr>
<tr>
<td>CuSO(_4) (50µg/g)</td>
<td>24.5±2.1</td>
<td>6.1±0.5</td>
<td>32.2±2.1</td>
<td>60.1±4.2</td>
<td>1.6±0.1</td>
<td>6.3±0.5</td>
</tr>
<tr>
<td>FeSO(_4) (50µg/g)</td>
<td>26.7±2.5</td>
<td>6.9±0.2</td>
<td>37.8±3.0</td>
<td>52.5±2.8</td>
<td>1.9±0.1</td>
<td>5.1±0.5</td>
</tr>
<tr>
<td>Silica(^c) + CuSO(_4) (50µg/g)</td>
<td>24.9±2.3</td>
<td>7.7±0.5</td>
<td>34.1±3.1</td>
<td>55.8±2.1</td>
<td>2.1±0.1</td>
<td>5.8±0.3</td>
</tr>
<tr>
<td>Silica(^c) + FeSO(_4) (50µg/g)</td>
<td>25.6±2.0</td>
<td>8.1±0.5</td>
<td>38.4±2.5</td>
<td>51.8±3.0</td>
<td>1.7±0.1</td>
<td>7.3±0.4</td>
</tr>
<tr>
<td>Potato starch</td>
<td>22.1±1.9</td>
<td>7.6±0.3</td>
<td>32.1±2.9</td>
<td>57.5±3.3</td>
<td>2.5±0.1</td>
<td>14.1±1.4</td>
</tr>
<tr>
<td>Hydrolyzed starch</td>
<td>20.2±1.8</td>
<td>8.9±0.6</td>
<td>32.0±2.7</td>
<td>56.3±2.7</td>
<td>2.8±0.2</td>
<td>15.2±1.1</td>
</tr>
<tr>
<td>(Starch+glucose+silica)(^d)</td>
<td>23.7±1.5</td>
<td>13.3±0.9</td>
<td>36.4±2.1</td>
<td>44.7±3.0</td>
<td>4.5±0.2</td>
<td>12.0±1.1</td>
</tr>
<tr>
<td>Institutional frying(^e)</td>
<td>24.9±2.0</td>
<td>15.6±1.2</td>
<td>32.6±1.6</td>
<td>41.1±2.9</td>
<td>9.6±0.4</td>
<td>13.3±0.8</td>
</tr>
</tbody>
</table>

\(^a\) - Apart from the salts, all components were added at 10% of the oil weight; \(^b\) - Canola oil heated at the frying test conditions; \(^c\) - Added silica gel containing 40% water. \(^d\) - A formulated food containing gelatinized starch, glucose, silica gel and water at 4:1:1:65 w/w. \(^e\) - Values from the 7\(^{th}\) day of actual frying using canola oil. \(^f\) – Testing conditions: temperature 185 ± 5°C; stirring at 500rpm; time 2h; the ratio of oil surface to volume at 0.42. OxTAG – Oxidized triacylglycerides; DG – Diacylglycerides; RTOC – Residual tocopherol.
Fig. 1: Polar components formation during the actual (F) and the test (T) frying using different oils. CAN- canola oil, HOLLCAN – high oleic low linolenic canola oil; HOSUN – high oleic sunflower oil.
Fig. 2: Contribution of polymers in the total polar compounds formed during the actual and the test frying using different oils. For oil abbreviations and symbols explanation see Fig 1.
Fig. 3: Contribution of dimers in the total polar compounds formed during the actual and the test frying utilizing different oils. For oil abbreviations and symbols explanation see Fig 1.
Fig. 4: Contribution of oxidized triacylglycerides (OxTAG) in the total amounts of polar compounds formed during the actual and the test frying using different oils. For oil abbreviations and symbols explanation see Fig 1.
Fig. 5: Contribution of diacylglycerides in the total amount of polar compounds formed during the actual and test frying in different oils. For oil abbreviations and symbols explanation see Fig 1.
Fig. 6: Changes of tocopherols during the actual (F) and the test (T) frying in the different oils. For oil abbreviations and symbols explanation see Fig 1.
Frying performance of canola oil triacylglycerides as affected by vegetable oils minor components

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Abstract: The endogenous minor components from canola, rice bran, sesame and palm oils including selected phospholipids, and various combinations of tocopherol isomers were tested during frying using canola oil triacylglycerols as frying medium. Thermo-oxidative degradation was assessed by measurement of total polar components, the rate of volatile carbonyl compounds and 4-hydroxynonenal formation. All the tested minor components protected to different extend canola triacylglycerides from thermo-oxidative degradation during frying. No significant differences were observed in protection of the triacylglycerides among all the tested tocopherol isomers and their mixtures. Irrespective of the composition of tocopherol homologous, an increase in the added amounts above 1000 µg/g did not improve protection. Minor components isolated from rice bran and sesame oils offered better protection during canola triacylglycerides frying than endogenous minor components isolated from canola oil. When 0.2% phosphatidylcholine or phosphatidylethanolamine was added to the canola triacylglycerides, the amount of formed polar components decreased twice compared to the tocopherol isomers. Accordingly, by optimizing the composition and the concentration of the endogenous minor components, the frying performance of oil can be significantly enhanced.

Keywords: Minor components, canola oil, rice bran oil, palm oil, tocopherols, sterols, phospholipids, frying performance.
Introduction

Under frying conditions, large number of volatile and non-volatile compounds is produced. Not only these compounds adversely affect the stability of the frying oil, but the food fried in the deteriorated oil acquires a significant amount of decomposition products that may have potentially adverse effects on the safety, flavour, nutritional value, and stability of a fried food. Moreover, several studies have shown that a number of volatile compounds formed during frying exhibit carcinogenic, mutagenic and genotoxic properties [1 – 3]. In consequence, the thermal stability of frying oils is a vital criterion in the selection of frying medium [4]. The search for oil with improved frying stability has led to the several modifications of the fatty acid composition of many commodity oils [5 – 9]. On the other hand, a body of research has demonstrated the significant role of endogenous minor components on frying stability of oil [10 – 14].

The amount and relative contribution of linoleic, linolenic and other unsaturated fatty acids in canola oil makes it desirable as a good source for essential fatty acids. However, the susceptibility of these acids to oxidation demands measures that will increase the oil’s frying stability without compromising it nutritional value. Previous work in this area focused mainly on the influence of added tocopherols on the frying stability, with relatively fewer reports on the use of other natural antioxidants [14–17]. So far, there has been no report on the effect of endogenous minor components from rice bran, sesame and palm oils on the frying stability of canola oil. Although, a few papers have reported the frying performance of canola oil blends with palm olein, olive and corn, rice bran, and sesame oils [18-22]. These blends had changed fatty acid, triacylglycerols and minor components compositions and make it impossible to assess the effect of the endogenous minor components [23,24].

In the present study, the effects of selected phospholipids, various combinations of tocopherol homologues, and minor components isolated from canola (CO), rice bran (RBO), sesame (SO) and palm oils (PO) on the frying performance of antioxidant stripped canola oil triacylglycerides were assessed. The vegetable oils chosen for this study contain unique endogenous minor components which are not found in canola oil, namely, tocotrienols and oryzanol in RBO, lignans in SO, and tocotrienols and carotenoids in PO.
Materials and Methods

Materials
Commercially processed regular canola oil was donated by Richardson Oilseed Limited (Lethbridge, Canada). High oryzanol rice bran (RBO) and red palm oils were gifts from Rito, Inc. (Stuttgart, Arkansas, USA) and Golden Jomalina Food Industries (Kuala Langat, Malaysia), respectively. Neutral alumina (70-230 mesh), silica gel 60Å (70 – 230 mesh), D-glucose, and potato starch were purchased from VWR (Edmonton, Canada). Phosphatidylcholine (>99%), phosphatidylethanolamine (~98%), β-carotene and 4-hydroxynonenal (HNE) were purchased from Sigma-Aldrich (St. Louis, MO, USA). BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide, TMCS (trimethylchlorosilane), and pyridine were obtained from Supelco (Bellefonte, PA, USA). Standards of tocopherols were purchased from Calbiochem-Novabiochem (San Diego, CA, USA). Standard of γ-oryzanol was provided by Oryza Oil and Fat Chemical Co. Ltd (Ichinomiya-City, Japan). Standards of volatile carbonyl compounds were purchased from Bedoukian Research (Danbury, CT, USA), all were subsequently derivatized to dinitrophenylhydrazones [25].

Preparation of antioxidant stripped canola triacylglycerides and isolation of minor components.
Isolation of endogenous minor components and triacylglycerides from canola and rice bran oils including further purification of fractions in Scheme 1 is presented. Canola oil was stripped of its endogenous minor components including antioxidants via adsorption chromatography, following the procedure described by Lampi and Kamal-Eldin [26] with modifications. A slurry of alumina (1 kg; activated at 103 °C for 16 h and at 200 °C for 8 h) in 2 L of hexane was loaded to a glass chromatography column (950 × 50 mm i.d.). The hexane was allowed to flow helping with proper and even packing. Canola oil (500 g) dissolved in 500 mL hexane was loaded into the packed column. The first 800 mL of the eluting hexane was discarded, followed by 3.7 L hexane containing 431 g of purified canola triacylglycerols (CTG). The canola oil minor components (CMC) were recovered from the column by elution with 2.5 L of 10% methanol in methyl tert butyl ether. Both the column and collection vessels were wrapped in aluminum foil to prevent photo-oxidation. The CTGs were stored as hexane solution at -16 °C until used in the experiments. From rice bran oil minor components were similarly isolated forming rice bran triglyceride (RBTG) and minor components fraction (RBMC).
Purification of minor components
The recovered minor components from canola and rice bran oils were further separated into two fractions by adsorption chromatography. Conditioned silica gel was prepared by heating it for 24 h at 160 °C, and the water content adjusted to 5% by addition of stoichiometric amount of water. Slurry of 120 g of the conditioned silica gel in 150 mL of hexane was loaded into a glass chromatography column (600 × 45 mm i.d.) allowing constant flow of hexane until the packing was evenly packed. Minor components, 20 g, dissolved in 30 mL of hexane were introduced into the column. Tocopherol fraction was subsequently eluted with 750 mL of 5% methyl tert butyl ether (MTBE) in hexane while sterols fraction with 750 mL of 50% MTBE in hexane. The purity of fractions was monitored by thin layer chromatography. Collected fractions were concentrated under vacuum using a rotary evaporator (BÜCHI, Flawil, Switzerland) providing 2.5 g of golden oil for tocopherol fraction and 15.3 g of yellow oil for sterol fraction. Both the column and collection vessels were wrapped in aluminum foil to prevent photo-oxidation. The fractions were stored in hexane at -16 °C until used in the experiments.

Purification of tocopherol and sterol fractions
Tocopherol fraction from canola and rice bran oils were further purified by preparative thin layer chromatography (PTLC) using MTBE/hexane (2:8, v/v) as developing solvent. Bands corresponding to the tocopherols were scrapped off and eluted three times with 10 mL of MTBE. Combined extracts were concentrated under vacuum using a rotary evaporator, flushed with nitrogen and kept in hexane at -16 °C until used in the experiments. From 1 g of starting tocopherol fraction 157 and 313 mg of purified tocopherols were obtained from canola (TCAN) and rice bran (TRBO) oils, respectively. Sterol fractions from both oils were similarly purified by PTLC using MTBE/hexane (65:35, v/v) as the developing solvent. From 1 g of initial sterol fraction 219 and 402 mg of purified sterols fractions was isolated from canola (SCAN) and rice bran (SRBO) oils, respectively.

Solvent extraction of endogenous minor components
Minor components were extracted directly from rice bran oil according to Miraliakbari and Shahidi [27] procedure with modifications. Briefly, 200 g of oil was placed in a 500 mL capacity separatory funnel and 200 mL methanol added followed by agitation for 5 min with periodic venting. The mixture was allowed to sit at room temperature for 1 h to allow separation of oil from methanol. The extraction was repeated 12 times with the same volume of fresh methanol. Extracts were combined
and excess of methanol evaporated under vacuum using a rotary evaporator. The 9.4 g of light yellow gummy material coded RBOS was flushed with nitrogen and stored at -16 °C until used in the experiments. Minor components from sesame and palm oils were extracted in the same way and coded SOS and POS, respectively.

Formulation of food for frying
A mixture of selected food ingredients was prepared to mimic as closely as possible typical frying food providing similar degradation processes as usually happening during French fries frying. Slurry of 4 g of potato starch, 1 g of glucose and 1 g of silica gel in 5 mL of cold distilled water was prepared then to it 15 mL of boiling water was added. The mixture was placed onto a hot plate preset at 110 °C, and heated for 2 min with continuous mixing. The resulted gel was left uncovered to cool to room temperature and used as formulated food (FF) in all frying experiments.

Frying test
An oil or purified triacylglycerides (12.0 g) were weighed into a clean, acid-washed glass beaker (30 mL, Pyrex, USA). Clean octagonal stir bars (9.5 × 25 mm, Fischer Scientific, USA) was placed into the glass vessel with oil, altering the surface-to-volume ratio of oil to 0.42. The vessel was heated at 185 ± 5 °C for 10 min, then 1.2 g of FF was added. The heating was continued for another 20 min without mixing and continuous stirring initiated at 500 rpm. Heating and stirring were maintained for additional 90 min. About 0.5 g of oil was withdrawn at the 60th, 80th, 100th, and 120th min for analysis. Selected sampling intervals reflect frying time at standard conditions for 1, 3, 5 and 7 days of actual frying using institutional fryer (General Electric Company, NY, USA) and are based on the amount and composition of polar components formed.

Total Polar components
The amount of polar components (TPC) was determined by gravimetric method following AOAC Method 982.27, using column chromatography to separate non–polar from polar fraction as described in Schulte modification [28, 29].

6
Tocopherols

Tocopherols were analyzed according to AOCS Official Method Ce 8-89 [30]. Briefly, 50 mg of oil samples were weighed directly into autosampler vials and dissolved in 1 mL hexane. Analyses were performed on a Finnigan Surveyor HPLC (Thermo Electron Corp., Waltham, MA, USA) with a Finnigan Surveyor Autosampler Plus and Finnigan Surveyor FL Plus fluorescence detector, the later was set for excitation at 292 nm and emission 325 nm. The column was a normal phase Diol column (5µm; 250 × 4.6 mm; MonoChrom, Varian, CA, USA). Of each sample, 10 µL was injected. Mobile phase consisted of 7% methyl-tert-butyl-ether in hexane with a flow rate of 0.6 mL/min. The amounts of tocopherols were quantified using external calibration for each isomer separately.

Phytosterols

Compositions of phytosterols were analyzed using the procedure described by Rudzińska et al [31]. Briefly, lipid samples were saponified with 1 M KOH in methanol at room temperature for 18 h, then water was added and unsaponifiables multi extracted with diethyl ether. Dry residues were silylated with BSTFA containing 1% TMCS. Derivatives of the sterols were separated on a Hewlett-Packard 6890 gas chromatograph with an HP-5 capillary column (30 m × 0.32 mm × 0.25 µm; J&W Scientific, Folsom, CA, USA). Split injection with split ratio 1:25 was used. Separation was done isothermally at 290 °C, with a helium flow rate of 1.6 mL/min. The injector and detector temperatures were set at 310°C. An internal standard, 5-α-cholestane, was used for quantification. Phytosterols were identified by comparison of retention data and by GCMS using a Finnigan Trace 2000 gas chromatograph coupled to a Finnigan Polaris Q quadrupole ion-trap mass spectrometer after separation on a DB-5 capillary column (50 m × 0.2 mm × 0.32 µm; J&W). Helium was used as carrier gas at a flow rate of 0.6 mL/min. All mass spectra were recorded using electron impact ionization mode at 70 eV and masses were scanned in the range of 100–650 Da. Ion source was held at 200 °C and injector at 300°C. A combination of the NIST Mass Spectra Library and collected spectra in lab were used to identify sterols.

Gamma-Oryzanol

RBOS and RBMC were analyzed for γ-oryzanol by HPLC as previously reported [32]. A Finnigan Surveyor Plus HPLC system (Thermo Electron, Waltham, MA, USA) was used. A 20 µL sample
was injected onto a C18 column (4 µm; 300 × 3.9 mm; Novapak, Waters, MA) held at 30°C. Separation was achieved by using acetonitrile/water (65:35, v/v) eluant. Finnigan Surveyor photodiode-array detector (PDA) was at 325 nm. The amount of γ-oryzanol is expressed as a sum of all esters separated and quantified by using the external calibration.

Carotenoids
Carotenoids were analysed according to the method of Khachik et al [33] with modifications. Analyses were carried out on Finnigan Surveyor Plus HPLC system (Thermo Electron, Waltham, MA, USA). A 10 µL sample was injected onto a C18 column (4µm; 300 × 3.9 mm; Novapak, Waters, MA) held at 25°C. Gradient system was used as follows: (1) 15% methanol, 75% acetonitrile, 5% methylene chloride and 5% hexane was held for 2 minutes; (2) at 20 minutes eluant was changed to 17% methanol , 60% acetonitrile, 11.5% methylene chloride and 11.5% hexane; (3) at 35 minute eluant was: 15% methanol, 40% acetonitrile, 22.5% methylene chloride, and 22.5% hexane; followed by returning to the initial eluant composition within 5 minutes with additional 5 min of equilibration. The flow rate was 0.5 mL/min. Carotenoids were quantified using external calibration using β-carotene as standard.

Analysis of minor components composition
The isolated minor components were separated into lipid classes according to Silversand and Haux [34] method with modifications. Separation was performed on a Finnigan Surveyor LC (Thermo Electron, Waltham, MA, USA). Components were separated on a normal phase Diol column (5 µm, 250 × 4.6 mm; Monochrom, Varian, CA, USA). The binary gradient was used consisting of: (A) hexane–acetic acid (99:1.0, v/v) and (B) hexane–isopropanol–acetic acid (84:15:1.0, v/v) solvents. The samples of 20 µL were injected and the gradient changed from 0% to 100% of the solvent B within 40 minutes. The final gradient was kept for 2 min and then returned to initial composition within 3 min followed by 5 min of equilibration. The flow rate was 0.6 mL/min and the column was kept at 45°C. Components were detected with a Sedex 75 evaporative light scattering detector (Sedere, Alfortville, France), operated at 30 °C with an air pressure of 1.5 bar. Triolein, diolein, monoolein, oleic acid, and stigmasterol linoleate, were used as standards for external calibration to assess the amount of triacylglycerols (TAG), diacylglycerols (DAG), monoacylglycerols (MAG), free fatty acids (FFA), and sterol esters (SE), respectively.
Quantification of 4-hydroxynonenal

Isolation and quantification of HNE was carried out according to the method developed in our laboratory and published elsewhere [35]. Briefly, polar fraction obtained during polar components analysis was utilized. When the non-polar fraction was eluted, polar fraction was removed from the column with four consecutive elutions using 5 mL of methanol. The combined eluants were subsequently evaporated under gentle stream of nitrogen to 5 mL. The cloudy solution was centrifuged at 2300 rpm for 5 min, and the clear supernatant analyzed by HPLC.

Analysis of HNE was carried out using a Finnigan Surveyor Plus HPLC (Thermo Electron, Waltham, MA, USA) with a Finnigan Surveyor Autosampler Plus and a Finnigan Surveyor UV-Vis Plus detector. A 20 µL sample was injected onto a C18 column (4 µm; 300 × 3.9 mm; Novapak, Waters, MA). HNE was detected at 223 nm after elution with acetonitrile/water (30:70, v/v) at a constant flow of 0.75 mL/min. Identification of HNE was done by comparison of retention data and by co-elution of HNE standard with selected samples. To further verify identity of the HNE peak identification was carried out on a QSTAR Elite mass spectrometer (AB SCIEX, Concord, ON, Canada) equipped with an APCI interface operated in a positive mode. Analyst QS 2.0 software was used for data acquisition and analysis. The conditions of mass spectrometric analysis were optimized for 4-hydroxynonenal as follows: the APCI source temperature was set at 450°C, the curtain gas at 25, the declustering potential at 45V, the focus potentials at 150V, and the ion source gas 1 and 2 at 20 and 60 psi, respectively. Quantification of HNE was carried out using external calibration.

Sampling and quantification of volatile carbonyl compounds

Volatile carbonyl compounds (VCC) generated during the frying experiments was trapped by a Sep-Pak DNPH-Silica cartridge (Waters, Milford, MA) where they were converted to the stable dinitrophenylhydrazones. During trapping the cartridge was connected to a pump with flexible tubing through a flowmeter. The wider end of the cartridge was suspended about 0.5 cm above the upper edge of the beaker used for frying test, and the samples of vapours were drawn through the cartridge at a flow rate of 350 mL/min. The resulted dinitrophenylhydrazones were eluted from the cartridge with 5 mL of acetonitrile keeping its flow at 2 mL/min.

Dinitrophenylhydrazones were separated and quantified by HPLC using a Finnigan Surveyor Plus HPLC system (Thermo Electron, Waltham, MA, USA). A 20 µL sample was injected onto a
C18 column (4µm; 300 × 3.9 mm; Novapak, Waters, MA) held at 30°C. Separation was achieved by using the following gradient: (1) 40% acetonitrile and 60% water was held for 5 min; (2) followed by 100% acetonitrile within 40 min and maintained it for 5 min; (3) for 5 min eluant returned to the initial composition followed by 5 min equilibration. The flow rate was 0.5 mL/min, Finnigan Surveyor UV-Vis Plus detector was set at 360 nm. VCC were identified by comparison of retention data with standards and their identity assessed by HPLC-MS (Exactive Bench-Top; Thermo Fischer Scientific, West Palm Beach, FL). The HPLC conditions were similar to the above. The mass spectrometer was equipped with an APCI ion source, operated in the negative mode. The spectra were collected using a mass scan range from 100 to 1000 Da.

Treatments
Performance of canola oil triacylglycerides was assessed with addition of the following components:

1. Canola oil triacylglycerol (CTG) - control
2. Phospholipids (phosphatidylcholine and phosphatidylethanolamine)
3. Combinations of tocopherol isomers
4. Canola oil tocopherol fraction, TCAN
5. Rice bran oil tocopherol fraction, TRBO
6. Canola oil sterol fraction, SCAN
7. Rice bran oil sterol fraction, SRBO
8. Solvent extracted minor components from rice bran oil, RBOS
9. Minor components isolated by chromatography from rice bran oil, RBMC
10. Solvent extracted minor components from sesame oil, SOS
11. Solvent extracted minor components from palm oil, POS

Statistical analysis
Results were statistically analyzed by single factor analysis of variance (ANOVA) and regression analyses using Minitab 2000 statistical software (Minitab Inc, PA, ver. 13.2). Statistically significant differences between means were determined by Duncan’s multiple range tests. Statistically significant differences were determined at P ≤ 0.05.
Results and Discussion

Minor components
In Table 1 composition of minor components isolated for this study is presented. TCAN and TRBO are fractions rich in tocopherols recovered from canola oil and rice bran oil, respectively. Both fractions contained more than 80% tocochromanols, with insignificant contribution of sterol ester. The small amounts of sterols are mainly coming from the sterol esters present in oils and having similar chromatographic properties as tocopherols. The corresponding SCAN and SRBO are fractions containing only phytosterols, where presence of tocopherols was not detected. RBOS and POS are fractions of minor components isolated by solvent extraction from rice bran and palm oils, respectively. Both fractions contained both tocopherols and sterols, reflecting composition of these compounds in the starting oils. In RBOS γ-oryzanols were detected, while as should be expected carotenoids in POS (Table 1). The composition of RBMC is not included in Table 1 because its composition is essentially the combination of compounds present in TRBO and SRBO fractions.

Tocopherols
All the indices of performance indicated that tocopherols at all tested concentrations and homologous compositions protected CTG under frying conditions. For instance, the amount of TPC formed in CTG containing a combination of 100, 450 and 450 µg/g of α-, γ-, and δ-tocopherol, respectively was 28% lower than formed in unprotected CTG at the end of the frying (Fig. 1). A statistically significant increase in the frying performance was observed when the concentration of tocopherol was increased from 500 to 1000 µg/g (Fig 1). However, further increase in the tocopherols amount to 2000 µg/g did not result in concomitant increase in frying stability of CTGs despite the increase in residual tocopherols amount at the end of frying (Figs. 1 and 2). This observation was consistent with the results of previous study by Lampi and Kamal-Eldin [26]. At the concentrations employed in the present work, results indicated that varying the relative proportions of tocopherol homologues in the mixture did not have significant effect on their ability to protect the CTGs during frying. Results by Warner and Moser also showed that varying the ratio of tocopherol homologues has no significant impact on their antioxidant activity when α-, γ-, δ-tocopherols were present together in the oil [36].

Furthermore, as observed for pure tocopherol homologous mixtures (Fig. 1), tocopherols fractions isolated from canola and rice bran oils offered significant level of protection to CTGs
during frying (Fig. 3). It is interesting that the presence of tocotrienols in rice bran tocochromanol mixtures did not lead to any enhancement in frying performance of CTG’s fortified with these components over other tested tocopherol compositions. Indeed, Romero et al [14] found no increase in the antioxidant activity when a mixture of α-tocopherol and α-tocotrienol was added to antioxidants stripped canola oil as compared to when only tocotrienol was added. They however, reported a reduced activity for the mixture as compared to α-tocopherol alone.

Volatile carbonyl compounds (VCC) are important secondary oxidation products formed during frying causing pollution in frying facility. Twenty one aldehydes including 9 alkanals, 9 alkenals and 3 alkadienals were detected and quantified in the present study (Table 2, Fig. 4). Propanal and pentenal were the most abundant volatile products formed during thermo-oxidative degradation of linolenic acid, whereas hexanal and pentanal were the major products formed from linoleic acid. Nonanal and octanal, on the other hand, were the major volatile degradation products formed from oleic acid (Table 2). The relatively lower concentrations of 2,4-decadienal and 2,4-heptadienal, the respective oxidation products of linoleic and linolenic acids might be due to further degradation of these compounds to produce other volatile compounds such as ethanal and 2-propenal, under the frying conditions used in the present study [37,38]. Similarly to TPC, the rate of VCC formation during degradation of unprotected CTGs was significantly higher when the tocopherol isomers mixtures were applied. Compared to other tested tocopherol compositions, the system containing 450-α, 450-γ and 100-δ generated the least amount of VCC. An increase in the tocopherol amount from 1000 to 2000 µg/g resulted in a significant increase in rate of VCC formation, with the increase more pronounced when higher amounts of α-tocopherol were present (Table 2). Whereas equivalent amounts of tocopherols were used, the rate of VCC formation was lower with TCAN, lowering it further when TRBO was applied. However, the VCC profile was independent of the composition of tocopherol isomers, indicating similar mechanism of degradation [14, 26]. Furthermore, irrespective of the minor components added the CTG, the same trends were observed for the major individual carbonyl compounds such as propanal and hexanal, and the total volatile carbonyl compounds (VCC).

Results indicated that the formation of HNE was significantly reduced in the presence of tocopherols compared to control (Fig. 5). At the end of the frying test, the amount of HNE accumulated in oils without tocopherols was 11.7 µg/g, compared to a maximum of 6.2 µg/g in sample containing mixture of tocopherol isomers. In agreement with the amount of TPC, no
significant differences were observed in the amount of HNE formation when all tested tocopherol homologous mixtures were applied (Fig. 5). The ability of tocopherols to restrict the formation of HNE during heating soybean oil at frying temperature has been observed [39].

Phytosterols
Separating tocopherols from sterols furnished a mixture of endogenous phytosterols without tocopherols to study their effect on the frying stability of CTGs. Sterol fractions SCAN and SRBO provided a concentration dependent protection for CTGs during frying (Fig. 3). When 500 µg/g of SCAN was added, no protective activity was observed. On the contrary, at this low concentration the protection by SRBO was comparable to the observed for endogenous tocopherols isolated from canola or rice bran oils (Fig. 3). This is presumably due to the presence of higher amount of sterols with known antioxidant activity such as an avenasterol in RBO (Table 1). When 3000 µg/g was added, the protection offered by SCAN was 20% better than tocopherols as measured by the amount of TPC at the end of the frying period (Fig. 3). Gertz et al [4] assessed antioxidative activity using the OSET procedure and reported superior antioxidant activity of canola oil sterols over tocopherols.

CTG fortified with a mixture of SCAN or SRBO and tocopherol isomers accumulated significantly higher amount of TPC throughout the frying period than present individually each of these fraction (results not included). Indeed, whenever tocopherols were added to the sterols fraction, the amount of TPC formed was similar to the amounts accumulated when CTGs contained only tocopherols. This indicates lack of synergistic interaction between these components. Thus, the antioxidant effect of sterols might not be realized in the presence of tocopherols, presumably due to the lower activation energy of the latter [26].

The emission rate of VCC was significantly lower when CTGs were heated in the presence of sterols at 3000 µg/g compared to unprotected CTGs (Table 2). However, it is contrary to the formation of TPC and no significant difference in the emission rate of VCC when CTGs were heated with sterols and tocopherols. Furthermore, no significant difference in both the amounts and profiles of volatile carbonyl compounds produced during heating CTGs with SCAN and SRBO fractions at the higher concentration (Table 2, Fig. 4).

The amount of HNE formed during heating CTGs fortified with SCAN was 60% lower comparing to control. In agreement with the TPC and VCC formation, no significant differences
were observed in HNE formation when SCAN and SRBO fraction were used for protection of CTGs (Fig. 6).

Solvent extracted minor components
Fortification of CTGs with RBOS, RBMC, SOS and POS at 0.5% resulted in significantly lower amounts of polar compounds formed, indicating lower oxidative degradation (Fig. 7). Comparing to RBMC, CTGs fortified with RBOS or SOS formed about 19% less TPC under frying. However, no statistically significant differences were observed in the stability of CTG’s fortified with RBMC, POS, and any of the tocopherol isomers mixtures (Fig 7). Thus, distinctive endogenous minor components such as carotenoids and tocotrienols present in POS and RBMC did not enhance the antioxidant effectiveness of the minor components during frying with CTGs. RBOS contained 10.5% γ-oryzanol and 11.8% wax as measured by HPLC and preparative TLC. RBMC, on the other hand, contained insignificant amounts of these minor compounds. Presumably, the significantly higher protective efficiency of RBOS over RBMC could be attributed to the presence of γ-oryzanol and waxes, in addition to tocopherols or potential synergistic effect between these components. Mezouari and Eichner [23] reported a significant antioxidant activity of RBO waxes during frying in sunflower oil. Similarly, the significant protection offered by SOS was possible due to the activity of the sesame lignans such as sesamol, sesaminol, sesamin and sesamolin. The improved frying stability of blends of sesame and canola oils over canola oil alone was attributed to the activity of the sesame oil lignans [20 – 22]. In a related study, the improved stability of a blend of soybean and sesame oils, and the food fried in it over soybean oil alone was assigned to the sesame oil lignans [40,41]. According to Hemalatha and Ghafoorunissa [42], addition of 1.2% of sesame lignans significantly increased the radical scavenging activity and the frying performance of soybean and sunflower oils.

The rate of VCC generation during frying in CTGs fortified with RBOS was 1.3 and 2.7 times lower than in CTG’s containing POS and control, respectively (Table 2). The rate of VCC generation during frying in CTGs containing RBOS was 9.8% lower than CTGs contained SOS; however, these differences were within the experimental error. No differences were observed in the VCC profiles for CTGs fortified with either of RBMC, RBOS, POS and SOS.
The results for HNE formation parallel those of VCC. At the end of the frying period, the amount of HNE detected in CTGs fortified with RBOS was 2.7 times lower than the amounts in control (Fig. 6).

Phospholipids
The effects of two phospholipids, namely phosphatidylcholine (PC) and phosphatidylethanolamine (PE) on the frying stability of CTGs were examined. As measured by TPC, no significant differences were observed in the ability of both phospholipids to protect CTGs during frying (Fig. 8). At concentration of 500 µg/g, neither PC nor PE offered any protection to CTGs. However, at concentration of 1000 µg/g, the antioxidant effect of phospholipids became evident (Fig. 8). At the end of the frying test, the amount of TPC accumulated in control was 2.5 and 2.3 times higher than the amount detected in CTG’s fortified with PC and PE, respectively (Fig. 8). Furthermore, the results from this study showed that CTGs containing 2000 µg/g PC were 1.9 times more stable than CTGs fortified with any tested tocopherol homologous mixtures.

At low phospholipids concentration tested, similar effect to sterols was observed when tocopherols were added. However, when the higher amounts of both phospholipids were added, the antioxidant activity was not impeded by the presence of tocopherols. Contrary to some literature reports, lack of synergy was observed between any of the studied phospholipids and tocopherols under the conditions employed in the present study [43,44]. On the other hand, a statistically significant synergy was observed between the tested phospholipids and RBOS (Fig. 8). This may be due to an interaction between the phospholipids and the phenolic compounds such as γ-oryzanol present in the RBOS. Ramadan [45] reported that quercetin increased the antioxidant activity of soybean lecithin in a triolein model system.

There is rather scanty information available on the application of phospholipids as antioxidants under frying conditions, probably because of their reportedly adverse effects on colour and foaming of oils [46]. Kourimska et al [47] found no significant increase in foaming of olive oil and no effect on the sensory properties of the French fries when lecithin was applied at 0.1%. In the present work, CTGs oil darkened at the faster rate when either or both phospholipids were utilized.

Compared to control, the rate of VCC generation during the frying was 3.1 and 3.5 times slower in the CTG’s containing PC and PE, respectively (Table 2). Similarly, both PC and PE significantly inhibited the formation of HNE during the frying test. At the end of the frying test, only 2.8 and 2.3
µg/g of HNE were observed in CTGs fortified with these phospholipids, respectively, compared to 11.7 µg/g in control (Fig. 6). These results are consistent with the abilities of soybean phospholipids to inhibit HNE formation in fried fish fillets during storage [48]. Despite the higher amount of TPC observed in the samples fortified with PE as compared to PC, the amount of carbonyl compounds, such as VCC and HNE, were generally lower when oil contained PE. This observation was in agreement with previous reports by Hidalgo et al [49]. Therefore, the possibility of a reaction between the amine group on PE and the carbonyl group is verified, which possibly stimulates drastic color changes [49].

Conclusions

The influence of various endogenous minor components on the frying performance of canola oil triacylglycerols was evaluated. The present study showed that both the composition and the concentration of the minor components exerted profound influence on the frying performance of the oil. Tocopherols remain the major antioxidants in the frying oils, whereas, the results from the present study indicated that there might be a need to look beyond tocopherols in order to design canola oil with remarkably improved frying performance. Phosphatidylcholine, phosphatidylethanolamine, and minor components isolated from rice bran and sesame oils particularly enhanced the frying performance of canola oil triacylglycerol.

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References


35. Aladedunye F, Przybylski, R. Carbon dioxide blanketing impedes the formation of 4-hydroxynonenal and acrylamide during frying. Eur J Lipid Sci Tech (Accepted)
Table 1. Composition of endogenous minor components isolated from canola, rice bran and palm oils

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentration (mg/g)</th>
<th>TCAN</th>
<th>TRBO</th>
<th>SCAN</th>
<th>SRBO</th>
<th>RBOS</th>
<th>POS</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-tocopherol</td>
<td>269.8±19</td>
<td>139.1±11</td>
<td>ND</td>
<td>ND</td>
<td>18.1±1.5</td>
<td>25.4±2.3</td>
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</tr>
<tr>
<td>β-tocopherol</td>
<td>ND</td>
<td>9.8±0.5</td>
<td>ND</td>
<td>ND</td>
<td>0.4±0.1</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>γ-tocopherol</td>
<td>505.1±39</td>
<td>178.9±11</td>
<td>ND</td>
<td>ND</td>
<td>37.2±1.4</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>δ-tocopherol</td>
<td>47.3±3</td>
<td>34.5±2.5</td>
<td>ND</td>
<td>ND</td>
<td>10.7±0.4</td>
<td>48.6±1.9</td>
<td></td>
</tr>
<tr>
<td>α-tocotrienol</td>
<td>ND</td>
<td>83.5±4.4</td>
<td>ND</td>
<td>ND</td>
<td>17.5±1.6</td>
<td>59.8±4.3</td>
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<tr>
<td>β-tocotrienol</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>γ-tocotrienol</td>
<td>ND</td>
<td>317.4±24</td>
<td>ND</td>
<td>ND</td>
<td>51.8±5.0</td>
<td>97.4±5.4</td>
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</tr>
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<td>δ-tocotrienol</td>
<td>ND</td>
<td>27.9±2.1</td>
<td>ND</td>
<td>ND</td>
<td>3.6±0.2</td>
<td>53.8±3.7</td>
<td></td>
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<tr>
<td>TOTAL</td>
<td>822.2±57</td>
<td>791.1±40</td>
<td>139.3±7.2</td>
<td>285.0±15</td>
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<td></td>
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<td>Brassicasterol</td>
<td>4.9±0.3</td>
<td>ND</td>
<td>115.0±9.3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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</tr>
<tr>
<td>Campesterol</td>
<td>11.9±1.0</td>
<td>7.9±0.4</td>
<td>264.1±16</td>
<td>143.5±10</td>
<td>63.8±4.1</td>
<td>44.7±2.3</td>
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<tr>
<td>Stigmasterol</td>
<td>1.8±0.1</td>
<td>6.0±0.4</td>
<td>14.5±1.2</td>
<td>157.2±11</td>
<td>78.1±4.9</td>
<td>1.5±0.1</td>
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<tr>
<td>β-Sitosterol</td>
<td>16.0±1.2</td>
<td>29.1±1.3</td>
<td>488.5±38</td>
<td>476.2±24</td>
<td>262.3±18</td>
<td>202.8±12</td>
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<tr>
<td>Δ^5-Avenasterol</td>
<td>1.9±0.1</td>
<td>3.3±0.2</td>
<td>10.3±0.7</td>
<td>10.8±0.6</td>
<td>13.5±0.9</td>
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<tr>
<td>Δ^7-Avenasterol</td>
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<td>5.1±0.6</td>
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<td>16.0±1.3</td>
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<td>24-Methylene-cycloartenol</td>
<td>ND</td>
<td>7.5±0.6</td>
<td>ND</td>
<td>22.9±2.1</td>
<td>27.5±1.8</td>
<td>ND</td>
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<tr>
<td>Cycloartenol</td>
<td>ND</td>
<td>6.7±0.5</td>
<td>ND</td>
<td>8.3±0.6</td>
<td>10.4±0.8</td>
<td>ND</td>
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<td>Citrostadienol</td>
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<td>7.5±0.3</td>
<td>ND</td>
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<td>15.6±1.2</td>
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<td></td>
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<tr>
<td>Unknown</td>
<td>4.9±0.3</td>
<td>ND</td>
<td>4.2±0.3</td>
<td>ND</td>
<td>10.7±0.9</td>
<td>ND</td>
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<tr>
<td>TOTAL</td>
<td>36.5±2.1</td>
<td>76.4±3.7</td>
<td>897.5±70</td>
<td>862.6±51</td>
<td>497.9±30</td>
<td>249.3±12</td>
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<td>Total γ-Oryzanol</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>105.4±7.4</td>
<td>ND</td>
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<tr>
<td>Total Carotenoids</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>27.3±1.8</td>
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<td>Triacylglycerols</td>
<td>48.3±3.9</td>
<td>51.6±3.3</td>
<td>ND</td>
<td>ND</td>
<td>125.1±12</td>
<td>186.8±19</td>
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<tr>
<td>Diacylglycerols</td>
<td>23.1±1.8</td>
<td>37.4±2.4</td>
<td>13.9±1.0</td>
<td>20.9±3.2</td>
<td>56.4±4.5</td>
<td>94.6±8.1</td>
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<td>Monoacylglycerols</td>
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<td>ND</td>
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<td>5.9±0.3</td>
<td>8.5±3.2</td>
<td>15.8±2.7</td>
<td></td>
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<tr>
<td>Free fatty acids</td>
<td>ND</td>
<td>2.8±0.2</td>
<td>6.3±0.6</td>
<td>8.7±1.1</td>
<td>13.9±1.4</td>
<td>27.2±1.9</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>69.9±5.4</td>
<td>40.7±2.6</td>
<td>81.2±4.7</td>
<td>101.9±8.4</td>
<td>53.5±4.8</td>
<td>114.0±11</td>
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</tbody>
</table>

*aSamples from two different experiments were analyzed in triplicate (n=6); values are reported as Mean ± SD. TCAN and TRBO - Tocopherol fractions, and SCAN and SRBO - Sterol fractions isolated from canola and rice bran oils, respectively; RBOS and POS – solvent extracted minor components from rice bran and palm oils, respectively; ND = not detected; Others – Undefined components*
Scheme 1. Schematic of isolation and purification of concentrated endogenous minor components from canola and rice bran oils. CC, column chromatography; PTLC, preparative thin layer chromatography; MTBE, methyl tert butyl ether; CO, canola oil; RBO, rice bran oil; CTG, canola oil triacylglycerols; CMC and RBMC are minor components isolated by column chromatography from canola and rice bran oils, respectively; TCAN and TRBO are tocopherol fractions isolated from canola and rice bran oils, respectively; SCAN and SRBO are sterol fractions isolated from canola and rice bran oils, respectively. For chromatographic conditions see the text.
Figure 1. Formation of polar components during test frying with antioxidant-free canola triacylglycerides containing different combinations and amounts of tocopherol homologous. CTG - canola triacylglycerides. Tocopherols concentrations are in µg/g. For details see text.
Figure 2. Changes of tocopherols during test frying of antioxidant-free canola triacylglycerides containing different combinations and amounts of tocopherol homologous mixtures. Tocopherols concentrations are in µg/g. For details see text.
Figure 3. Formation of polar components during test frying of antioxidant-free canola triacylglycerides containing different amounts of tocopherol and sterol fractions isolated from canola and rice bran oils. CTG - canola triacylglycerides. For details see text.
Table 2. Rate of volatile carbonyl compounds emission (µg/g/hr) during test frying with antioxidant-free canola triacylglycerides fortified with various minor components

<table>
<thead>
<tr>
<th>VCC</th>
<th>CTGs</th>
<th>Canola (RBD)</th>
<th>25α+25γ+50δ</th>
<th>450α+450γ+100δ</th>
<th>1000α+1000γ+450δ</th>
<th>900α+900γ+2000δ</th>
<th>900α+2000γ+9000δ</th>
<th>2000α+9000γ+9000δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanal</td>
<td>20.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.8&lt;sup&gt;d&lt;/sup&gt;</td>
<td>21.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>15.8&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>15.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>19.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Propanal</td>
<td>257.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>100.1&lt;sup&gt;c&lt;/sup&gt;</td>
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<sup>a</sup> Samples from two different frying experiments were analyzed in triplicate (n=6). Values with the same superscript in the same row are not significantly different at p < 0.05.
Figure 4. Typical HPLC chromatogram of dinitrophenylhydrazone derivatives of volatile carbonyl compounds. a = ethanal; b = propenal; c = propanal; d = 2-butenal; e = butanal; f = 2-pentenal; g = pentanal; h = 2-hexenal; i = hexanal; j = unknown; k = 2,4-heptadien; l = 2-heptenal; m = heptanal; n = 2-octenal; o = 2,4-nonadienal; p = octanal; q = 2-nonenal; r = nonanal; s = 2,4-decadienal; t = 2-decenal; u = decanal; v = 2-undecenal; w = unknown. See the text for HPLC conditions.
**Figure 5.** Formation of HNE during test frying of antioxidant-free canola triacylglycerides containing different combinations of tocopherol homologous mixtures. CTG - canola triacylglycerides. For details see text.
Figure 6. Formation of HNE during test frying of antioxidant-free canola triacylglycerides containing different amounts of endogenous minor components. CTG - canola triacylglycerides. For details see text.
Figure 7. Formation of polar components during test frying of antioxidant-free canola triacylglycerides containing 0.5% of endogenous minor components isolated from different oils. CTG - canola triacylglycerides. For details see text.
Figure 8. Formation of polar components during test frying of antioxidant-free canola triacylglycerides containing different amounts of phosphatidylcholine and phosphatidylethanolamine. CTG - canola triacylglycerides. For details see text.
Antioxidative properties of phenolic acids and interaction with endogenous minor components during frying

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Running title: Phenolic acids and frying performance

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**Keywords:** Canola oil / Phenolic acids / Frying test / HNE / Total polar components / Volatile carbonyl compounds

**Abbreviations:** HNE, 4-hydroxynonenal; TPC, total polar components; VCC, volatile carbonyl compounds; SC, canola sterol fraction; TC, canola tocopherol fraction; FA, ferulic acid; GA, gallic acid; CA, caffeic acid; HCA, dihydrocaffeic acid; VA, vanillic acid; EF, ethyl ferulate; BSTFA, N,O-bis(trimethylsilyl)trifluoroacetamide; TMCS, trimethylchlorosilane; DNPH, dinitrophenylhydrazine; MTBE, methyl-tert-butyl ether; PTLC, preparative thin layer chromatography; CTG, canola triacylglycerides.
Abstract

The ability of selected phenolic acids to improve the frying performance of canola oil triacylglycerides was evaluated in a frying test. The frying performance was assessed by the analysis of total polar components, formation of 4-hydroxynonenal and volatile carbonyl compounds. Ferulic, caffeic, dihydrocaffeic, gallic, and vanillic acids significantly increased the frying stability of canola oil triacylglycerols. At the end of the frying test, the amount of polar components formed in the canola triacylglycerides was at 22.9% compared to a maximum of 18.8% when the phenolic acids were added. Similarly, the level of HNE was reduced by up to 45% when triacylglycerols were supplemented with these compounds. The results showed that ethyl ferulate was more efficient as antioxidant than its acid form, while dihydrocaffeic acid offered a better protection than caffeic acid. Ferulic acid, a cinnamic acid derivative, was more efficient than its benzoic analog, vanillic acid. A positive interaction between phenolic acids and the sterol fraction isolated from canola oil was observed which contributed to the formation of steryl esters of the phenolic acids, such as steryl ferulate during the frying.

**Practical applications:** The poor thermal stability of polyunsaturated oils limits their application for prolonged frying. Polyunsaturated fatty acids offer important health benefits and can improve nutritional value of fried foods. Contrary to the commonly applied synthetic antioxidants, the phenolic acids tested in this study often are part of endogenous oil components present in oilseeds and also in some oils, and are known for their positive health benefits. Thus, the simple phenolic acids, especially the cinnamic acid derivatives may be applied as potent antioxidants to protect oils during thermal processes used for food production.
1. Introduction

Thermo-oxidative alterations of oils is the main degradation phenomenon happening in every food product containing lipids, particularly accelerated under the conditions employed during frying, leading to the formation of a variety of volatile and non-volatile compounds. The intensive mass exchange between the frying oil and the fried food occurring during frying, causes that degradation products are fast transferred into the food product [1]. Apart from the negative impacts on the physical, flavor and nutritional quality of the oils and the fried food, many of these products are known for their toxicity. For instance, 4-hydroxynonenal, a secondary oxidation product formed by oxidative degradation of omega-6 polyunsaturated fatty acids has been shown to exhibit mutagenic, cytotoxic and genotoxic properties, which are related to pathogenesis of several human diseases such as Alzheimer’s and atherosclerosis [2, 3]. Furthermore, several studies have shown that a number of volatile compounds are formed during frying which may exhibit carcinogenic, mutagenic and genotoxic properties [4 – 6]. Thus, the oxidative stability of frying oils is of paramount importance in the selection of frying medium and the quality of fried foods [7].

The contribution of linoleic and linolenic acids to food products containing or fried in canola oil makes it desirable as a good source of essential fatty acids. However, the susceptibility of these acids to thermo-oxidative degradation demand for measures that will increase the oil’s frying stability without compromising the availability of its essential fatty acids. Previous work in this area focused mainly on the influence of added tocopherols on the frying stability, with relatively fewer reports on the use of other natural antioxidants [8–11].

Like tocopherols, phenolic acids are widely distributed in oilseeds and are known to be efficient cell antioxidants [12]. Besides their high antioxidant activity, they are also known to possess interesting biological properties such as: antiviral, antimicrobial, antithrombosis, antifibrosis, anti-inflammatory, and anti-cancer activities of some of the phenolic acids have been documented [13 – 15]. Their ability to inhibit oxidation of edible oils under storage conditions have been established, although their effectiveness under thermo-oxidative conditions during frying remains controversial [16 – 18]. Higher oxidative stability was reported for canola oil supplemented with fraction rich in phenolic acids isolated from canola [19, 20], and evening primrose meals [21].
The type of chemical reactions occurring during frying is different from those happening at ambient storage conditions, which affects the behavior of antioxidants [22, 23]. Thus, evaluation of the antioxidant activity of individual phenolic acids under frying conditions remains an interesting task. To the best of our knowledge, the effect of simple phenolic acids, such as: caffeic, dihydrocaffeic, ferulic, gallic, and vanillic on the frying performance of canola oil has not been studied. So far, there are no reports on the capacity of these phenolic acids to inhibit the formation of toxic hydroxyalkenals during frying. Frying oils contain a number of endogenous minor components such as tocopherols, phytosterols, free fatty acids, which are involved in the oxidation process and may affect the activity of added antioxidants [18, 24]. Therefore, in the present study, the ability of the phenolic acids to improve the frying stability and inhibit the formation of HNE during test frying in canola oil triacylglycerols was evaluated. Also an interaction between phenolic acids and selected canola oil endogenous minor components was explored.

2. Materials and Methods

2.1 Materials

Commercially refined, bleached and deodorized regular canola oil was donated by Richardson Oilseed Limited (Lethbridge, Canada). Neutral alumina (70-230 mesh), silica gel 60Å (70 – 230 mesh), D-glucose, and potato starch, were obtained from VWR (Edmonton, Canada). Ferulic (FA), caffeic (CA), dihydrocaffeic (HCA), gallic (GA), vanillic acids (VA), ethyl ferulate (EF), and 4-hydroxynonenal (HNE) were purchased from Sigma-Aldrich (St. Louis, MO). BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide, TMCS (trimethylchlorosilane), and pyridine were obtained from Supelco (Bellefonte, PA, USA). γ-Oryzanol standard was supplied by Oryza Oil and Fat Chemical Co. Ltd (Ichinomiya-City, Japan). Volatile carbonyl compounds standards were purchased from Bedoukian Research (Danbury, CT).

2.2 Preparation of antioxidant stripped triacylglycerides and isolation of endogenous minor components

Canola oil was stripped of its endogenous minor components including antioxidants via adsorption chromatography following procedure described by Lampi and kamal-Eldin [25] with modifications. A slurry of 1 kg of alumina (activated at 103°C for 16 h and 200°C for 8 h) in 2 L
of hexane was loaded into a glass chromatography column (950 × 50 mm i.d.) keeping continuous hexane flow until bed in the column was evenly packed. In 500 mL of hexane 500 g of canola oil was dissolved and mixture loaded onto the packed column with continuous flow of hexane. Purified canola triacylglycerols were eluted with 3.7 L of hexane, discarding first 800 mL of hexane as containing only solvent. The polar components were eluted with 2.5 L of 10% methanol in methyl-tert-butyl ether. Both the column and collection vessels were wrapped in aluminum foil to prevent photo-oxidation. The collected fractions were stored as hexane solution at -16°C until used in the experiments.

2.3 Isolation of tocopherol and sterol fraction
The recovered minor components from canola oil were separated by adsorption chromatography into two fractions [26]. Water conditioned silica gel was prepared by heating it for 24 h at 160°C, and adjusting the water content to 5%. Slurry of 120 g of conditioned silica gel in 150 mL hexane was loaded into a glass chromatography column (600 × 45 mm i.d.) allowing hexane continuous flow until column was evenly packed. Minor components, 20 g, dissolved in 30 mL hexane were introduced into the column, the hexane was allowed to flow through the column and then discarded. Tocopherol fraction was subsequently eluted with 750 mL of 5% MTBE in hexane while sterol fraction with 750 mL of 50% MTBE in hexane. Purity of fractions was monitored by thin layer chromatography. Collected fractions were concentrated under vacuum using a rotary evaporator (BÜCHI Labortechnik AG, Flawil, Switzerland) at 35°C.

Tocopherol fraction was further purified by preparative thin layer chromatography (PTLC) using MTBE/hexane (2:8, v/v) as the developing solvent. Bands corresponding to tocopherols were scrapped and extracted three times with 10 mL of MTBE. Combined extracts were concentrated under vacuum using a rotary evaporator, flushed with nitrogen and coded TC. Similarly, sterol fraction was purified by PTLC using MTBE/hexane (65:35, v/v) as the developing solvent following procedure described for tocopherols. Purified sterol fraction is coded SC.

2.4 Preparation of frying food
Food as closely as possible mimicking most often fried foods containing mainly starch was prepared by mixing 4 g of potato starch, 1 g of glucose and 1 g of silica gel with 5 mL of cold
water. To this slurry 15 mL of boiling water was added. The mixture was transferred onto a hot plate preset at 110°C, and heated for 2 min with continuous mixing. The resulted gel was left uncovered to cool to ambient temperature and is coded FF.

2.5 Addition of antioxidants to canola oil triacylglycerides
A stock solution of the examined antioxidant in ether was added to canola oil triacylglycerides to deliver the target concentrations. After mixing, the solvent was evaporated under vacuum using a rotary evaporator (BÜCHI Labortechnik AG, Flawil, Switzerland) at 40°C. Residual solvent was removed under a gentle stream of nitrogen. The optimum concentrations for the examined antioxidants used in this study (500 µg/g for phenolic acids; 1000 µg/g for tocopherols; 3000 µg/g for sterols) were established by preliminary studies [26]. Moreover, in a typical refined, bleached and deodorized canola oil, the amount of sterols is at least 3 times that of tocopherols.

2.6 Frying test procedure and oil sampling
Vegetable oil (12.0 g) was weighed into an acid-washed glass beaker (30 mL; Pyrex, USA). Clean stirring bar (9.5 × 25 mm; Fischer Scientific, USA) was placed in the beaker, increasing the surface-to-volume ratio of an oil to 0.42. The beaker was heated at 185 ± 5°C for 10 min, and 1.2 g of FF was added. The heating was continued for another 20 min and thereafter, stirring at 500 rpm was initiated. Both stirring and heating were maintained for another 90 min. About 0.5 g of oil was withdrawn at the 60th, 80th, 100th and 120th min of heating for analyses. Selected sampling points are equivalent to the frying at standard conditions using institutional fryer (General Electric Company, NY, USA) for 1, 3, 5 and 7 days, respectively, based on the amount and composition of polar components formed [27].

2.7 Total polar components
Total polar components (TPC) were assessed by gravimetric procedure following AOAC Method 982.27. Column chromatography was utilized for separation of non–polar from polar fraction applying Schulte modifications [28, 29].

2.8 Tocopherols
Tocopherols were analyzed according to AOCS Method Ce 8-89 [30]. Briefly, 50 mg of oil samples were weighed directly into autosampler vials and dissolved in 1 mL of hexane. Analyses were performed on a Finnigan Surveyor HPLC (Thermo Electron Corp., Waltham, MA, USA) with a Finnigan Surveyor Autosampler Plus and Finnigan Surveyor FL Plus fluorescence detector, the later set for excitation at 292 nm and emission 325 nm. The column was a normal phase Diol column (5µm; 250 × 4.6 mm; MonoChrom, Varian, CA, USA). Of each sample, 10 µL was injected. Mobile phase consisted 7% MTBE in hexane and a flow rate of 0.6 mL/min. The amounts of tocopherols were quantified using external calibration for each isomer separately.

2.9 Phytosterols

Compositions of phytosterols were analyzed using the procedure described by Rudzińska et al. [31]. Briefly, lipid samples were saponified with 1 M KOH in methanol at room temperature for 18 h, then water was added and unsaponifiables extracted with diethyl ether. Dry residues were silylated with BSTFA containing 1% TMCS. Derivatives of the sterols were separated on a Hewlett-Packard 6890 gas chromatograph with an HP-5 capillary column (30 m × 0.32 mm × 0.25 µm; J&W Scientific, Folsom, CA, USA). Split injection with split ratio 1:25 was used. Separation was done isothermally at 290°C, and at a helium flow rate of 1.6 mL/min. The injector and detector temperatures were set at 310°C. An internal standard, 5-α-cholestane, was used for quantification. Phytosterols were identified by comparison of retention data and by GC–MS using a Finnigan Trace 2000 gas chromatograph coupled to a Finnigan Polaris Q quadrupole ion-trap mass spectrometer after separation on a DB-5 capillary column (50 m × 0.2 mm × 0.32 µm; J&W). Helium was used as carrier gas at a flow rate of 0.6 mL/min. All mass spectra were recorded using electron-impact ionization mode at 70 eV and scanning mass in the range of 100–650 D. Ion source was held at 200°C and injector at 300°C. A combination of the NIST Mass Spectra Library and collected spectra of sterols were used to identify the sterols.

2.10 Steryl ferulates

Analysis for steryl ferulates (γ-oryzanol) was carried out as previously reported [32]. A Finnigan Surveyor Plus HPLC system (Thermo Electron, Waltham, MA, USA) was used. A 20 µL sample was injected onto a C18 column (4 µm; 300 × 3.9 mm; Novapak, Waters, MA) held at 30°C.
Separation was achieved by using acetonitrile-water (65:35, v/v). Detection was at 325 nm using a Finnigan Surveyor photodiode-array detector (PDA). Total amounts of gamma-oryzonals is expressed as a group of esters and quantified by using external calibration method with standard oryzanol sample.

2.11 **Analysis of minor components composition**
The isolated minor components were separated into lipid classes according to Silversand and Haux [33] method with modifications. Separation was performed on a Finnigan Surveyor LC (Thermo Electron, Waltham, MA, USA). Components were separated on a normal phase Diol column (5 µm, 250 × 4.6 mm; Monochrom, Varian, CA, USA). The binary gradient was used consisting of: (A) hexane–acetic acid (99:1.0, v/v) and (B) hexane–isopropanol–acetic acid (84:15:1.0, v/v) solvents. The samples of 20 µL were injected and the gradient changed from 0% to 100% of the solvent B within 40 minutes. The final gradient was kept for 2 min and then returned to initial composition within 3 min followed by 5 min of equilibration. The flow rate was 0.6 mL/min and the column was kept at 45°C. Components were detected with a Sedex 75 evaporative light scattering detector (Sedere, Alfortville, France), operated at 30°C with an air pressure of 1.5 bar. Triolein, diolein, monoolein, oleic acid, and stigmasterol linoleate, were used as standards for external calibration to assess the amount of triacylglycerols (TAG), diacylglycerols (DAG), monoacylglycerols (MAG), free fatty acids (FFA), and sterol esters (SE), respectively.

2.12 **Quantification of HNE**
An analysis of HNE was carried out according to a method developed in our laboratory and published elsewhere [34]. First, frying oil sample was separated on non-polar and polar fractions following AOAC Method 982.27 with Schulte modifications [28, 29]. The polar fraction was eluted four times with 5 mL of methanol. The excess of solvent was evaporated with gentle stream of nitrogen reducing volume to 5 mL. The cloudy solution was centrifuged at 2300 rpm for 5 min, and the clear supernatant analyzed for HNE by HPLC.

Analysis of HNE was carried out using a Finnigan Surveyor Plus HPLC (Thermo Electron, Waltham, MA, USA) consisting of a Finnigan Surveyor Autosampler Plus and a Finnigan Surveyor UV-VIS Plus detector. A 20 µL sample was injected onto a C18 column (4
µm; 300 × 3.9 mm; Novapak, Waters, MA). HNE was detected at 223 nm and sample separated using acetonitrile/water (30:70, v/v) as mobile phase at a constant flow of 0.75 mL/min. HNE was identified by comparison of retention data and co-elution with HNE standard. Identity of HNE was assessed by HPLC-MS as described elsewhere [34] Quantification of HNE was carried out using external calibration with HNE standard.

2.13 Sampling and quantification of volatile carbonyl compounds (VCC)

Volatile carbonyl compounds generated during the frying experiments were trapped in a Sep-Pak DNPH-Silica cartridge (Waters, Milford, MA) where these compounds were converted to the stable dinitrophenylhydrazones [26]. The cartridge was connected to an air pump through a flowmeter with flexible tubing. The wider end of the cartridge was suspended about 0.5 cm above the top edge of the beaker, and the volatiles were drawn through the cartridge at a flow rate of 350 mL/min. The sampling time was 2 h and the resulted dinitrophenylhydrazones were eluted from the cartridge with 5 mL of acetonitrile keeping its flow at 2 mL/min.

Dinitrophenylhydrazones were separated and quantified using a Finnigan Surveyor Plus HPLC system (Thermo Electron, Waltham, MA, USA). A 20 µL of sample was injected onto a C18 column (4µm; 300 × 3.9 mm; Novapak, Waters, MA) held at 30°C. Separation was achieved by applying the following gradient: (1) Acetonitrile/water (40:60, v/v) was held for 5 min; (2) Within 40 min acetonitrile contribution increased to 100% and was held for 5 min; (3) In 10 min gradient returned to initial composition which was held for 5 min for equilibration. The flow rate was 0.5 mL/min. Finnigan Surveyor UV-VIS Plus detector was at 360 nm. VCC were identified by comparison of the retention data with the standards and by HPLC-MS (Exactive Bench-Top; Thermo Fischer Scientific, West Palm Beach, FL). The HPLC conditions were similar to the above. The mass spectrometer was equipped with an APCI ion source operated in the negative mode. The spectra were collected in a mass range from 100 to 1000 Da at a scan rate of 1 scan/sec. The derivatization of the carbonyl standards into dinitrophenylhydrazones was achieved using the method by Possanzini and Dipalo [35]
2.14 Statistical analysis

Samples from two repetitions of each frying experiments were analyzed in triplicate. Data are presented as mean ± SD. Data were analyzed by single factor analysis of variance (ANOVA) and regression analysis using Minitab 2000 statistical software (Minitab Inc, PA, ver. 13.2). Statistically significant differences between means were determined by Duncan’s multiple range tests. Statistically significant differences were determined at P ≤ 0.05.

3. Results and discussions

3.1 Composition of TC and SC

The major components of TC and SC are presented in Table 1. TC fraction contained over 82% of tocopherols, and insignificant amount of sterols was also detected. This amount of sterols is related mainly to the presence of sterol esters which have similar chromatographic properties as tocopherols. Conversely, SC fraction composed of nearly 90% phytosterols, and no detectable amount of tocopherols was observed (Table 1).

3.2 Total polar components

Assessment of the amount of TPC is considered as the single and the most reliable measurement of thermo-oxidative oil degradation during frying [23, 36]. As measured by the amount of TPC formed, all the phenolic acids examined in the present study offered outstanding protection to CTG during the frying (Fig. 1). At the end of the frying period, the amount of TPC in unprotected CTG was at 22.9%, compared to 17.0, 16.8, 15.4, 15.9, 17.8, and 18.8% when CTG were fortified with FA, CA, HCA, EF, GA, and VA, respectively. In the first 60 min of frying test, about 1 day of actual frying, CTGs containing gallic and caffeic acids showed formation of lower amounts of TPC compared to ferulic acid. However, as frying proceeded, a marked reduction in the activity of GA was observed. At the end of the frying period, no significant differences (P ≥ 0.18) were observed in the activity of CA, FA, and GA regarding inhibition of polar components formation (Fig. 1). The observed reduced ability of gallic acid could be explained by its inherent tendency to oligomerization. Wang et al. [37] observed that although gallic acid was quite effective during assessment of Oxidative Stability Index (OSI), however it did not prevent changes in viscosity.
The role of the 2,3-double bond configuration in the structure of CA on its antioxidant activity remains controversial [17, 18]. In the present study, HCA offered significantly better protection (P ≤ 0.04) than CA during frying test using canola oil triacylglycerols (Fig. 1). Nenadis et al. reported a superior antioxidant activity of HCA over CA during triolein oxidation at 45 and 120°C [18]. Thus, the reduced antioxidant activity of CA may be related to the electron withdrawing characteristics of the 2,3-double bond [18].

Compared to ferulic acid (FA), ethyl ferulate (EF) offered a significantly better protection (P ≤ 0.04) against thermo-oxidative degradation during frying as measured by TPC (Fig. 1). At the end of the frying period, the amounts of TPC in CTG fortified with FA and EF were 17.0 and 15.9%, respectively. According to a study by Warner and Laszlo [38], the ferulic acid completely disappeared after 15 h of frying in soybean oil, while 55% of an ethyl ferulate was still found for the same period of frying. Thus, the observed higher activity of EF over FA during the frying test with CTG can be related to its higher thermal stability and/or differences in oil solubility. Among all of the tested phenolic acids, VA was the least effective and at the end of the frying period, the amount of TPC was at 18.8%, compared to 17.0% when the structurally related ferulic acid was applied (Fig. 1). According to Marinova et al. [16], derivatives of cinnamic acid were better antioxidants than the corresponding analogues of benzoic acid. It is well known that the presence of an alkyl chain the carboxyl group and the phenolic ring increases the radical scavenging activity of the phenolic acids [14, 15].

An interesting interaction was observed during frying between canola oil endogenous minor components, namely sterols, and the added phenolic acids. The sterol and tocopherol fractions were added in the amounts delivering 3000 µg/g of sterol and 1000 µg/g of tocopherol, respectively. Concentration of 1000 µg/g has previously been established as the optimum concentration for tocopherol antioxidant activity under frying conditions while sterols required a much higher concentration to be effective [26]. As measured by the amount of TPC formed, no significant interaction was observed between the phenolic acids and the tocopherol fraction isolated from canola oil (Fig. 1). For instance, at the end of the frying period, the amount of TPC in CTG fortified with TC, FA, and a mixture of FA and TC were at 17.8, 17.0, and 17.1%, respectively. On the contrary, a positive interaction was observed between tested phenolic acids and the sterol components isolated from canola oil. At the end of the frying period, the amount of
TPC in CTG containing a mixture of FA and SC was 26 and 11% lower compared to the amounts when these components were added individually (Fig. 1). Preliminary studies indicated that 500 µg/g was the optimum concentration for phenolic acid antioxidant activity under the conditions employed in this study, no additional protection was observed even at phenolic acid concentration of 5000 µg/g (results not shown). Thus, the improvement in antioxidant activity observed between sterols and the phenolic acids is most likely due to interactions between these components rather than the effect of an increase in concentration. The analysis for steryl ferulates indicated that up to 59 µg/g of γ-oryzanol was formed during the frying (Fig. 2). The formation of γ-oryzanal progressed with the frying time reaching maximum at 80 min of frying test and lowering to the value of 15.9 µg/g at the end of the frying period (Fig. 2). Formation of these components during frying might affect antioxidant capacity and improve protection of the frying oil. Sitostanyl ferulate improved protection of high oleic sunflower during frying [39]. The high thermal stability and improved antioxidant capacity of γ-oryzanol was established when steryl ferulates present in rice bran oil were assessed as frying oil protectants [7, 39].

3.3 Volatile carbonyl compounds (VCC)

VCC are important secondary oxidation products formed during frying which may contribute to the pollution in frying facilities. Apart from their potential toxicity, many of the volatile carbonyl compounds are directly responsible for off-odor and off-flavor development originating from lipids oxidation. Twenty one aldehydes including 9 alkanals, 9 alkenals and 3 alkadienals were identified and quantified in the present study (Table 2, Fig. 3). All the tested additives exhibited significant antioxidant activity as the measured amounts of VCC decreased at the end of the frying period when additives were added (Table 2). Compared to pure CTG, the rate of VCC formation was reduced by 42, 58, 54, 52, 57, 58, 55, 56, 60, and 51% when VA, EF, GA, FA, CA, HCA, SC, TC, FA+SC, and FA+TC were added to oil, respectively. In agreement with the TPC results, the rate of VCC formation was significantly lower when CTG contained ethyl ferulate compared to a ferulic acid. Furthermore, no significant differences (P ≥ 0.14) in the formation of VCC were observed when CTG were fortified with a mixture of phenolic acid and TC compared to CTG containing either of the constituents (Table 2). Conversely, an improved antioxidant activity was observed when CTG contained the tested phenolic acids and SC. For instance, the rate of VCC formation was at 203, 190, and 171 µg/g/hr at the end of the frying
period when CTG were fortified with FA, SC, and FA+SC, respectively (Table 2). Again, the similar pattern of changes was observed between TPC and VCC formation when CTG were fortified with VA and FA. These results support earlier observations that the derivatives of cinnamic acid offer better antioxidant capacity than the corresponding benzoic acid analogues [16].

3.4 Hydroxynonenal

Hydroxyalkenals is a group of polar secondary oxidation products formed during oxidative degradation of polyunsaturated fatty acids, particularly at elevated temperatures. Principally, HNE has attracted much attention not only because of its unusually high toxicity, but also because it could be formed at the concentrations posing health concerns when food is processed at elevated temperatures and containing n-6 polyunsaturated fatty acids. As shown in Fig. 4, all applied additives inhibited the formation of HNE during frying when canola oil triacylglycerols were used. At the end of the frying period the following amounts of HNE were found: 11.2 µg/g for purified CTG, 5.9, 7.1, 6.0, 5.7, 6.0, 6.3, 5.8, 4.9, 4.3, 4.5, and 5.6 µg/g when CTG comprised EF, VA, CA, HCA, FA, GA, TC, SC, FA+SC, CA+SC, and FA+TC, respectively. The amounts of HNE found in CTG fortified with VA were significantly higher than observed for CTG containing any of the phenolic acids. However, no significant differences in HNE formation were observed when individually FA, CA, EF, HCA and GA were applied. In agreement with other measured indices, CTG fortified with the mixture of phenolic acids and SC accumulated significantly lower amounts of HNE, indicating improved thermo-oxidative protection of these components (Fig. 4).

4. Conclusions

The present study substantiated that the frying performance of canola oil could be improved by adding simple phenolic acids. In this study, no interaction was observed between phenolic acids and endogenous canola oil tocopherols in improving protection of the oil during frying. On the other hand, a significant positive interaction was observed between phenolic acids and endogenous canola sterols; this synergy could be due to the formation of γ-oryzanol during the frying process.
Acknowledgement

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Conflict of interest statement

The authors have declared no conflict of interest
References


Table 1. Composition of tocopherol and phytosterol in isolated fraction of endogenous minor components from canola oil

<table>
<thead>
<tr>
<th>Components</th>
<th>TC (mg/g)</th>
<th>SC (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-tocopherol</td>
<td>269.8 ± 19.2</td>
<td>nd</td>
</tr>
<tr>
<td>γ-tocopherol</td>
<td>505.1 ± 38.9</td>
<td>nd</td>
</tr>
<tr>
<td>δ-tocopherol</td>
<td>47.3 ± 3.1</td>
<td>nd</td>
</tr>
<tr>
<td>Brassicasterol</td>
<td>4.9 ± 0.3</td>
<td>115.0 ± 9.3</td>
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<tr>
<td>Campesterol</td>
<td>11.9 ± 1.0</td>
<td>264.1 ± 16.1</td>
</tr>
<tr>
<td>Stigmasterol</td>
<td>1.8 ± 0.1</td>
<td>14.5 ± 1.2</td>
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<tr>
<td>β-Sitosterol</td>
<td>16.0 ± 1.2</td>
<td>488.5 ± 38.4</td>
</tr>
<tr>
<td>Δ⁵-Avenasterol</td>
<td>1.9 ± 0.1</td>
<td>10.3 ± 0.7</td>
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<td>Δ⁷-Avenasterol</td>
<td>nd</td>
<td>5.1 ± 0.6</td>
</tr>
<tr>
<td>Triacylglycerols</td>
<td>48.3 ± 3.9</td>
<td>nd</td>
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<tr>
<td>Diacylglycerols</td>
<td>23.1 ± 1.8</td>
<td>13.9 ± 1.0</td>
</tr>
<tr>
<td>Monoacylglycerols</td>
<td>nd</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>Free fatty acids</td>
<td>nd</td>
<td>6.3 ± 0.6</td>
</tr>
<tr>
<td>Steryl esters</td>
<td>29.7 ± 1.2</td>
<td>nd</td>
</tr>
<tr>
<td>Others</td>
<td>69.9 ± 5.4</td>
<td>81.2 ± 4.7</td>
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</table>

⁸Samples from two different frying experiments were analyzed in triplicate (n=6); values are reported as Mean ± SD. TC – tocopherol-rich fraction; SC – sterol-rich fraction; nd = not detected
Table 2: Volatile carbonyl compounds rate of emission (µg/g/hr) during frying canola oil triacylglycerides with various phenolic compounds

<table>
<thead>
<tr>
<th>VCC</th>
<th>CTG</th>
<th>VA</th>
<th>EF</th>
<th>GA</th>
<th>FA</th>
<th>CA</th>
<th>HCA</th>
<th>SC</th>
<th>TC</th>
<th>FA+SC</th>
<th>FA+TC</th>
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<td>Ethanal</td>
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<td>18.6bd</td>
<td>15.0c</td>
<td>19.2bd</td>
<td>18.9bd</td>
<td>17.6de</td>
<td>16.3ce</td>
<td>13.2f</td>
<td>16.0ce</td>
<td>13.1f</td>
<td>15.8ce</td>
</tr>
<tr>
<td>Propanal</td>
<td>257.0b</td>
<td>127.1d</td>
<td>91.9eh</td>
<td>101.8cf</td>
<td>107.2cf</td>
<td>97.1eh</td>
<td>94.8ce</td>
<td>99.4eh</td>
<td>93.9ce</td>
<td>85.9c</td>
<td>111.9f</td>
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<tr>
<td>2-propenal</td>
<td>40.9b</td>
<td>30.6c</td>
<td>14.3b</td>
<td>17.1ef</td>
<td>20.1d</td>
<td>16.2ef</td>
<td>16.1ef</td>
<td>17.7e</td>
<td>19.2de</td>
<td>20.9d</td>
<td>20.0d</td>
</tr>
<tr>
<td>2-butenal</td>
<td>0.3b</td>
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<td>0.1c</td>
<td>0.1c</td>
<td>0.2d</td>
<td>0.2d</td>
<td>0.1c</td>
<td>0.2d</td>
<td>0.1c</td>
<td>0.2d</td>
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<tr>
<td>Butanal</td>
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<td>1.1b</td>
<td>0.7c</td>
<td>0.9d</td>
<td>0.9d</td>
<td>1.1b</td>
<td>1.0bd</td>
<td>0.8c</td>
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<tr>
<td>2-pentenal</td>
<td>15.4b</td>
<td>8.6c</td>
<td>6.2d</td>
<td>7.2f</td>
<td>6.1d</td>
<td>5.5e</td>
<td>5.8de</td>
<td>6.4d</td>
<td>5.8de</td>
<td>6.1de</td>
<td>6.1de</td>
</tr>
<tr>
<td>Pentanal</td>
<td>39.2b</td>
<td>31.0d</td>
<td>27.0c</td>
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<td>25.5ce</td>
<td>25.0ce</td>
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<td>29.3ce</td>
<td>24.9ce</td>
<td>28.9ed</td>
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<td>2-hexenal</td>
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<td>1.2d</td>
<td>1.4e</td>
<td>1.7f</td>
<td>1.3de</td>
<td>1.2d</td>
<td>1.4e</td>
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<tr>
<td>Hexanal</td>
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<td>8.1g</td>
<td>7.2f</td>
<td>9.8d</td>
<td>8.4g</td>
<td>7.8fg</td>
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<td>2,4-heptadienal</td>
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<td>0.7d</td>
<td>0.7d</td>
<td>0.4e</td>
<td>0.6f</td>
<td>0.6f</td>
<td>0.4e</td>
<td>0.6f</td>
<td>0.6f</td>
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<tr>
<td>2-heptenol</td>
<td>2.2b</td>
<td>1.1c</td>
<td>0.9d</td>
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<td>0.6e</td>
<td>0.8f</td>
<td>0.8f</td>
<td>1.1c</td>
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<td>0.8f</td>
<td>1.1c</td>
</tr>
<tr>
<td>Heptanal</td>
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<td>1.9c</td>
<td>1.1d</td>
<td>1.1d</td>
<td>1.3e</td>
<td>1.1d</td>
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<td>1.7g</td>
</tr>
<tr>
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<tr>
<td>Octanal</td>
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<td>1.7c</td>
<td>1.2d</td>
<td>1.2d</td>
<td>1.3d</td>
<td>1.2d</td>
<td>1.3d</td>
<td>1.5e</td>
<td>1.6c</td>
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<tr>
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<tr>
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<tr>
<td>Total</td>
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<td>248.9c</td>
<td>178.8df</td>
<td>197.2e</td>
<td>203.2e</td>
<td>183.3d</td>
<td>178.0de</td>
<td>189.7de</td>
<td>189.5de</td>
<td>170.6f</td>
<td>207.8e</td>
</tr>
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</table>

*aSamples from two different frying experiments were analyzed in triplicate (n=6). Values with same superscript in the same row are not significantly different at p < 0.05. See Fig. 1 for details.*
Figure 1. Polar components formation during test frying canola triacylglycerides fortified with different phenolic acids and minor components isolated from canola oil. CTG – canola triacylglycerides; HCA – dihydrocaffeic acid (500 µg/g); CA – caffeic acid (500 µg/g); GA – gallic acids (500 µg/g); VA – vanillic acid (500 µg/g); FA – ferulic acid (500 µg/g); EF – ethyl ferulate (500 µg/g); SC – canola oil sterol fraction (3000 µg/g); TC – canola oil tocopherol fraction (1000 µg/g).
Figure 2. Amount of steryl ferulates detected in CTGs fried with a mixture of ferulic acid and sterol fraction isolated from canola oil. CTGs – canola oil triacylglycerides; FA+SC – ferulic acid with canola sterol fraction.
Figure 3. Chromatogram of volatile carbonyl dinitrophenylhydrazones. See text for HPLC conditions. a = ethanal; b = propenal; c = propanal; d = 2-butenal; e = butanal; f = 2-pentenal; g = pentanal; h = 2-hexenal; i = hexanal; j = unknown; k = 2,4-heptadienal; l = 2-heptenal; m = heptanal; n = 2-octenal; o = 2,4-nonadienal; p = octanal; q = 2-nonenal; r = nonanal; s = 2,4-decadienal; t = 2-decenal; u = decanal; v = 2-undecenal; w = unknown.
Figure 4. Formation of 4-hydroxynonenal during test frying using canola triacylglycerides containing different phenolic acids and minor components isolated from canola oil. CTG – canola triacylglycerides; HCA – dihydrocaffeic acid (500 µg/g); CA – caffeic acid (500 µg/g); GA – gallic acids (500 µg/g); VA – vanillic acid (500 µg/g); FA – ferulic acid (500 µg/g); EF – ethyl ferulate (500 µg/g); SC – canola sterol fraction added at 3000 µg/g; TC – canola tocopherol fraction added at 1000 µg/g.
Synthesis, Radical Scavenging Activity, Protection during Storage and Frying by Novel Antioxidants

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Abstract
Novel antioxidants, derivatives of trolox and selected phenolic acids have been prepared in good yields and fully characterized by $^1$H NMR, $^{13}$C NMR, and MS. Their antioxidant activities have been assessed by DPPH and ORAC assays, and during frying and accelerated storage tests. Novel phenolic compounds exhibited higher radical scavenging activities than both trolox and α-tocopherol. Trolox hydroxybenzoate showed a significantly higher protection than α-tocopherol under storage conditions. All new antioxidants performed better than α-tocopherol under frying conditions. Moreover, their outstanding thermal stability makes them more valuable than α-tocopherol for frying applications.

KEYWORDS: Antioxidant; Synthesis; Frying; Phenolics; Radical scavenging activity; trolox; storage stability; frying performance
INTRODUCTION

Free radicals are involved in inflammatory and cardiovascular diseases, cancer and stimulate aging (1-7). In food, rancidity is one of the major concerns and mainly related to oxidative degradation of polyunsaturated fatty acids. For years antioxidants have been used to prevent degradation of food (8). Phenolic derivatives are one of the most effective and commonly used antioxidants. These derivatives slow down the degradation of food ingredients by inhibiting their oxidation (9-10). Among this family of compounds, both synthetic antioxidants such as BHT, BHA, TBHQ and natural ones such as tocopherols, phenolic acids, herbal extracts are used to protect against oxidative degradation. Although synthetic antioxidants have shown good efficiency, their use has been limited because of their possible detrimental effect on human health (8). As a consequence, there is a growing interest in the development of new antioxidants that are based on natural components and exhibit low toxicity.

Among the natural antioxidants α-tocopherol (vitamin E) is the most effective; however its activity is affected by the environment and conditions in which these compounds operate (11). Distribution and type of substitutes on the chromanol ring is mainly responsible for its effectiveness. Indeed, the methyl groups activate the aromatic ring and the geometry adopted by the heterocyclic ring results in a stabilization of the phenoxyl radical (11). Natural phenolic acids are components of food and their antioxidative activity have been used for food protection. Besides, some are also known for health stimulating properties. As an example, gallic acid and its derivatives have been shown to exhibit the following health affecting properties: cardioprotective (12), neuroprotective (13), anti-inflammatory (14), antimitagenic (15) and anticancerogenic (16).

The objective of this work was the synthesis of new phenolic compounds with higher antioxidant activities than the common natural antioxidants and improved stability during storage and frying. Given that very often antioxidants are assessed for their free radical scavenging and this assessment has a limited value in predicting antioxidant effectiveness in real food system (17), we applied accelerated storage and frying as measures of their efficacy.

MATERIALS AND METHODS

General Procedures. Column chromatography was performed using EMD silica gel Si 60 (40-63 µm). \(^1\)H NMR and \(^13\)C NMR were recorded on a 300 MHz Bruker Avance II spectrometer (Bruker BioSpin Corporation, Billerica, MA) with TMS as an internal standard. NMR data are presented in the following order: chemical shift in ppm, multiplicity (s-singlet;
d-doublet; t-triplet; m-multiplet), coupling constant in Hertz, assignment broad band $^1$H decoupling. Melting points (Mp) were measured with an Electrothermal MEL-TEMP 3.0 (Barnstead). Analyses of residual antioxidants after frying and storage tests were performed on a Finnigan Surveyor LC (Thermo Electron Corp., Waltham, MA, USA) with a Finnigan Surveyor Autosampler Plus and Finnigan Surveyor FL Plus fluorescence detector. The column was a normal phase Diol column (5µm; 250 × 4.6 mm; Monochrom, Varian, CA). A Beckman DU-65 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA) was used in the DPPH assay as well as for the determination of PV. For ORAC assay, fluorescence was measured on a Cary Eclipse fluorescence spectrophotometer (Varian, Palo Alto, CA). High-resolution mass spectra were obtained with a QSTAR Elite mass spectrometer (AB SCIEX, Concord, ON, Canada) equipped with an electrospray source operated in positive ion mode.

Chemicals. AAPH-2,2'-azobis (2-amidinopropane) dihydrochloride; DCC-N,N'-dicyclohexylcarbodiimide; DMAP-dimethylaminopyridine; DPPH-2,2-diphenyl-1-picrylhydrazyl; fluorescein disodium salt, trolox and other phenolic acids were purchased from Sigma-Aldrich (St. Louis, MO). THF - tetrahydrofuran and DCM – dichloromethane were purified using a MBraun Solvent Purification System (M. Braun Incorporated, Stratham, NH). DMF- dimethylformamide and other solvents used in this work were of HPLC grade and obtained from VWR (Edmonton, Canada).

Syntheses. Compounds 3a, 3d, 4a, 4d, 4e, 5, and 6 were synthesized according to a method described by Tranchimand et al (18) while compound 7 according to procedure described by Muller et al (19).

General procedure for synthesis of benzoic acid esters (3b and 3c). Benzoic esters 3b and 3c were prepared according to a procedure described by Tranchimand et al (18) Briefly, potassium carbonate (77.2 mmol, 3.0 equiv) and benzyl bromide (77.2 mmol, 3.0 equiv) were added to a solution of the desired benzoic acid derivative (25.7 mmol) dissolved in 100 mL of dry DMF under an argon blanket. The mixture was stirred for 15 hrs and transferred into distilled water (150 mL). Compound of interest was extracted thrice with diethyl ether (100 mL). The combined extracts were washed with distilled water (100 mL), dried on magnesium sulfate and concentrated under vacuum using a rotary evaporator.

Data for benzyl 3-methoxy-4-benzyloxybenzoate (3b; Scheme 1). The crude benzoic ester was purified by recrystallization in hexanes. Aspect: white solids. Yield = 89%. The spectral results were in agreement with published data (20).
**Data for benzyl 3,5-dimethoxy-4-benzyloxybenzoate (3c; Scheme 1).** The crude benzoic ester was purified by hexane recrystallization. Aspect: white solids. Yield = 76%. $^1$H NMR (300.0 MHz, CDCl$_3$): $\delta$: 3.73 (s, 6H, OCH$_3$); 4.96 (s, 2H, OCH$_2$Ph); 5.23 (s, 2H, OCH$_2$Ph); 7.18-7.35 (m, 12H, CH$_{Ar}$). $^{13}$C NMR (75.0 MHz, CDCl$_3$): $\delta$ 56.2 (OCH$_3$); 66.8 (COOCH$_2$Ph); 75.9 (OCH$_2$Ph); 106.9 (C$_{Ar}$); 125.3 (C$_{Ar}$); 128.0 (C$_{Ar}$); 128.2 (C$_{Ar}$); 128.3 (C$_{Ar}$); 128.4 (C$_{Ar}$); 128.5 (C$_{Ar}$); 128.6 (C$_{Ar}$); 136.1 (C$_{Ar}$); 137.3 (C$_{Ar}$); 141.1 (C$_{Ar}$); 153.3 (C$_{Ar}$); 166.2 (C=O).

**General procedure for preparation of carboxylic acids (4b and 4c; Scheme 1).** Carboxylic acids were prepared according to the procedure described by Tranchimand et al (18). Briefly, potassium hydroxide (72.0 mmol, 5.0 equiv) was added to a solution of the corresponding benzoic ester 3b or 3c (14.4 mmol) in a mixture of distilled water (53 mL) and ethanol (210 mL). The mixture was refluxed for 2 hours and solvent evaporated under a vacuum using a rotary evaporator. The residue obtained was dissolved in distilled water (200 mL). The aqueous solution was extracted twice with diethyl ether (50 mL) and acidified with concentrated sulfuric acid until white solids were formed. The suspension was then extracted three times with ethyl acetate (200 mL). The combined extracts were washed with distilled water and dried on magnesium sulfate and finally concentrated using a rotary evaporator.

**Data for 3-methoxy-4-benzyloxybenzoic acid (4b; Scheme 1)** Aspect: white solids. Yield = 87%. The spectral results matched published data (21).

**Data for 3,5-dimethoxy-4-benzyloxybenzoic acid (4c; Scheme 1).** Aspect: white solids. Yield = 90%. $^1$H NMR (300.0 MHz, DMSO-d6): $\delta$: 3.84 (s, 6H, OCH$_3$); 5.00 (s, 2H, OCH$_2$Ph); 7.25 (s, 2H, CH$_{Ar}$); 7.27-7.47 (m, 5H, CH$_{Ar}$); 12.96 (s, 1H, COOH). $^{13}$C NMR (75.0 MHz, DMSO-d6): $\delta$ 56.4 (OCH$_3$); 74.4 (OCH$_2$Ph); 106.9 (C$_{Ar}$); 126.5 (C$_{Ar}$); 128.3 (C$_{Ar}$); 128.5 (C$_{Ar}$); 128.6 (C$_{Ar}$); 138.0 (C$_{Ar}$); 140.6 (C$_{Ar}$); 153.3 (C$_{Ar}$); 167.4 (C=O).

**Procedure for esters 8a to 8e syntheses.** DCC (3.06 mmol, 2.0 equiv) and DMAP (0.23 mmol, 0.15 equiv) were added under an argon blanket to an alcoholic solution of compound 7 (1.53 mmol) and the desired benzoic acid derivative (3.06 mmol, 2.0 equiv) in dry DCM (40 mL). The mixture was stirred for 15 hrs at room temperature and distilled water (50 mL) added. The organic layer was removed and washed once more with distilled water (20 mL), then dried over anhydrous magnesium sulfate and concentrated. Finally, the crude product was purified by flash column chromatography with silica gel and solvents used for elution are described for the particular compounds.
Data for (6'-benzoyloxy-2',5',7',8'-tetramethylchroman-2'-yl) methyl 4-benzyloxybenzoate (8a; Scheme 2). Eluent = DCM/hexanes (7:3 v/v). Aspect: highly viscous oil. Yield = 70%. 1H NMR (300.0 MHz, CDCl3): δ 1.40 (s, 3H, CH3); 1.81-2.10 (m, 2H, C=C-CH2-CH2); 2.11 (s, 3H, CH3); 2.18 (s, 3H, CH3); 2.22 (s, 3H, CH3); 2.67 (t, J = 6.9 Hz, 2H, C=C-CH2); 4.29 (d, J = 11.4 Hz, 1H, CH2OCO); 4.38 (d, J = 11.4 Hz, 1H, CH2OCO); 4.69 (s, 2H, OCH2Ph); 5.13 (s, 2H, OCH2Ph); 7.00 (d, J = 8.7 Hz, 1H, CH Ar); 7.28-7.53 (m, 10H, CH Ar); 8.00 (d, J = 8.7 Hz, 1H, CH Ar). 13C NMR (75.0 MHz, CDCl3): δ 11.9 (CH3); 12.0 (CH3); 12.9 (CH3); 20.3 (C=C-CH2); 22.4 (CH3); 28.8 (C=C-CH2-CH2); 68.9 (CH2OCO); 70.1 (OCH2Ph); 73.8 (OCH2OCO); 74.8 (OCH2Ph); 114.5 (C Ar); 127.5 (C Ar); 127.7 (C Ar); 127.8 (C Ar); 128.2 (C Ar); 128.3 (C Ar); 128.5 (C Ar); 128.7 (C Ar); 131.7 (C Ar); 136.2 (C Ar); 147.4 (C Ar); 148.6 (C Ar); 162.6 (C Ar); 166.1 (C=O). MS (m/z): calculated for C53H56O5 = 537.2636; found 537.2637 [M + H]+.

Data for (6'-benzoyloxy-2',5',7',8'-tetramethylchroman-2'-yl) methyl 3-methoxy-4-benzyl-oxybenzoate (8b; Scheme 2). Eluent = Ethyl acetate/hexanes (25:75 v/v). Aspect: highly viscous oil. Yield = 75%. 1H NMR (300.0 MHz, CDCl3): δ 1.39 (s, 3H, CH3); 1.81-2.09 (m, 2H, C=C-CH2-CH2); 2.11 (s, 3H, CH3); 2.17 (s, 3H, CH3); 2.22 (s, 3H, CH3); 2.67 (t, J = 6.9 Hz, 2H, C=C-CH2); 3.94 (s, 3H, OCH3); 4.26 (d, J = 11.4 Hz, 1H, CH2OCO); 4.39 (d, J = 11.4 Hz, 1H, CH2OCO); 4.69 (s, 2H, OCH2Ph); 5.23 (s, 2H, OCH2Ph); 6.90 (d, J = 8.10 Hz, 1H, CH Ar); 7.27-7.65 (m, 12H, CH Ar). 13C NMR (75.0 MHz, CDCl3): δ 11.9 (CH3); 12.0 (CH3); 12.9 (CH3); 20.3 (C=C-CH2); 22.4 (CH3); 28.8 (C=C-CH2-CH2); 56.1 (OCH3); 68.9 (CH2OCO); 70.8 (OCH2Ph); 73.8 (OCH2OCO); 74.8 (OCH2Ph); 112.5 (C Ar); 112.6 (C Ar); 117.4 (C Ar); 122.9 (C Ar); 123.2 (C Ar); 123.5 (C Ar); 126.1 (C Ar); 127.2 (C Ar); 127.7 (C Ar); 128.1 (C Ar); 128.3 (C Ar); 128.5 (C Ar); 128.7 (C Ar); 136.4 (C Ar); 147.4 (C Ar); 148.6 (C Ar); 149.2 (C Ar); 152.2 (C Ar); 166.2 (C=O). MS (m/z): calculated for C36H38O6 = 567.2741; found 567.2743 [M + H]+.

Data for (6'-benzoyloxy-2',5',7',8'-tetramethylchroman-2'-yl) methyl 3,5-dimethoxy-4-benzyl-oxybenzoate (8c; Scheme 2). Eluent = DCM/hexanes (4:1 v/v). Aspect: highly viscous oil. Yield = 71%. 1H NMR (300.0 MHz, CDCl3): δ 1.40 (s, 3H, CH3); 1.84-2.11 (m, 2H, C=C-CH2-CH2); 2.13 (s, 3H, CH3); 2.18 (s, 3H, CH3); 2.22 (s, 3H, CH3); 2.68 (t, J = 6.9 Hz, 2H, C=C-CH2); 3.87 (s, 6H, OCH3); 4.28 (d, J = 11.4 Hz, 1H, CH2OCO); 4.43 (d, J = 11.4 Hz, 1H, CH2OCO); 4.69 (s, 2H, OCH2Ph); 5.09 (s, 2H, OCH2Ph); 7.25-7.53 (m, 12H, CH Ar). 13C NMR (75.0 MHz, CDCl3): δ 11.8 (CH3); 12.0 (CH3); 12.9 (CH3); 20.2 (C=C-CH2); 22.3 (CH3); 28.9 (C=C-CH2-CH2); 56.2 (OCH3); 69.2 (CH2OCO); 73.8 (OCH2OCO); 74.8 (OCH2Ph); 75.0 (OCH2Ph); 106.9 (C Ar); 117.3 (C Ar); 123.1 (C Ar); 125.3 (C Ar); 126.1 (C Ar);
127.7 (C_{Ar}); 127.9 (C_{Ar}); 128.0 (C_{Ar}); 128.2 (C_{Ar}); 128.4 (C_{Ar}); 128.5 (C_{Ar}); 128.6 (C_{Ar}); 137.4 (C_{Ar}); 137.9 (C_{Ar}); 141.2 (C_{Ar}); 147.3 (C_{Ar}); 148.7 (C_{Ar}); 153.3 (C_{Ar}); 166.1 (C=O). MS (m/z): calculated for C_{37}H_{48}O_{7} = 597.2847; found 597.2831 [M + H]^+.

Data for (6'-benzylxy-2',5',7',8'-tetramethylchroman-2'-yl) methyl 3,4-di-benzylxybenzoate (8d; Scheme 2). Eluent = Ethyl acetate/hexanes (1:4 v/v). Aspect: highly viscous oil. Yield = 78%. $^1$H NMR (300.0 MHz, CDCl$_3$): δ 1.41 (s, 3H, CH$_3$); 1.83-2.13 (m, 2H, C=C-C$_2$-CH$_2$); 2.15 (s, 3H, CH$_3$); 2.22 (s, 3H, CH$_3$); 2.26 (s, 3H, CH$_3$); 2.70 (t, J = 6.9 Hz, 2H, C=C-CH$_2$); 4.30 (d, J = 11.4 Hz, 1H, CH$_2$OCO); 4.39 (d, J = 11.4 Hz, 1H, CH$_2$OCO); 4.74 (s, 2H, OCH$_2$Ph); 5.25 (s, 2H, OCH$_2$Ph); 5.27 (s, 2H, OCH$_2$Ph); 6.98 (d, J = 8.7 Hz, 1H, CH$_3$Ar); 7.29-7.70 (m, 17H, CH$_3$Ar). $^{13}$C NMR (75.0 MHz, CDCl$_3$): δ 11.9 (CH$_3$); 12.0 (CH$_3$); 12.9 (CH$_3$); 20.3 (C=C-C$_2$-CH$_2$); 22.4 (CH$_3$); 28.8 (C=C-CH$_2$-CH$_2$); 68.9 (CH$_2$OCO); 70.8 (OCH$_2$Ph); 71.2 (OCH$_2$Ph); 73.8 (OCCCH$_2$OCO); 74.8 (OCH$_2$Ph); 113.2 (C$_2$Ar); 115.5 (C$_7$Ar); 117.4 (C$_{Ar}$); 123.0 (C$_{Ar}$); 123.2 (C$_{Ar}$); 124.1 (C$_{Ar}$); 126.1 (C$_{Ar}$); 127.1 (C$_{Ar}$); 127.4 (C$_{Ar}$); 127.8 (C$_{Ar}$); 128.0 (C$_{Ar}$); 128.1 (C$_{Ar}$); 128.3 (C$_{Ar}$); 128.5 (C$_{Ar}$); 128.6 (C$_{Ar}$); 128.7 (C$_{Ar}$); 136.6 (C$_{Ar}$); 136.9 (C$_{Ar}$); 137.9 (C$_{Ar}$); 147.4 (C$_{Ar}$); 148.3 (C$_{Ar}$); 148.6 (C$_{Ar}$); 153.0 (C$_{Ar}$); 166.0 (C=O). MS (m/z): calculated for C$_{40}$H$_{48}$O$_{7}$ = 643.3054; found 643.3087 [M + H]$^+$.

Data for (6'-benzylxy-2',5',7',8'-tetramethylchroman-2'-yl) methyl 3,4-tri-benzylxybenzoate (8e; Scheme 2). Eluent = DCM/hexanes (4:1 v/v). Aspect: light yellow solids. Yield = 80%. $^1$H NMR (300.0 MHz, CDCl$_3$): δ 1.36 (s, 3H, CH$_3$); 1.77-2.06 (m, 2H, C=C-C$_2$-CH$_2$); 2.12 (s, 3H, CH$_3$); 2.19 (s, 3H, CH$_3$); 2.23 (s, 3H, CH$_3$); 2.66 (t, J = 6.6 Hz, 2H, C=C-CH$_2$); 4.26 (d, J = 11.4 Hz, 1H, CH$_2$OCO); 4.36 (d, J = 11.4 Hz, 1H, CH$_2$OCO); 4.70 (s, 2H, OCH$_2$Ph); 5.14 (s, 6H, OCH$_2$Ph); 7.22-7.55 (m, 22H, CH$_3$Ar). $^{13}$C NMR (75.0 MHz, CDCl$_3$): δ 11.9 (CH$_3$); 12.1 (CH$_3$); 12.9 (CH$_3$); 20.2 (C=C-C$_2$-CH$_2$); 22.4 (CH$_3$); 28.7 (C=C-C$_2$-CH$_2$); 69.1 (CH$_2$OCO); 71.2 (OCH$_2$Ph); 73.7 (OCCCH$_2$OCO); 74.8 (OCH$_2$Ph); 75.2 (OCH$_2$Ph); 109.1 (C$_{Ar}$); 117.3 (C$_{Ar}$); 123.1 (C$_{Ar}$); 125.1 (C$_{Ar}$); 126.1 (C$_{Ar}$); 127.5 (C$_{Ar}$); 127.6 (C$_{Ar}$); 127.8 (C$_{Ar}$); 127.9 (C$_{Ar}$); 128.0 (C$_{Ar}$); 128.1 (C$_{Ar}$); 128.2 (C$_{Ar}$); 128.4 (C$_{Ar}$); 128.5 (C$_{Ar}$); 128.6 (C$_{Ar}$); 136.7 (C$_{Ar}$); 137.4 (C$_{Ar}$); 137.9 (C$_{Ar}$); 142.5 (C$_{Ar}$); 147.3 (C$_{Ar}$); 148.6 (C$_{Ar}$); 152.5 (C$_{Ar}$); 165.9 (C=O). MS (m/z): calculated for C$_{40}$H$_{48}$O$_{7}$ = 749.3473; found 749.3485 [M + H]$^+$.

General procedure for antioxidants 1a to 1e preparation (Fig 1). Palladium on charcoal (10% w/w) was added to a solution of the desired benzylated compound, 0.7 mmol in 8 mL of dry THF. The mixture was stirred at room temperature under an atmosphere of hydrogen for 24 hrs, then filtrated on celite and concentrated on a rotary evaporator under reduced pressure. The residue was purified by flash column chromatography with silica gel and solvents used for elution are described for each individual component below.
Data for (6'-hydroxy-2',5',7',8'-tetramethylchroman-2'-yl) methyl 4-hydroxybenzoate (1a). Eluent = Ethyl acetate/hexanes (2:3 v/v). Aspect: white solids. Yield = 90%. Mp = 139-140 °C. 1H NMR (300.0 MHz, CDCl3): δ 1.42 (s, 3H, CH3); 1.80-2.09 (m, 2H, C=C-CH2-CH3); 2.10 (s, 3H, CH3); 2.11 (s, 3H, CH3); 2.15 (s, 3H, CH3); 2.67 (t, J = 6.6 Hz, 2H, C=C-CH2); 4.21 (s, 1H, OH); 4.27 (d, J = 11.4 Hz, 1H, CH2OCO); 4.34 (d, J = 11.4 Hz, 1H, CH2OCO); 5.40 (s, 1H, OH); 6.84 (d, J = 8.7 Hz, 1H, CHAr); 7.95 (d, J = 8.7 Hz, 1H, CHAr). 13C NMR (75.0 MHz, CDCl3): δ 11.3 (CH3); 11.8 (CH3); 12.2 (CH3); 20.3 (C=C-CH2); 22.2 (CH3); 28.9 (C=C-CH2-CH2); 68.9 (CH2OCO); 73.5 (OCCH2OCO); 115.3 (CAr); 117.1 (CAr); 118.6 (CAr); 121.3 (CAr); 122.5 (CAr); 122.8 (CAr); 132.0 (CAr); 145.0 (CAr); 160.0 (CAr); 166.3 (C=O). MS (m/z): calculated for C21H22O5 = 357.1697; found 357.1696 [M + H]+.

Data for (6'-hydroxy-2',5',7',8'-tetramethylchroman-2'-yl) methyl 3-methoxy-4-hydroxybenzoate (1b). Eluent = Ethyl acetate/hexanes (1:1 v/v). Aspect: white solids. Yield = 70%. Mp = 171-172 °C. 1H NMR (300.0 MHz, DMSO-d6): δ 1.03 (s, 3H, CH3); 1.43-1.75 (m, 2H, C=C-CH2-CH2); 1.75 (s, 3H, CH3); 1.78 (s, 3H, CH3); 1.82 (s, 3H, CH3); 2.25-2.38 (m, 2H, C=C-CH2); 3.57 (s, 3H, OCH3); 3.90 (d, J = 11.4 Hz, 1H, CH2OCO); 3.99 (d, J = 11.4 Hz, 1H, CH2OCO); 6.17 (s, 1H, OH); 6.58 (d, J = 8.1 Hz, 1H, CHAr); 7.20 (s, 1H, CHAr); 7.24 (d, J = 8.1 Hz, 2H, CHAr); 8.48 (s, 1H, OH). 13C NMR (75.0 MHz, DMSO-d6): δ 12.1 (CH3); 12.3 (CH3); 13.2 (CH3); 20.2 (C=C-CH2); 22.2 (CH3); 28.8 (C=C-CH2-CH2); 56.0 (OCH3); 68.5 (CH2OCO); 73.7 (OCCH2OCO); 112.9 (CAr); 115.6 (CAr); 117.2 (CAr); 120.8 (CAr); 120.9 (CAr); 121.6 (CAr); 123.2 (CAr); 123.9 (CAr); 144.4 (CAr); 146.0 (CAr); 147.9 (CAr); 152.1 (CAr); 165.8 (C=O). MS: calculated for C22H25O6 = 387.1802; found 387.1781 [M + H]+.

Data for (6'-hydroxy-2',5',7',8'-tetramethylchroman-2'-yl) methyl 3,5-dimethoxy-4-hydroxybenzoate (1c). Eluent = Ethyl acetate/hexanes (2:3 v/v). Aspect: white solids. Yield = 81%. Mp = 144-145 °C. 1H NMR (300.0 MHz, CDCl3): δ 1.38 (s, 3H, CH3); 1.79-2.06 (m, 2H, C=C-CH2-CH2); 2.11 (s, 3H, CH3); 2.12 (s, 3H, CH3); 2.15 (s, 3H, CH3); 2.69 (t, J = 6.9 Hz, 2H, C=C-CH2); 3.93 (s, 6H, OCH3); 4.22 (s, 1H, OH); 4.27 (d, J = 11.4 Hz, 1H, CH2OCO); 4.40 (d, J = 11.4 Hz, 1H, CH2OCO); 5.90 (s, 1H, OH); 7.33 (s, 2H, CHAr). 13C NMR (75.0 MHz, CDCl3): δ 12.1 (CH3); 12.3 (CH3); 13.2 (CH3); 20.2 (C=C-CH2); 22.2 (CH3); 28.9 (C=C-CH2-CH2); 56.5 (OCH3); 68.7 (CH2OCO); 73.7 (OCCH2OCO); 107.2 (CAr); 117.2 (CAr); 119.6 (CAr); 120.8 (CAr); 121.6 (CAr); 123.2 (CAr); 141.3 (CAr); 144.4 (CAr); 146.0 (CAr); 148.0 (CAr); 165.8 (C=O). MS: calculated for C25H28O7 = 417.1908; found 417.1902 [M + H]+.

Data for (6'-hydroxy-2',5',7',8'-tetramethylchroman-2'-yl) methyl 3,4-dihydroxybenzoate (1d). Eluent = Ethyl acetate/hexanes (1:1 v/v). Aspect: white solids. Yield = 76%. Mp = 182-
183 °C. $^1$H NMR (300.0 MHz, CDCl$_3$): δ 1.38 (s, 3H, CH$_3$); 1.86-2.08 (m, 2H, C=C-CH$_2$-CH$_2$); 2.09 (s, 3H, CH$_3$); 2.11 (s, 3H, CH$_3$); 2.14 (s, 3H, CH$_3$); 2.66 (t, $J = 6.6$ Hz, 2H, C=C-CH$_2$); 4.31 (s, 2H, CH$_2$OOC); 4.35 (s, 1H, OH); 6.31 (s, 1H, OH); 6.40 (s, 1H, OH); 6.89 (d, $J = 8.4$ Hz, 1H, CH$_3$Ar); 7.50 (s, 1H, CH$_3$Ar); 7.55 (d, $J = 8.4$ Hz, 1H, CH$_3$Ar). $^{13}$C NMR (75.0 MHz, CDCl$_3$): δ 11.3 (CH$_3$); 11.8 (CH$_3$); 12.2 (CH$_3$); 20.4 (C=C-CH$_2$); 39.1 (C=C-CH$_2$); 69.3 (CH$_2$OOC); 73.6 (OCCCH$_2$OOC); 114.9 (C$_{Ar}$); 116.5 (C$_{Ar}$); 117.4 (C$_{Ar}$); 118.6 (C$_{Ar}$); 121.4 (C$_{Ar}$); 122.2 (C$_{Ar}$); 122.9 (C$_{Ar}$); 123.9 (C$_{Ar}$); 143.0 (C$_{Ar}$); 144.9 (C$_{Ar}$); 145.3 (C$_{Ar}$); 148.9 (C$_{Ar}$); 166.4 (C=O). MS: calculated for C$_{21}$H$_{24}$O$_6$ = 373.1646; found 373.1663 [M + H]$^+$.

Data for (6'-hydroxy-2',5',7',8'-tetramethylchroman-2'-yl) methyl 3,4,5-trihydroxybenzoate (1e). Eluent = Ethyl acetate/hexanes (1:1 v/v). Aspect: white solids. Yield = 77%. Mp = 212-213 °C. $^1$H NMR (300.0 MHz, DMSO-d$_6$): δ 1.30 (s, 3H, CH$_3$); 1.73-2.00 (m, 2H, C=C-CH$_2$-CH$_2$); 2.00 (s, 3H, CH$_3$); 2.04 (s, 3H, CH$_3$); 2.05 (s, 3H, CH$_3$); 2.59 (t, $J = 6.9$ Hz, 2H, C=C-CH$_2$); 4.20 (s, 2H, CH$_2$OOC); 6.99 (s, 2H, CH$_2$Ar); 7.47 (s, 1H, OH); 9.10 (s, 3H, OH). $^{13}$C NMR (75.0 MHz, DMSO-d$_6$): δ 12.2 (CH$_3$); 12.3 (CH$_3$); 13.2 (CH$_3$); 20.2 (C=C-CH$_2$); 22.0 (CH$_3$); 28.7 (C=C-CH$_2$-CH$_2$); 68.3 (CH$_2$OOC); 73.7 (OCCCH$_2$OOC); 109.0 (C$_{Ar}$); 117.0 (C$_{Ar}$); 119.7 (C$_{Ar}$); 120.8 (C$_{Ar}$); 121.6 (C$_{Ar}$); 123.2 (C$_{Ar}$); 139.0 (C$_{Ar}$); 144.4 (C$_{Ar}$); 145.9 (C$_{Ar}$); 146.0 (C$_{Ar}$); 166.0 (C=O). MS: calculated for C$_{21}$H$_{24}$O$_7$ = 389.1595; found 389.1580 [M + H]$^+$.

**DPPH Radicals Scavenging Assay.** The DPPH assay was performed according to Nenadis and Tsimidou (22). Briefly, to 2960 µL of 0.1 mM ethanolic solution of DPPH 40 µL of synthesized antioxidant solution in ethanol was added at the following concentrations: 0.37, 0.74, 1.11, 1.85, 3.7, and 5.2 µM forming the ratios between the molar amounts of antioxidant to the molar amount of DPPH radicals at 0.05, 0.10, 0.15, 0.25, 0.5 and 0.7, respectively. The decrease of absorbance at 516 nm was measured at 25 °C after 20 min of reaction time. The blank solution contained the same amount of DPPH and 40 µL of ethanol. Each test was performed in triplicate. The results are expressed as the %DPPH inhibition calculated according to the following equation:

$$\% \text{DPPH} = \frac{(Ac - At)}{Ac} \times 100$$
Where Ac and At are the absorbances of the control sample and the test sample, respectively. All standard deviations for DPPH tests were below 3.0%. Both trolox and α-tocopherol were used as references. The IC₅₀ represents the concentration of antioxidant required to decrease the initial amount of DPPH by 50%.

**ORAC Assay.** ORAC assays were performed according to Szydlowska-Czerniak et al (23). Briefly, fluorescein disodium salt and AAPH solutions were prepared in 75 mM phosphate buffer (pH=7.4). The antioxidant solutions, 1 mM of each compound were dissolved in methanol and specific volume of it dissolved in the buffer to provide the required amount of antioxidant within a range of 3.125 - 25.00 µM. Four different concentrations were tested for each antioxidant. Solution of fluorescein, 3.0 mL (0.0816 µM) was mixed with 0.5 mL of antioxidant solution directly in a quartz cuvette. The mixture was kept at 37 °C for 10 min and 0.5 mL of the AAPH solution (153.0 µM) added. The fluorescence was measured at 37 °C for 30 min at 30 sec intervals. The emission and excitation were set at 525 nm and 485 nm, respectively. For a blank, phosphate buffer replaced the antioxidant solution. Each antioxidant solution was prepared in duplicate and three measurements were performed for each sample. A calibration curve was generated using trolox as the reference antioxidant.

The area under the fluorescence decay curve (AUC) was calculated as follows:

\[
AUC = \sum_{t=0.5}^{30} \frac{f_t + f_{t-0.5}}{2}
\]

Where \(f_t\) is the fluorescence at time \(t\) (min).

The net AUC corresponding to the sample was calculated using the following equation:

\[
AUC_{\text{net}} = AUC - AUC_{\text{blank}}
\]

For each antioxidant, a regression between \(AUC_{\text{net}}\) and the compound concentrations was calculated and the results were expressed as trolox equivalents (TE).

**Canola Oil Triacylglycerides Isolation.** Canola oil was stripped of its endogenous minor components including antioxidants via adsorption chromatography, following the procedure described by Lampi et al. (24).

**Accelerated Storage.** The ability of the new antioxidants to protect oil against oxidative degradation was determined using Schaal Oven test. To 1 g of pure canola triacylglycerols (CTG) 350 ppm of the tested antioxidant was added in a vial (National Scientific Target DP Vials; 2mL, 12 × 32 mm). The samples were stored in the dark for five days at 60 °C, providing the surface area to volume ratio at 0.78. Samples were examined at 24 h intervals for peroxide value and the residual amounts of antioxidant. The effectiveness of the new
compounds was compared with α-tocopherol as natural antioxidant and butylated hydroxytoluene (BHT) as a synthetic antioxidant. Experiments were set up in two repetitions for each tested antioxidant and samples from each repetition were analyzed in duplicate.

**Peroxide Value (PV).** A modified method originally published by Hornero-Mendeza et al. was used (25). Briefly, 200 mg of oil was dissolved in 5 mL of hexane; to 200 µL of the sample solution 5 mL of methanol/chloroform/HCL (1:1:0.012, v/v/v.), then 100 µL of NH₄SCN (30% w/w in water) and 100 µL of ferrous chloride (0.4% in water) were added. After 5 minutes incubation at room temperature the absorbance at 480 nm was measured.

**Test Frying.** The effectiveness of the developed antioxidants to protect CTG under frying conditions was assessed using a frying test system. CTG (12.0 g), fortified with 500 µg/g of the studied antioxidant, was weighed into a glass beaker (Pyrex, USA). Octagonal stir bar (ThermoFischer Scientific, USA) was placed into the vessel, evolving the final surface-to-volume ratio to 0.42. Then vessel was heated at 185 ± 5 °C for 10 minutes and 1.2 g of formulated starch (a mixture of gelatinized potato starch with glucose and silica gel, 4:1:1 w/w) was added. The heating was continued for another 20 minutes without mixing and then was stirred at 500 rpm. Heating and stirring were afterwards maintained for 90 minutes. About 0.5 g of oil sample was withdrawn at 60th, 80th, 100th and 120th minutes of heating. Selected sampling points reflect frying time based on the amount of polar components formed and correspond to 1, 3, 5 and 7 days of actual frying time using an institutional fryer (General Electric Company, NY, USA). Frying performance of oils was assessed by the measurement of total polar compounds (TPC) and the amount of retained tocopherol and added antioxidant. Samples from two repetitions of frying test were analyzed in duplicate for TPC and residual antioxidant.

**Total Polar Compounds.** The amounts of polar compounds were determined by gravimetric procedure following AOAC Method 982.27 with Schulte modification (27, 28).

**Residual Tocopherol and Novel Antioxidant.** Tocopherols and the new antioxidants were analyzed according to AOCS Official Method Ce 8–89 (29). Briefly, oil samples (50.0 mg) were weighed directly into autosampler vials and dissolved in hexanes (1.0 mL). For tocopherol, the mobile phase consisted of 7% methyl tert–butyl ether in hexanes with a flow rate of 0.6 mL/min and the fluorescence detector was set for excitation at 292 nm and emission at 325 nm. For the new antioxidants the mobile phase was changed to 65% methyl tert–butyl ether in hexane and the fluorescence detector emission set to 394 nm. For each run 10 µL of sample was injected.
**Statistical Analysis.** Data were analyzed by single factor analysis of variance (ANOVA) and regression using Minitab 2000 statistical software (Minitab Inc., PA, ver. 13.2). Significant differences between means were determined by Duncan’s multiple range tests. Statistically significant differences were determined at the P < 0.05 level.

**RESULTS AND DISCUSSION**

**Synthesis.** In order to produce the new antioxidants 1a-1e (Fig 1), benzoic acids 4a-4e have been prepared in two steps using commercially available derivatives of benzoic acids 2a-2d (Scheme 1). Benzoic derivatives, 2a-2d, have been benzylated using benzyl bromide and potassium carbonate in dimethylformamide (DMF) to produce 3a-3d esters with the following yields: 68% (3a), 89% (3b), 76% (3c) and 94% (3d). Saponification of 3a-3d esters with potassium carbonate in methanol/water solution has formed the desired benzoic acids derivatives with the yields of 87% (4a, 4b), 90% (4c), and 95% (4d). Gallic acid derivative 4e, has been synthesized from methyl gallate following the reaction described in Scheme 2 (18). Derivatives of trolox 1a-1e have been prepared in four steps as described in Scheme 3. Alcohol 7 has been obtained according to procedure described by Muller et al (19). A Steglich esterification between compound 7 and benzoic acid derivatives 4a-4e formed esters 8a-8e (30). These esters have been purified by flash chromatography, and were produced with the following yields: 70% (8a), 75% (8b), 71% (8c), and 80% (8d, 8e). Antioxidants 1a-1e has been obtained by hydrogenation over palladium catalyst compounds 8a-8e. Isolated antioxidants formed white solids and were produced with the following yields: 90% (1a), 70% (1b), 81% (1c), 76% (1d) and 77% (1e).

The structures of antioxidants 1a-1e have been confirmed by $^1$H NMR, $^{13}$C NMR, $^{31}$P NMR and MS. As an example, the $^1$H NMR spectrum of antioxidant 1b in Figure 2 is included. The $^1$H NMR spectrum is characterized by four methyl groups at 1.03 ppm, 1.75 ppm, 1.78 ppm and 1.82 ppm (s), by methylene groups at 1.43-1.75 ppm (m) and 2.25-2.38 ppm (m), and by a methoxy group at 3.57 ppm (s). The inequivalent H$_3$ protons next to the ester group are easily identified by two doublets at 3.90 ppm and 3.99 ppm. As for the aromatic protons, they are identified by two doublets at 6.58 ppm and 7.24 ppm and by a singlet at 7.20 ppm. The complete disappearance of the characteristic signals for the benzyl groups of ester 8b (two singlets at 4.69 ppm and 5.23 ppm as well as a multiplet at 7.27-7.65 ppm) clearly demonstrate that the removal of benzyl protection of the alcohol groups has been completed.

**Antioxidant Capacity Assays**
DPPH Assay. The radical scavenging properties of the synthesized antioxidants 1a to 1e, trolox (A1) and α-tocopherol (A2) have been evaluated by the DPPH assay. Results of this study in Table 1 are included. These results attest that the radical scavenging activities of the new compounds 1d (IC₅₀ = 0.82 µM) and 1e (IC₅₀ = 0.63 µM) were significantly higher than the commercially available trolox (IC₅₀ = 1.75 mM) and tocopherol (IC₅₀ = 1.61 mM). As for antioxidant 1a (IC₅₀ = 1.92 mM), its scavenging activity towards DPPH radicals was the lowest among those synthesized (Table 1). Antioxidants 1b (IC₅₀ = 1.69 mM) and 1c (IC₅₀ = 1.64 mM) provided similar scavenging activity as trolox and α-tocopherol. In the case of this test it was established that the number of hydroxyl groups on the aromatic ring define radical scavenging activity. When comparing activities for compounds 1a (2 OH), 1d (3 OH) and 1e (4 OH), where the only structural differences are in the number of hydroxyl groups on the ring, statistically significant differences have been observed in the DPPH radicals scavenging potency in the decreasing order: 1e>1d>1a. It is well established that phenolic compounds scavenged radicals by proton donation (10). Consequently, this paper results are consistent with this observation because higher number of hydroxyl group resulted in the higher capacity of proton donation. A dimeric antioxidant containing two hydroxyl groups in fused double chromanol rings had better DPPH radical scavenging activity, and provided double reducing power compared to α-tocopherol (31, 32). Furthermore, it is well established fact that configuration of hydroxyl group on ring in ortho position increases the radical scavenging activity (33). Our results also confirmed that the configuration of methoxy substituents to the hydroxyl group on the ring in ortho position increased the radical scavenging activity of the antioxidant (34). Indeed, 1b (1 methoxy group, IC₅₀ = 1.69 mM) and 1c (2 methoxy groups, IC₅₀ = 1.64 mM) were more efficient than 1a (IC₅₀ = 1.92 mM). Component 1c was slightly more effective in radical scavenging activity than 1b however the differences were not statistically significant. This trend can be related to the structure differences discussed above. Similar trends discussed above related to half life of DPPH radicals were observed for inhibition (Table 1).

ORAC Assay. The antioxidant capacities of the synthesized antioxidants using caffeic acid as reference have been assessed by the ORAC assay. Figure 3 depicts the decay curves for different concentrations (3.125-25.0 µM) of antioxidant 1b. A regression coefficient above 99% has been achieved for correlation between the AUC₅₀ and the antioxidant concentration for each compound tested (Results not included). Antioxidant capacities of each compound, expressed in trolox equivalent are given in Table 1. According to these results, all the synthesized phenolic compounds exhibited a higher antioxidant capacity than trolox. An
improved antioxidant activity was reported for a synthesized compound containing hydroxytyrosol attached to the chromanol ring which inhibited formation of malondialdehyde in rat liver microsomal membrane when oxidation was induced by AAPH (35). The data obtained for caffeic acid (4.46 TE) was similar to the one published by Gomez-Ruiz et al. (4.52; 36). Antioxidant 1b (5.10 TE) was the most effective radical scavenger among the studied compounds whereas 1e (2.26 TE) was the least active. It is worth mentioning that the ORAC assessment gives different results from the DPPH assay. As an example, 1e was the least efficient radical scavenger in the ORAC assay whereas it was the most effective scavenger for the DPPH radicals (Table 1). Contrary to the DPPH results, no trend has been observed in ORAC test. There is as well as lack of relation between the numbers of hydroxyl or methoxy groups on the chromanol ring. Each of the tests utilizes different stable free radicals and a different mechanism of scavenging is involved making data not comparable and not transferrable to the food system (37-39). Both DPPH and ORAC assays confirmed that the new antioxidants 1a-1e bestowed similar or higher radical scavenging activity as some commercially available antioxidants. Therefore, developed phenolic compounds are good candidates to improve food stability.

Accelerated storage stability (Schaal Oven Test). Since scavenging activity is not directly indicative of antioxidant effectiveness in food system we also tested developed compounds in real food applications (39). The ability of the phenolic derivatives 1a to 1e to protect CTG from oxidative degradation was assessed under Schaal Oven test (SOT) conditions. All synthesized antioxidants are well soluble in an oil and fat system. In order to determine the amount of antioxidant needed for an optimal protection of the oil, α-tocopherol, BHT and 1e have been added to CTG at two concentrations, 0.7 µM and 1.86 µM. Results of the SOT indicated that an antioxidant concentration of 0.7 µM, equivalent to 350 ppm, was sufficient (Fig 4). Indeed, when 1.86 µM of antioxidant was added, the PV’s were not significantly different from 0.7 µM (Fig 4). Results also demonstrated that new antioxidant 1e was significantly more effective that α-tocopherol at protecting CTG from oxidative degradation. The amounts of hydroperoxides formed at the end of the storage period were significantly lower for CTG fortified with 1e (PV = 9.4 meq/kg) than α-tocopherol (PV = 37.1 meq/kg) and activity was similar to BHT at the same concentration (Fig 4). In order to compare the efficiency of all novel antioxidants, 1a to 1e, each compound has been added to CTG at 0.7 µM and subjected to accelerated storage. All compounds discussed in this paper have very good solubility in canola oil. Results showed that all newly developed antioxidants (1a to 1e) significantly inhibited CTG oxidative degradation under accelerated storage.
condition compared to unprotected canola triacylglycerols (Fig 5). After 5 days of storage, PV of CTG was at 111 meq/kg whereas for triacylglycerides fortified with antioxidants 1a to 1e were between 9.4 and 44.4 (meq/kg) (Fig 5). PV values for oils containing compounds 1b, 1c and 1d were not statistically different. Hence, the effectiveness to protect CTG from oxidation by 1b, 1c and 1d was lower than for 1e. Among these compounds 1a was the least effective in canola triacylglycerides protection against oxidative degradation and less efficient than α-tocopherol. The phenolic compound 1e exhibits the highest antioxidant potency among the novel compounds in both accelerated oil storage and in scavenging the DPPH radicals. This observation demonstrates that DPPH scavenging activity test closely describes the behaviour of a particular compound in oil whereas the ORAC assay did not show any relation (Table 1 and Fig 5).

Protection during Frying. Although many compounds are known to display antioxidant activities at ambient temperature, the stringent conditions such as high temperature (~185°C) and prolonged exposure of oil to oxygen during frying tallying an additional demand on antioxidants. Consequently, antioxidant for institutional and industrial frying should not only be effective at frying conditions but thermally stable and retain low volatility to prevent their evaporation (40). As an example, BHT is not efficient under frying conditions due to it evaporative losses at elevated temperatures (41). The results from this study demonstrated that all developed antioxidants protected CTG from degradation during frying (Fig 6). At the end of the frying operation, the amount of total polar compounds accumulated in CTG was 25.7% while the amounts accumulated in triacylglycerides fortified with the new antioxidants ranged from 17.0% to 18.8%. Thus, antioxidants 1a to 1e were less efficient than BHT in the Schaal Oven test, the opposite occurred under frying conditions. At the initial stage of frying (70th minute; 1 day of actual frying), no significant difference was observed in the effectiveness of antioxidants 1a to 1e and α-tocopherol (Fig 6). However, as frying progressed, CTG fortified with antioxidants 1a to 1e has accumulated significantly lower amounts of polar compounds compared to CTG with α-tocopherol, indicating more efficient protection against oxidative degradation. Antioxidants 1a to 1e protected triacylglycerols at the same level whereas 1d and 1e were significantly more efficient. Hence, it seems that a higher number of hydroxyl groups on the chromanol aromatic rings tends to improve the ability of the antioxidant to prevent oxidation of the oil during frying. The presence of methoxy substituents in ortho position to the hydroxyl group did not affect the efficiency of the antioxidant.

Antioxidant Stability. In order to investigate the stability of 1a to 1e, the amounts of antioxidant remaining at different stages during storage and frying have been measured
(Figures 7 and 8). All novel antioxidants exhibited significantly higher stability than \( \alpha \)-tocopherol. Indeed, under accelerated storage 69.2\% (1a), 76.3\% (1b), 74.8\% (1c), 70.2\% (1d) and 72.5\% (1e) of added antioxidants have been observed at the end of storage time, compared to 35\% for CTG containing \( \alpha \)-tocopherol (Figure 7). \( \alpha \)-Tocopherol has been completely depleted at the 80\textsuperscript{th} minute of the frying test, whereas 27.0\% (1a), 37.5\% (1b), 49.1\% (1c), 18.1\% (1d) and 24.5\% (1e) were still remained (Figure 8). It is noteworthy that, in frying test, 1e exhibited a significantly higher stability than the other compounds. As a consequence, it is expected that significantly higher amounts of antioxidant will be carried over to fried foods when frying in oil containing one of the newly developed phenolic compounds. Hence, foods fried in such oils will possess higher storage stability than foods fried in oils containing \( \alpha \)-tocopherol at the same concentration level. Additionally, fried food containing higher amounts of antioxidants can offer better nutritional quality.

A convenient method has been developed for the synthesis of novel antioxidants. These phenolic components have been prepared in four steps from trolox and phenolic acids and shown to be produced in good yields. The radical scavenging activities of these compounds were different when assessed by DPPH and ORAC assays indicating lack of compatibility of these tests in assessing antioxidant activity. These studies have demonstrated that each prepared phenolic antioxidant offered a higher antioxidant activity than trolox and \( \alpha \)-tocopherol. Although a trend has not been observed between chemical structure and data from the ORAC assay, however results obtained with the DPPH assay can be explained by chemical structure of the components. Furthermore, the new antioxidants were better at protecting oil from oxidation during storage and frying when compared to standard antioxidants used today. The antioxidant 1e displayed superior protection of oil under accelerated storage conditions when compared to other novel antioxidants and \( \alpha \)-tocopherol. Results also indicated that novel antioxidants were significantly better in protecting frying oil when compared to \( \alpha \)-tocopherol. Antioxidants 1d and 1e were the most effective at frying temperatures. Phenolic compound 1e was the most efficient antioxidant under storage and frying conditions. The remarkably high thermal stability of novel antioxidants makes them very valuable under frying conditions and these compounds may also improve nutritional quality of fried foods.

Further investigations are underway to get a better understanding of the relationship between structure and antioxidant activity of these novel antioxidants. The nature of the
degradation products from the new antioxidants will also be studied to better understand what
degradation products may form.

**Abbreviations:** AAPH: 2,2'-azobis(2-amidinopropane) dihydrochloride; CTG: Canola
triacylglyceride; DCC: N,N'-dicyclohexylcarbodiimide; DCM: dichloromethane; DMAP: 4-
dimethylaminopyridine; DMF: dimethylformamide; DPPH: 2,2-Diphenyl-1-Picrylhydrazyl;
THF: tetrahydrofuran; TPC: total polar components; PV: peroxide value.
References


Figure and Scheme Captions

Figure 1. Structure of novel antioxidants 1a-1e

Figure 2. $^1$H NMR spectrum of antioxidant 1b

Figure 3. Fluorescence decay profiles induced by AAPH for new 1b antioxidant at different concentrations. All standard deviations for this assessment were below 3.0%

Figure 4. Changes in peroxide value during accelerated storage of canola triacylglycerols fortified with different amounts of α-tocopherol, BHT and the novel antioxidant 1e. CTG – canola triacylglycerides. For details see text.

Figure 5. Changes in peroxide formation during storage of canola triacylglycerols with different antioxidants added at 300 ppm. CTG – Canola triacylglycerols. For details see text

Figure 6. Polar components formation during canola triacylglycerides test frying with α-tocopherol and novel antioxidants 1a – 1e added at 500 ppm. For details see text.

Figure 7. Percentage of remaining antioxidant during accelerated storage of canola triacylglycerols fortified with α-tocopherol and novel antioxidants 1a-1e added at 300 ppm.

Figure 8: Percentage of remaining antioxidant during canola triacylglycerol test frying fortified with α-tocopherol and novel antioxidants at 500 ppm.

Scheme 1. Reagents and conditions: (i) BnBr, K$_2$CO$_3$, DMF; (ii) KOH, H$_2$O/MeOH, reflux

Scheme 2. Reagents and conditions: (i) BnBr, K$_2$CO$_3$, DMF; (ii) KOH, H$_2$O/MeOH, reflux

Scheme 3. Reagents and conditions: (i) BnBr, K$_2$CO$_3$, DMF; (ii) LiAlH$_4$, THF, 0 °C; (iii) 4a-4e, DCC, DMAP, DCM; (iv) H$_2$, Pd/C 10%, THF
Table 1. DPPH and ORAC tests results for novel antioxidants

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>DPPH Test</th>
<th>ORAC Test</th>
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<tbody>
<tr>
<td></td>
<td>IC₅₀ (µM)</td>
<td>Inhibition (%)</td>
</tr>
<tr>
<td>1a</td>
<td>1.92 ± 0.01</td>
<td>29.9 ± 1.2</td>
</tr>
<tr>
<td>1b</td>
<td>1.69 ± 0.06</td>
<td>32.2 ± 2.3</td>
</tr>
<tr>
<td>1c</td>
<td>1.64 ± 0.04</td>
<td>34.4 ± 1.8</td>
</tr>
<tr>
<td>1d</td>
<td>0.82 ± 0.01</td>
<td>65.0 ± 2.1</td>
</tr>
<tr>
<td>1e</td>
<td>0.63 ± 0.01</td>
<td>88.1 ± 2.2</td>
</tr>
<tr>
<td>Troloxᵇ</td>
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<tr>
<td>α-Tocopherolᵇ</td>
<td>1.61 ± 0.02</td>
<td>34.6 ± 1.7</td>
</tr>
<tr>
<td>Caffeic acidᵇ</td>
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ᵃCompounds 1a–1e, novel antioxidants, for their structure see Fig 1
ᵇReference antioxidants.
Figure 1. Structure of novel antioxidants 1a - 1e. For details see text.
Figure 2. $^1$H NMR spectrum of novel antioxidant 1b. For details see text.
Figure 3. Fluorescence decay profiles induced by AAPH for new 1b antioxidant at different concentrations.
Figure 4. Changes in peroxide value during accelerated storage of canola triacylglycerols fortified with different amounts of α-tocopherol, BHT and the novel antioxidant 1e. CTG – canola triacylglycerides. For details see text.
Figure 5. Changes in peroxide formation during storage of canola triacylglycerols with different antioxidants added at 300 ppm. CTG – Canola triacylglycerols. For details see text.
Figure 6. Polar components formation during canola triacylglycerides test frying with α-tocopherol and novel antioxidants 1a – 1e added at 500 ppm. For details see text.
Figure 7. Percentage of remaining antioxidant during accelerated storage of canola triacylglycerols fortified with α-tocopherol and novel antioxidants 1a-1e added at 300 ppm.
Figure 8: Percentage of remaining antioxidant during canola triacylglycerol test frying fortified with α-tocopherol and novel antioxidants at 500 ppm.
Scheme 1. Reactions, reagents and conditions for syntheses of benzoic acid derivatives: (i) BnBr, K₂CO₃, DMF; (ii) KOH, H₂O/MeOH, reflux
**Scheme 2.** Reactions, reagents and conditions for synthesis benzylated esters: (i) BnBr, K$_2$CO$_3$, DMF; (ii) KOH, H$_2$O/MeOH, reflux. For details see text.
**Scheme 3.** Reactions, reagents and conditions for syntheses of antioxidant 1 and 8: (i) BnBr, K₂CO₃, DMF; (ii) LiAlH₄, THF, 0 °C; (iii) 4a-4e, DCC, DMAP, DCM; (iv) H₂, Pd/C 10%, THF. For details see text.
Radical Scavenging Activity and Performance of Novel Phenolic Antioxidants in Oils during Storage and Frying

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Abstract  Novel phenolic antioxidants: 2a (6′-hydroxy-2′,5′,7′,8′-tetramethylchroman-2′-yl)methyl 3-methoxy-4-hydroxycinnamate, 2b (6′-hydroxy-2′,5′,7′,8′-tetramethylchroman-2′-yl)methyl 3,5-dimethoxy-4-hydroxycinnamate, 2c (6′-hydroxy-2′,5′,7′,8′-tetramethylchroman-2′-yl)methyl 3,4-dihydroxycinnamate, and 3 (6-hydroxy-2,5,7,8-tetramethylchroman-2-yl)methyl (6′-hydroxy-2′,5′,7′,8′-tetramethylchroman-2′-carboxylate) have been prepared in good yields and fully characterized by $^1$H and $^{13}$C NMR, and HRMS. Their radical scavenging activities have been evaluated by DPPH and ORAC assays. Each of the synthesized antioxidants exhibited significantly higher radical scavenging activities than trolox and α-tocopherol. These novel antioxidants efficiently protected canola oil triacylglycerides (CTG) during accelerated storage and frying. Compounds 2c and 3 were significantly more efficient than α-tocopherol protecting CTG under accelerated storage. All new antioxidants were more efficient than α-tocopherol under frying conditions and present significantly higher thermal stability.

Keywords: Phenolic antioxidants, synthesis, canola oil, frying, storage stability, trolox, cinnamic acid, caffeic acid
Introduction

Oxidation of polyunsaturated lipids is one of the main causes of food degradation [1]. Degraded lipids deteriorate its flavor and forms free radicals which may stimulate and/or initiate development of some health problems [1]. Free radicals are also involved in inflammatory and cardiovascular diseases, cancer and aging [2–5]. To prevent development of rancidity in foods, antioxidants have been utilized for years. Antioxidants are efficient scavengers of radicals, the later are formed during the initiation or the propagation stages of oxidative degradation [3].

Phenolic compounds including phenolic acids, tocopherol and flavonoids are widely distributed in the plants and are the efficient cell antioxidants [6]. Besides their high antioxidant activity, some phenolic derivatives present interesting biological properties. Caffeic acid and its analogues provide antiviral, anti-thrombosis, anti-hypertension, anti-fibrosis and antitumor properties [7]. Ferulic acid and its derivatives, the most abundant phenolic acid in plants, has been shown to possess antioxidant, antimicrobial, anti-inflammatory, anti-thrombosis and anti-cancer activities [8].

Synthetic antioxidants such as BHT, BHA and TBHQ have been used in some food applications; however their safety has been challenged due to possible negative effect on human health [9, 10]. Consequently, the synthesis of new antioxidants derived from natural ingredients, offering low toxicity, interesting biological properties as well as high antioxidant effectiveness are of current interest. In this context, our group recently took an interest in the preparation of new phenolic compounds presenting higher antioxidant efficiency than those of the common natural antioxidants [11]. We focused our work on the preparation of esters of common phenolic acids and components bearing a chromanol ring, the later is responsible for the antioxidant activity.

Phenolic antioxidants break the free radical chain and a stable phenoxyl radical is formed, stability of it is achieved by the delocalization of unpaired electrons on the aromatic ring [12]. It is well known that an electron donating groups such as hydroxyl and methoxy present in the ortho or para position to hydroxyl substituent on the aromatic ring increases the antioxidant effectiveness. Placing an electron acceptor substituent in the same position, such as an ester group, is lowering antioxidant potency of a compound [12]. Therefore, in order to improve the antioxidant capacities of synthesized phenolic compounds, we focused on the preparation of the new derivatives fulfilling discussed structural functions (Figure 1).
According to structure activity principles, the presence of an alkyl chain between the ester function and the aromatic ring should increase the radical scavenging activity of the compounds [7, 8]. The high activity of α-tocopherol is defined by the substituents present on the chromanol ring, we applied this consideration when preparing compound 3, where two chromanol rings have been joint by alkyl chain (Figure 1).

The synthesis and radical scavenging activities of the novel antioxidants: 2a, 2b, 2c, and 3 is reported. Furthermore, the ability of these compounds to protect CTG under accelerated storage and frying has been investigated.

Materials and Methods

Materials

Refined, bleached and deodorised regular canola oil was obtained from Richardson Oilseeds (Lethbridge, Canada). 2,2'-azobis (2-amidinopropane) hydrochloride (AAPH), N,N'-dicyclohexylcarbodiimide (DCC), dimethylaminopyridine (DMAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH), fluorescein disodium salt, trolox and phenolic acids were purchased from Sigma-Aldrich (St. Louis, MO). THF and dichloromethane (DCM) were purified using MBraun Solvent Purification System (M. Braun Incorporated, Stratham, NH). Dimethylformamide (DMF) and other solvents used in this work were of HPLC grade and were obtained from VWR (Edmonton, Canada).

Methods

Synthesis of Benzyl 3-methoxy-4-benzyloxyccinnamate (5a) and Benzyl 3,5-dimethoxy-4-benzyloxyccinnamate (5b) (Scheme 1)

Potassium carbonate (10.7 g, 77.2 mmol, 3.0 equiv) and benzyl bromide (9.2 mL, 77.2 mmol, 3.0 equiv) were added to a solution of the desired cinnamic acid derivative (25.7 mmol), the later dissolved in 100 mL of dry DMF, and kept under an argon blanket. The mixture was stirred for 15 h and then transferred into distilled water (150 mL). The compound of interest was extracted thrice with diethyl ether (100 mL). The combined extracts were washed with distilled water (100 mL), dried on magnesium sulfate, and concentrated under vacuum using a rotary evaporator.
Data of compounds 5a and 5b

**Benzyl 3-methoxy-4-benzyloxyccinnamate (5a):** The crude ester was purified by crystallization in hexanes. Aspect: White solids. Mp = 89 – 90°C. Yield = 86%. The spectral results were in agreement with data published by Leschot et al [13].

**Benzyl 3,5-dimethoxy-4-benzyloxyccinnamate (5b):** The crude oily product was purified by washing thrice with hot hexanes (100 mL). Aspect: Yellow oil. Yield = 92%.  

1H NMR (300.0 MHz, CDCl3): δ: 3.84 (s, 6H, OCH3); 5.05 (s, 2H, OCH2Ph); 5.25 (s, 2H, OCH2Ph); 6.40 (d, 3JHH = 15.9 Hz, 1H, CH=CH-CO); 6.74 (s, 2H, CHAr); 7.26-7.49 (m, 10H, CHAr); 7.63 (d, 3JHH = 15.9 Hz, 1H, CH=CH-CO). 13C NMR (300.0 MHz, CDCl3): δ: 56.1 (OCH3); 66.4 (OCH2Ph); 75.1 (OCH2Ph); 105.3 (CAr); 117.1 (CH=CH-CO); 128.0 (CAr); 128.2 (CAr); 128.4 (CAr); 128.5 (CAr); 128.7 (CAr); 130.0 (CAr); 136.1 (CAr); 137.6 (CAr); 139.0 (CAr); 145.3 (CH=CH-CO); 153.8 (CAr); 166.8 (C=O). HRMS (m/z): calculated for C25H24O5 = 405.1697; found 405.1692 [M + H]+.

Synthesis of Benzyl 3,4-dibenzyloxyccinnamate 5c (Scheme 1)

Potassium carbonate (15.3 g, 111.0 mmol, 4.0 equiv) and benzyl bromide (13.2 mL, 111.0 mmol, 4.0 equiv) were added to a solution of caffeic acid (5.0 g, 27.7 mmol) dissolved in 100 mL of dry DMF. The mixture was stirred for 15 h under an argon blanket and then transferred into distilled water (150 mL). The compound of interest was extracted thrice with diethyl ether (100 mL). The combined extracts were washed with distilled water (100 mL), dried on magnesium sulfate, and concentrated under vacuum using a rotary evaporator. The crude product was purified by crystallization in hexanes. The desired benzoic ester was isolated as white solids with 91% yield.

**Data for Benzyl 3,4-dibenzyloxyccinnamate (5c):** Mp = 80-81 °C. 1H NMR (300.0 MHz, CDCl3): δ: 5.19 (s, 2H, OCH2Ph); 5.22 (s, 2H, OCH2Ph); 5.26 (s, 2H, OCH2Ph); 6.31 (d, 3JHH = 15.9 Hz, 1H, CH=CH-CO); 6.93 (d, 3JHH = 8.4 Hz, 1H, CHAr); 7.09 (d, 3JHH = 8.4 Hz, 1H, CHAr); 7.14 (s, 1H, CHAr); 7.29-7.50 (m, 15H, CHAr); 7.63 (d, 3JHH = 15.9 Hz, 1H, CH=CH-CO). HRMS (m/z): calculated for C30H26O4 = 451.1909; found 451.22 [M + H]+.

Synthesis of 3-methoxy-4-benzyloxyccinnamic acid (6a), 3,5-dimethoxy-4-benzyloxyccinnamic acid (6b), and 3,4-dibenzyloxyccinnamic acid (6c) (Scheme 1)
Potassium hydroxide (4.0 g, 72.0 mmol, 5.0 equiv) was added to a solution of the corresponding benzoic ester (14.4 mmol) in a mixture of distilled water (53 mL) and ethanol (210 mL). The mixture was refluxed for 2 h and the solvent evaporated under a vacuum using a rotary evaporator. The residue was dissolved in distilled water (200 mL) and the aqueous solution was washed twice with diethyl ether (50 mL) and acidified with concentrated sulfuric acid until white solids were formed. The suspension was then extracted thrice with ethyl acetate (200 mL). The combined extracts were washed with distilled water, dried on magnesium sulfate, and finally concentrated under vacuum using a rotary evaporator.

Data for compound 6a – 6c

3-Methoxy-4-benzyloxyccinnamic acid (6a): Aspect: White solids. Mp = 193 - 194 °C. Yield = 86%. The spectral results matched the published results by Muller et al [14].

3,5-Dimethoxy-4-benzyloxyccinnamic acid (6b): Aspect: White solids. Mp = 114-115 °C. Yield = 98%. \(^1\)H NMR (300.0 MHz, DMSO-d6): \(\delta\) = 3.83 (s, 6H, OCH\(_3\)); 4.95 (s, 2H, OCH\(_2\)Ph); 6.66 (d, \(^3\)J\(HH\) = 15.9 Hz, 1H, CH=CH-CO); 7.06 (s, 2H, CH\(_Ar\)); 7.31-7.48 (m, 5H, CH\(_Ar\)); 7.55 (d, \(^3\)J\(HH\) = 15.9 Hz, 1H, CH=CH-CO); 12.32 (s, 1H, COOH). \(^13\)C NMR (300.0 MHz, DMSO-d6): \(\delta\) = 56.4 (OCH\(_3\)); 74.5 (OCH\(_2\)Ph); 106.1 (C\(_Ar\)); 119.0 (CH=CH-CO); 128.2 (C\(_Ar\)); 128.4 (C\(_Ar\)); 128.5 (C\(_Ar\)); 130.5 (C\(_Ar\)); 138.2 (C\(_Ar\)); 138.6 (C\(_Ar\)); 144.7 (CH=CH-CO); 153.7 (C\(_Ar\)); 168.3 (C=O). HRMS (m/z): calculated for C\(_{18}\)H\(_{18}\)O\(_5\) = 315.1232; found 315.1256 [M + H]\(^+\).

3,4-Dibenzyloxyccinnamic acid (6c): Aspect: White solids. Mp = 202-203 °C. Yield = 90%. The spectral results matched one reported by Percec et al [15].

Compounds 7 and 8 were synthesized according to a procedure described by Muller et al [14].

Synthesis of (6'-benzloxy-2',5',7',8'-tetramethylchroman-2'-yl) methyl 3-methoxy-4-benzyloxyccinnamate (9a), (6'-benzloxy-2',5',7',8'-tetramethylchroman-2'-yl) methyl 3, 5-dimethoxy-4-benzyloxyccinnamate (9b), and (6'-benzloxy-2',5',7',8'-tetramethylchroman-2'-yl) methyl 3, 4-dibenzyloxyccinnamate (9c) (Scheme 2)

DCC (632 mg, 3.06 mmol, 2.0 equiv) and DMAP (19.5 mg, 0.23 mmol, 0.15 equiv) were added under an argon blanket to an alcoholic solution of component 8 (500 mg, 1.53 mmol)
and the desired cinnamic acid derivative (3.06 mmol, 2.0 equiv) in dry DCM (40 mL). The mixture was stirred for 15 h at room temperature, and then distilled water (50 mL) was added. The organic layer was removed and washed once with distilled water (20 mL), then dried over anhydrous magnesium sulfate, and concentrated. Finally, the crude product was purified by a flash chromatography using silica gel and DCM as eluant.

Data for compound 9a – 9c

(6′-benzyloxy-2′,5′,7′,8′-tetramethylchroman-2′-yl) methyl 3-methoxy-4-benzyloxyccinnamate (9a): Aspect: White solids. Yield = 71%. $^1$H NMR (300.0 MHz, CDCl$_3$): $\delta$: 1.36 (s, 3H, CH$_3$); 1.78-2.11 (m, 2H, C=C-CH$_2$-CH$_2$); 2.11 (s, 3H, CH$_3$); 2.18 (s, 3H, CH$_3$); 2.22 (s, 3H, CH$_3$); 2.66 (t, $^3$J$_{HH}$ = 6.9 Hz, 2H, C=C-CH$_2$); 3.93 (s, 3H, OCH$_3$); 4.22 (d, $^2$J$_{HH}$ = 11.4 Hz, 1H, CH$_2$OCO); 4.29 (d, $^2$J$_{HH}$ = 11.4 Hz, 1H, CH$_2$OCO); 4.69 (s, 2H, OCH$_2$Ph); 5.19 (s, 2H, OCH$_2$Ph); 6.33 (d, $^3$J$_{HH}$ = 15.9 Hz, 1H, CH=CH$_2$CO); 6.89 (d, $^3$J$_{HH}$ = 8.4 Hz, 1H, CH$_Ar$); 7.01-7.09 (m, 2H, CH$_Ar$); 7.26-7.52 (m, 10H, CH$_Ar$); 7.62 (d, $^3$J$_{HH}$ = 15.9 Hz, 1H, CH=CH$_2$CO).

$^{13}$C NMR (75.0 MHz, CDCl$_3$): $\delta$: 11.9 (CH$_3$); 12.0 (CH$_3$); 12.9 (CH$_3$); 20.2 (C=C-CH$_2$-CH$_2$); 22.2 (CH$_3$); 28.6 (C=C-CH$_2$-CH$_2$); 56.0 (OCH$_3$); 68.7 (CH$_2$OCO); 70.9 (OCH$_2$Ph); 73.8 (OCH$_2$Ph); 74.8 (OCH$_2$Ph); 110.2 (C$_Ar$); 113.4 (C$_Ar$); 115.7 (CH=CH$_2$CO); 117.3 (C$_Ar$); 122.5 (C$_Ar$); 123.2 (C$_Ar$); 126.1 (C$_Ar$); 127.7 (C$_Ar$); 127.8 (C$_Ar$); 127.9 (C$_Ar$); 128.0 (C$_Ar$); 128.3 (C$_Ar$); 128.5 (C$_Ar$); 136.6 (C$_Ar$); 145.0 (CH=CH$_2$CO); 147.4 (C$_Ar$); 148.6 (C$_Ar$); 149.8 (C$_Ar$); 150.3 (C$_Ar$); 167.1 (C=O). HRMS (m/z): calculated for C$_{38}$H$_{40}$O$_6$ = 593.2898; found 593.2886 [M + H]$^+$. 

(6′-benzyloxy-2′,5′,7′,8′-tetramethylchroman-2′-yl) methyl 3, 5-dimethoxy-4- benzyloxyccinnamate (9b): Aspect: Highly viscous oil. Yield = 80%. $^1$H NMR (300.0 MHz, CDCl$_3$): $\delta$: 1.39 (s, 3H, CH$_3$); 1.81-2.14 (m, 2H, C=C-CH$_2$-CH$_2$); 2.14 (s, 3H, CH$_3$); 2.21 (s, 3H, CH$_3$); 2.25 (s, 3H, CH$_3$); 2.70 (t, $^3$J$_{HH}$ = 6.9 Hz, 2H, C=C-CH$_2$); 3.88 (s, 6H, OCH$_3$); 4.27 (d, $^2$J$_{HH}$ = 11.4 Hz, 1H, CH$_2$OCO); 4.33 (d, $^2$J$_{HH}$ = 11.4 Hz, 1H, CH$_2$OCO); 4.72 (s, 2H, OCH$_2$Ph); 5.08 (s, 2H, OCH$_2$Ph); 6.40 (d, $^3$J$_{HH}$ = 15.9 Hz, 1H, CH=CH$_2$CO); 6.77 (s, 1H, CH$_Ar$); 7.28-7.55 (m, 10H, CH$_Ar$); 7.65 (d, $^3$J$_{HH}$ = 15.9 Hz, 1H, CH=CH$_2$CO). $^{13}$C NMR (75.0 MHz, CDCl$_3$): $\delta$: 11.9 (CH$_3$); 12.0 (CH$_3$); 12.9 (CH$_3$); 20.2 (C=C-CH$_2$-CH$_2$); 22.2 (CH$_3$); 28.6 (C=C-CH$_2$-CH$_2$); 56.2 (OCH$_3$); 68.9 (CH$_2$OCO); 73.8 (OCH$_2$Ph); 74.8 (OCH$_2$Ph); 105.3 (C$_Ar$); 117.0 (CH=CH$_2$CO); 117.3 (C$_Ar$); 123.2 (C$_Ar$); 126.1 (C$_Ar$); 127.7 (C$_Ar$); 127.9 (C$_Ar$); 128.0 (C$_Ar$); 128.2 (C$_Ar$); 128.3 (C$_Ar$); 128.4 (C$_Ar$); 128.5 (C$_Ar$); 128.6 (C$_Ar$);
130.0 (C$_{Ar}$); 137.5 (C$_{Ar}$); 137.9 (C$_{Ar}$); 139.1 (CH=CH-CO); 147.4 (C$_{Ar}$); 148.6 (C$_{Ar}$); 153.8 (C$_{Ar}$); 166.8 (C=O). HRMS (m/z): calculated for C$_{39}$H$_{42}$O$_{7}$ = 623.3003; found 623.2977 [M + H]$^+$. 

(6'-benzylxy-2',5',7',8'-tetramethylchroman-2'-yl) methyl 3, 4-dibenzyloxyccinnamate (9c): Aspect: Highly viscous oil. Yield = 88%. $^1$H NMR (300.0 MHz, CDCl$_3$): δ: 1.35 (s, 3H, CH$_3$); 1.79-2.10 (m, 2H, C=C-CH$_2$-CH$_2$); 2.11 (s, 3H, CH$_3$); 2.17 (s, 3H, CH$_3$); 2.22 (s, 3H, CH$_3$); 2.65 (t, $^3$J$_{HH}$ = 6.9 Hz, 2H, C=C-CH$_2$); 3.88 (s, 6H, OCH$_3$); 4.20 (d, $^2$J$_{HH}$ = 11.1 Hz, 1H, CH$_2$OCO); 4.28 (d, $^2$J$_{HH}$ = 11.1 Hz, 1H, CH$_2$OCO); 4.42 (d, $^2$J$_{HH}$ = 11.1 Hz, 1H, CH$_2$OCO); 7.28-7.51 (m, 10H, CH$_Ar$); 7.57 (d, $^3$J$_{HH}$ = 15.9 Hz, 1H, C=CH=CH$_1$CO). $^{13}$C NMR (75.0 MHz, CDCl$_3$): δ: 11.9 (CH$_3$); 12.0 (CH$_3$); 12.9 (CH$_3$); 20.2 (C=CH-CH$_2$); 22.2 (CH$_3$); 28.6 (C=CH-CH$_2$-CH$_2$); 68.7 (CH$_2$OCO); 71.0 (OCH$_2$Ph); 71.4 (OCH$_2$Ph); 73.8 (OC-CH$_2$OCO); 74.8 (OCH$_2$Ph); 113.8 (C$_{Ar}$); 114.3 (C$_{Ar}$); 115.8 (CH=CH-CO); 117.3 (C$_{Ar}$); 123.0 (C$_{Ar}$); 123.2 (C$_{Ar}$); 127.2 (C$_{Ar}$); 127.3 (C$_{Ar}$); 127.7 (C$_{Ar}$); 127.8 (C$_{Ar}$); 127.9 (C$_{Ar}$); 128.0 (C$_{Ar}$); 128.3 (C$_{Ar}$); 128.5 (C$_{Ar}$); 128.6 (C$_{Ar}$); 136.2 (C$_{Ar}$); 136.3 (C$_{Ar}$); 136.7 (C$_{Ar}$); 136.9 (C$_{Ar}$); 137.9 (C$_{Ar}$); 144.9 (CH=CH-CO); 147.4 (C$_{Ar}$); 148.6 (C$_{Ar}$); 149.0 (C$_{Ar}$); 151.1 (C$_{Ar}$); 153.2 (C$_{Ar}$); 167.0 (C=O). HRMS (m/z): calculated for C$_{44}$H$_{46}$O$_{6}$, 669.3211= found 669.3204 [M + H]$^+$. 

Synthesis of antioxidants (6'-hydroxy-2',5',7',8'-tetramethylchroman-2'-yl) methyl 3-methoxy-4-hydroxycinnamate (2a), (6'-hydroxy-2',5',7',8'-tetramethylchroman-2'-yl) methyl 3, 5-dimethoxy-4-hydroxycinnamate (2b), and (6'-hydroxy-2',5',7',8'-tetramethylchroman-2'-yl) methyl 3, 4-dihydroxycinnamate (2c) (Scheme 2) 

Palladium on charcoal (10% wt) was added to a solution of the desired benzylated compound (1.0 mmol in 10 mL of dry THF) then the mixture was stirred at room temperature when purged with hydrogen for 24 h. Reactants were filtrated on Celite, and concentrated on a rotary evaporator under reduced pressure. The residue was purified by flash chromatography with silica gel, and the solvents used for elution are described for each individual component below.

Data for antioxidants 2a - 2c
(6'-hydroxy-2',5',7',8'-tetramethylchroman-2'-yl) methyl 3-methoxy-4-hydroxycinnamate (2a): Eluant: ethyl acetate/hexanes (3:5:6.5 v/v). Aspect: Highly viscous oil. Yield = 78%. \(^1\)H NMR (300.0 MHz, CDCl\(_3\)): \(\delta\): 1.24 (s, 3H, CH\(_3\)); 1.65-1.95 (m, 2H, CH\(_2\)CH\(_2\)CH\(_3\)); 2.08 (s, 3H, CH\(_3\)); 2.10 (s, 3H, CH\(_3\)); 2.15 (s, 3H, CH\(_3\)); 2.53-2.67 (m, 4H, CH\(_2\)CH\(_2\)COO and CH\(_2\)CH\(_2\)COO); 2.89 (t, \(^3\)J\(_{HH}\) = 7.5 Hz, 2H, CH\(_2\)CH\(_2\)CH\(_3\)); 3.86 (s, 3H, OCH\(_3\)); 4.07 (d, \(^2\)J\(_{HH}\) = 11.1 Hz, 1H, CH\(_2\)O); 4.14 (d, \(^2\)J\(_{HH}\) = 11.1 Hz, 1H, CH\(_2\)O); 4.25 (s, 1H, OH); 5.49 (s, 1H, OH); 6.62-6.71 (m, 2H, CH\(_3\)); 6.82 (d, \(^3\)J\(_{HH}\) = 7.8 Hz, 2H, CH\(_2\)O). \(^13\)C NMR (75.0 MHz, CDCl\(_3\)): \(\delta\): 11.3 (CH\(_3\)); 11.8 (CH\(_3\)); 12.2 (CH\(_3\)); 20.2 (CH\(_2\)CH\(_2\)CH\(_3\)); 21.8 (CH\(_3\)); 28.6 (CH\(_2\)CH\(_2\)CH\(_3\)); 30.7 (s, CH\(_3\)); 36.2 (s, CH\(_3\)); 55.9 (OCH\(_3\)); 68.7 (CH\(_2\)O); 73.3 (OCH\(_2\)O); 110.9 (C\(_{Ar}\)); 114.3 (C\(_{Ar}\)); 117.0 (C\(_{Ar}\)); 118.5 (C\(_{Ar}\)); 120.8 (C\(_{Ar}\)); 121.3 (C\(_{Ar}\)); 122.7 (C\(_{Ar}\)); 132.3 (C\(_{Ar}\)); 144.1 (C\(_{Ar}\)); 144.9 (C\(_{Ar}\)); 145.0 (C\(_{Ar}\)); 146.4 (C\(_{Ar}\)); 172.8 (C=O). HRMS (m/z): calculated for C\(_{24}\)H\(_{30}\)O\(_6\) = 415.2115; found 415.2117 [M + H]\(^+\).

(6'-hydroxy-2',5',7',8'-tetramethylchroman-2'-yl) methyl 3, 5-dimethoxy-4-hydroxycinnamate (2b): Eluant: ethyl acetate/hexanes (2:3 v/v). Aspect: Highly viscous oil. Yield = 83%. \(^1\)H NMR (300.0 MHz, CDCl\(_3\)): \(\delta\): 1.29 (s, 3H, CH\(_3\)); 1.69-1.92 (m, 2H, CH\(_2\)CH\(_2\)CH\(_3\)); 2.08 (s, 3H, CH\(_3\)); 2.11 (s, 3H, CH\(_3\)); 2.15 (s, 3H, CH\(_3\)); 2.58-2.68 (m, 4H, CH\(_2\)CH\(_2\)COO and CH\(_2\)CH\(_2\)COO); 2.89 (t, \(^3\)J\(_{HH}\) = 7.5 Hz, 2H, CH\(_2\)CH\(_2\)CH\(_3\)); 3.86 (s, 6H, OCH\(_3\)); 4.07 (d, \(^2\)J\(_{HH}\) = 11.4 Hz, 1H, CH\(_2\)O); 4.15 (d, \(^2\)J\(_{HH}\) = 11.4 Hz, 1H, CH\(_2\)O); 4.24 (s, 1H, OH); 5.39 (s, 1H, OH); 6.42 (s, 2H, CH\(_2\)O); 13\(^\text{C}\) NMR (75.0 MHz, CDCl\(_3\)): \(\delta\): 11.3 (CH\(_3\)); 11.8 (CH\(_3\)); 12.2 (CH\(_3\)); 20.2 (CH\(_2\)CH\(_2\)CH\(_3\)); 21.8 (CH\(_3\)); 28.6 (CH\(_2\)CH\(_2\)CH\(_3\)); 31.2 (s, CH\(_2\)); 36.3 (s, CH\(_2\)); 56.2 (OCH\(_3\)); 68.7 (CH\(_2\)O); 73.3 (OCH\(_2\)O); 104.8 (C\(_{Ar}\)); 117.0 (C\(_{Ar}\)); 118.5 (C\(_{Ar}\)); 121.3 (C\(_{Ar}\)); 122.7 (C\(_{Ar}\)); 131.5 (C\(_{Ar}\)); 133.1 (C\(_{Ar}\)); 144.9 (C\(_{Ar}\)); 145.0 (C\(_{Ar}\)); 147.0 (C\(_{Ar}\)); 172.8 (C=O). HRMS (m/z): calculated for C\(_{25}\)H\(_{32}\)O\(_7\) = 445.2221; found 445.2208 [M + H]\(^+\).

(6'-hydroxy-2',5',7',8'-tetramethylchroman-2'-yl) methyl 3, 4-dihydroxycinnamate (2c): Eluant: ethyl acetate/hexanes (2:3 v/v). Aspect: Highly viscous oil. Yield = 80%. \(^1\)H NMR (300.0 MHz, CDCl\(_3\)): \(\delta\): 1.23 (s, 3H, CH\(_3\)); 1.64-1.91 (m, 2H, CH\(_2\)CH\(_2\)CH\(_3\)); 2.08 (s, 3H, CH\(_3\)); 2.09 (s, 3H, CH\(_3\)); 2.15 (s, 3H, CH\(_3\)); 2.55-2.63 (m, 4H, CH\(_2\)CH\(_2\)COO and CH\(_2\)CH\(_2\)COO); 2.82 (t, \(^3\)J\(_{HH}\) = 7.5 Hz, 2H, CH\(_2\)CH\(_2\)CH\(_3\)); 4.04 (d, \(^2\)J\(_{HH}\) = 11.4 Hz, 1H, CH\(_2\)O); 4.16 (d, \(^2\)J\(_{HH}\) = 11.4 Hz, 1H, CH\(_2\)O); 4.25 (s, 1H, OH); 5.11 (s, 1H, OH); 5.17 (s, 1H, OH); 6.60 (d, \(^3\)J\(_{HH}\) = 8.1 Hz, 1H, CH\(_2\)O); 6.73 (d, \(^3\)J\(_{HH}\) = 8.1 Hz, 1H, CH\(_2\)O). \(^13\)C NMR (75.0 MHz, CDCl\(_3\)): \(\delta\): 11.3 (CH\(_3\)); 11.8 (CH\(_3\)); 12.2 (CH\(_3\)); 20.2 (CH\(_2\)CH\(_2\)CH\(_3\)); 21.9 (CH\(_3\)); 28.6 (CH\(_2\)CH\(_2\)CH\(_3\)); 30.3 (s, CH\(_2\)); 36.1 (s, CH\(_2\)); 68.8
(CH₂OCO); 73.3 (OCCH₂OCO); 115.3 (C₆H₅); 115.4 (C₆H₅); 117.1 (C₆H₅); 118.7 (C₆H₅); 120.6 (C₆H₅); 121.4 (C₆H₅); 122.8 (C₆H₅); 133.2 (C₆H₅); 142.0 (C₆H₅); 143.4 (C₆H₅); 144.9 (C₆H₅); 145.0 (C₆H₅); 173.1 (C=O). HRMS (m/z): calculated for C₂₃H₂₈O₆ = 401.1959; found 401.1939 [M + H]^+.

Synthesis of 6-Benzyloxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (10) (Scheme 3)

Carboxylic acid 10 was prepared similarly to compounds 6a, 6b, and 6c. Benzoic ester 7 (3.0 g, 6.97 mmol) and potassium hydroxide (1.96 g, 34.8 5 mmol, 5.0 equiv) produced carboxylic acid 10 (2.13 g, 6.26 mmol).

Data for 6-Benzyloxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (10): Aspect: White solids. Mp = 151-153 °C. Yield = 90%. 1H NMR (300.0 MHz, CDCl₃): δ: 1.64 (s, 3H, CH₃); 1.88-2.02 (m, 1H, C=C-CH₂-C₆H₅); 2.15 (s, 3H, CH₃); 2.17 (s, 3H, CH₃); 2.23 (s, 3H, CH₃); 2.35-2.44 (m, 1H, C=C-CH₂-C₆H₅); 2.52-2.74 (m, 1H, C=C-CH₂-C₆H₅); 4.69 (s, 2H, CH₂OPh); 7.28-7.53 (m, 5H, C₆H₅). 13C NMR (75.0 MHz, CDCl₃): δ: 11.9 (CH₃); 12.1 (CH₃); 12.9 (CH₃); 20.7 (C=C-CH₂); 25.2 (CH₃); 30.1 (C=C-CH₂-CH₂); 74.7 (OCH₂Ph); 117.2 (C₆H₅); 123.0 (C₆H₅); 126.2 (C₆H₅); 127.8 (C₆H₅); 127.9 (C₆H₅); 128.5 (2* C₆H₅); 137.9 (C₆H₅); 147.4 (C₆H₅); 149.1 (C₆H₅); 179.5 (C=O). HRMS (m/z): calculated for C₂₁H₂₆O₄ = 341.1747; found 341.1737 [M + H]^+.

Synthesis of (6-benzyloxy-2,5,7,8-tetramethylchroman-2-yl) methyl (6’-benzyloxy-2’,5’,7’,8’-tetramethylchroman-2’-carboxylate) (11) (Scheme 3)

Ester 11 was prepared similarly to compounds 9a, 9b, and 9c. Alcohol 8 (0.50 g, 1.53 mmol) was mixed with carboxylic acid 10 (1.04 g, 3.06 mmol, 2.0 equiv), DCC (0.63 g, 3.06 mmol, 2.0 equiv), and DMAP (29.0 mg, 0.23 mmol, 0.15 equiv). Compound 11 was isolated as highly viscous oil, further purified by flash chromatography with silica gel. Two stereoisomers were observed on the NMR spectrum.

Data for (6-benzyloxy-2,5,7,8-tetramethylchroman-2-yl) methyl- 6’-benzyloxy-2’,5’,7’,8’-tetramethylchroman-2’-carboxylate (11): Eluant: ethyl acetate/hexanes (9:1 v/v). Aspect: Highly viscous oil. Yield = 73%. 1H NMR (300.0 MHz, CDCl₃): δ: 1.05 and 1.15 (2, 3H, CH₃); 1.40-1.60 (m, 2H, CH₂); 1.65 and 1.64 (2s, 3H, CH₃); 1.79-1.98 (m, 1H, CH₂); 1.98, 2.03, 2.05, 2.11, 2.12, 2.13, 2.15, 2.19, 2.20 and 2.21 (10s, 18H, CH₃); 2.27-2.68 (m, 5H,
Synthesis of (6-hydroxy-2,5,7,8-tetramethylchroman-2-yl) methyl (6'-hydroxy-2',5',7',8'-tetramethylchroman-2'-carboxylate) 3 (Scheme 3)

Antioxidant 3 was prepared similarly to compounds 2a, 2b, and 2c. Hydrogenation of the ester 11 (658.0 mg) using a palladium on charcoal catalyst (66.0 mg, 1.01 mmol, 10% wt) provided compound 3 (267.0 mg, 0.57 mmol). The crude compound was purified by flash chromatography on silica gel. Two stereoisomers were observed on the NMR spectrum.

Data for (6-hydroxy-2,5,7,8-tetramethylchroman-2-yl) methyl 6'-hydroxy-2',5',7',8'-tetramethylchroman-2'-carboxylate (3): Eluant: ethyl acetate/hexanes (7/3 v/v). Aspect: White solids. Mp = 79-81 °C. Yield = 57%. $^1$H NMR (300.0 MHz, CDCl$_3$): δ: 1.05 and 1.12 (2s, 3H, CH$_3$); 1.20-1.50 (m, 2H, CH$_2$); 1.62, 1.63 (2s, 3H, CH$_3$); 1.86 (s, CH$_3$); 2.00, 2.02, 2.03, 2.04, 2.06, 2.09, 2.12, 2.14 and 2.16 (9s, 18H, CH$_3$); 2.22-2.65 (m, 6H, CH$_2$); 3.80, 4.03, 4.13 and 4.26 (4d, $^2$J$_{HH}$ = 11.1 Hz, 2H, CH$_2$OCO); 4.05, 4.13, 4.18, 4.19 (4s, 2H, OH). $^{13}$C NMR (75.0 MHz, CDCl$_3$): δ: 10.9 (CH$_3$); 11.1 (CH$_3$); 11.2 (CH$_3$); 11.3 (CH$_3$); 11.7 (CH$_3$); 11.8 (CH$_3$); 11.9 (CH$_3$); 12.1 (CH$_3$); 12.2 (CH$_3$); 20.0, 20.1 (2s, C=C-CH$_2$); 21.0 (CH$_3$); 21.1, 21.6 (2s, C=C-CH$_2$); 25.7, 25.8 (2s, CH$_3$); 27.7, 27.8 (2s, C=C-CH$_2$-CH$_2$); 30.8, 30.9 (2s, C=C-CH$_2$-CH$_2$); 68.8, 69.2 (2s, CH$_2$OCO); 73.2 (OC-CH$_3$); 73.3 (OC-CH$_3$); 116.8 (C$_{Ar}$); 116.9 (C$_{Ar}$); 118.4 (C$_{Ar}$); 188.5 (C$_{Ar}$); 212.1 (C$_{Ar}$); 122.3 (C$_{Ar}$); 122.6 (C$_{Ar}$); 144.7 (C$_{Ar}$); 144.9 (C$_{Ar}$); 145.2 (C$_{Ar}$); 145.3 (C$_{Ar}$); 145.8 (C$_{Ar}$); 145.9 (C$_{Ar}$); 174.0, 174.1 (2s, C=O). HRMS (m/z): calculated for C$_{42}$H$_{48}$O$_6$ = 649.3524; found 649.3567 [M + H]$^+$.
NMR Spectroscopy

$^1$H and $^{13}$C NMR were recorded on a 300 MHz Bruker Avance II spectrometer (Billerica, MA, USA) with TMS as internal reference for $^1$H and $^{13}$C NMR chemical shifts. Data are presented in the following order: chemical shift in ppm, multiplicity (s, singlet; d doublet; t, triplet; m, multiplet), coupling constant in Hertz, assignment broad band $^1$H decoupling.

Melting Point

Melting points (Mp) were measured with a Barnstead Electrothermal MEL-TEMP 3.0 (Barnstead; Dubuque, IA, USA).

Mass spectrometry

High resolution mass spectra (HRMS) were obtained on a QSTAR Elite mass spectrometer (Applied Biosystems; Foster City, CA, USA) equipped with an electrospray and operated in positive ion mode.

High Performance Liquid Chromatography

Tocopherol and the new antioxidants were analyzed according to AOCS Official Method Ce 8–89 [16]. Analyses were performed on a Finnigan Surveyor LC (Thermo Electron Corp., Waltham, MA, USA) with a Finnigan Surveyor Autosampler Plus and Finnigan Surveyor FL Plus fluorescence detector. The column was a normal phase Diol column (5µm; 250 × 4.6 mm; Monochrom, Varian, CA). For tocopherol, the mobile phase consisted of 7% methyl-tert-butyl-ether in hexanes with a flow rate of 0.6 mL/min and the fluorescence detector was set for excitation at 292 nm and emission at 325 nm. For the analysis of novel antioxidants the mobile phase was changed to 65% methyl-tert-butyl-ether in hexane. The fluorescence detector was set for excitation at 292 nm and emission at 394 nm. For each sample 10 µL was injected.

DPPH Radical-Scavenging Assay
The DPPH assay was performed according to method described by Nenadis and Tsimidou [17]. Briefly, to 2960 µL of 0.1 mM ethanolic solution of DPPH, 40 µL of synthesized antioxidant solution in ethanol was added at the following concentrations: 0.37, 0.74, 1.11, 1.85, 3.7, and 5.2 mM forming the ratios between the molar amounts of antioxidant to the molar amount of DPPH radicals at 0.05, 0.10, 0.15, 0.25, 0.5 and 0.7, respectively. The decrease of absorbance at 516 nm was measured at 25 °C after 20 min of reaction. The blank solution contained the same amount of DPPH and 40 µL of ethanol. Each test was performed in triplicate. The results are expressed as the %DPPH inhibition calculated according to the following equation:

\[
\%\text{DPPH} = \frac{(A_c - A_t) \times 100}{A_c}
\]

Where \(A_c\) and \(A_t\) are the absorbances of the control and the test sample, respectively. All standard deviations for DPPH tests were below 3.0%. Both trolox and \(\alpha\)-tocopherol were used as references. Calculated \(\text{IC}_{50}\) represents the concentration of antioxidant required to decrease the initial amount of DPPH by 50%.

ORAC Assay

ORAC assays were performed according to Szydlowska-Czerniak et al [18]. Briefly, the fluorescein disodium salt and AAPH solutions were prepared in 75 mM phosphate buffer (pH=7.4). The antioxidant solutions, 1 mM of each compound, were dissolved in methanol, and specific volume of it dissolved in the buffer to provide the required amounts of antioxidant within a range of 3.125 - 25.00 µM. Four different concentrations were tested for each antioxidant. A solution of fluorescein disodium salt, 3.0 mL (0.0816 µM) was mixed with 0.5 mL of antioxidant solution directly in a quartz cuvette. The mixture was kept at 37 °C for 10 min and 0.5 mL of the AAPH solution (153.0 µM) added. The fluorescence was measured at 37 °C for 30 min at 30 sec intervals. The emission and excitation were set at 525 and 485 nm, respectively. For a blank, phosphate buffer replaced the antioxidant solution. Each antioxidant solution was prepared in duplicate and three measurements were performed for each sample. A calibration curve was generated using trolox as the reference antioxidant. The area under the fluorescence decay curve (AUC) was calculated as follows:

\[
\text{AUC} = \sum_{t=0}^{n} \frac{f(t) + f(t+\Delta t)}{2}
\]

Where \(f_t\) is the fluorescence at a time \(t\) (min).
The net AUC was calculated using the following equation:

$$AUC_{\text{net}} = AUC - AUC_{\text{blank}}$$

For each antioxidant, a regression between $AUC_{\text{net}}$ and the compound concentrations was calculated, and the results are expressed as trolox equivalents (TEq).

Canola Oil Triacylglycerides Isolation

Canola oil was stripped of its endogenous minor components including antioxidants via adsorption chromatography, following the procedure described by Lampi and Kamal-Eldin [19]

Accelerated Storage

The ability of the new antioxidants to protect oil against oxidative degradation was determined using Schaal Oven test. To 1 g of canola triacylglycerols (CTG) 350 µg/g of the tested antioxidant was added in a vial (National Scientific Target DP Vials; 2 mL, 12 × 32 mm). The samples were stored in the dark for five days at 60 °C, providing the surface area to volume ratio at 0.78. Samples were examined at 24 h intervals for peroxide value and the residual amounts of antioxidant. The effectiveness of the new compounds was compared to α-tocopherol. Experiments were set up in two repetitions for each tested antioxidant, and samples from each repetition were analyzed in duplicate.

Peroxide Value (PV)

To assess PV method published by Hornero-Mendez et al, and modified by Shantha and Decker was used [20, 21]. Briefly, 200 mg of oil was dissolved in 5 mL of hexane. To 200 µL of the sample solution, 5 mL of methanol/chloroform/HCl solution (1:1:0.012, v/v), then 100 µL of NH$_4$SCN (30% w/w in water), and 100 µL of ferrous chloride (0.4% in water) were added. After 5 min of incubation at room temperature, the absorbance measured at 480 nm.

Frying Test
The effectiveness of the developed antioxidants to protect CTG under frying conditions was assessed using a frying test developed in our laboratory. CTG (12.0 g), fortified with 500 µg/g of the studied compound, was weighed into a glass beaker (Pyrex, USA). An octagonal stir bar (ThermoFischer Scientific, USA) was placed into the vessel, altering the final surface to volume ratio to 0.42. The vessel with sample was heated at 185 ± 5 °C for 10 min, and 1.2 g of formulated starch (a mixture of gelatinized potato starch with glucose and silica gel, 4:1:1 w/w) was added. The heating was continued for another 20 min without mixing and then was stirred at 500 rpm. Heating and stirring were afterward maintained for another 90 min. About 0.5 g of oil sample was withdrawn at the 60th, 80th, 100th and 120th minutes of heating. Selected sampling points reflect the frying time based on the amount of polar components formed and correspond to 1, 3, 5, and 7 days of actual frying time using an institutional fryer (General Electric Company, NY, USA). The frying performance of oils was assessed by the measurement of total polar components (TPC) and the retained amounts of added antioxidant. Samples from two repetitions of frying test were analyzed in duplicate for TPC and residual antioxidant.

Total Polar Components (TPC)

The amounts of polar compounds were determined by gravimetric procedure following AOAC Method 982.27 with Schulte modification [22, 23]

Statistical Analysis

Data were analyzed by single factor analysis of variance (ANOVA) and regression analyses using Minitab 2000 statistical software (Minitab Inc., PA, ver. 13.2). Significant differences between means were determined by Duncan’s multiple range tests. Statistically significant differences were determined at P < 0.05.

Results and Discussion

Synthesis
In order to produce the new antioxidants 2a, 2b, and 2c, precursors 6a, 6b, and 6c have first been prepared in two steps from carboxylic acids 4a, 4b, and 4c, respectively (Scheme 1). Cinnamic acid derivatives, 4a–4c, have been benzylated using benzyl bromide and potassium carbonate in dimethylformamide (DMF) to produce 5a–5c with the following yields: 86% (5a), 92% (5b), and 91% (5c). Saponification of 5a–5c esters with potassium hydroxide in ethanol/water solution has formed the desired cinnamic acids derivatives with the yield of 86% (6a), 98% (6b), and 90% (6c). Antioxidants 2a, 2b, and 2c have been prepared in four steps from trolox as described in Scheme 2. First, alcohol 8 was isolated in two steps according to a synthetic pathway described by Percec et al [15]. A Steglich esterification between compound 8 and cinnamic acid derivatives 6a–6c formed esters 9a–9c. Finally, antioxidants 2a–2c has been obtained by hydrogenation of compounds 9a–9c using palladium as catalyst. Isolated antioxidants formed highly viscous oils and were produced with the following yield: 78% (2a), 83% (2b), and 80% (2c).

In order to prepare the antioxidant 3, synthesis of precursor 10 was first carried out. Carboxylic acid 10 has been easily synthesized by saponification of ester 7 (Scheme 3). A Steglich esterification, followed by a hydrogenation, finally made the desired antioxidant 3 in a moderate yield of 57% (Scheme 3).

All developed antioxidants showed good solubility in oils and fats.

DPPH assay

The radical scavenging properties of the synthesized antioxidants 2a, 2b, 2c and 3, trolox and \( \alpha \)-tocopherol have been evaluated by the DPPH assay. Results of this study are given in Table 1. These results confirm that the radical scavenging effectiveness of the novel antioxidants 2a (IC\textsubscript{50} = 1.52 mM), 2b (IC\textsubscript{50} = 1.05 mM), 2c (IC\textsubscript{50} = 0.76 mM) and 3 (IC\textsubscript{50} = 1.15 mM) were significantly higher (P \leq 0.001) than the trolox (IC\textsubscript{50} = 1.75 mM) and \( \alpha \)-tocopherol (IC\textsubscript{50} = 1.61 mM). Antioxidant 2c, which possesses the highest number of hydroxyl groups, was the most efficient. Furthermore, compared to trolox (one hydroxyl) and \( \alpha \)-tocopherol (one hydroxyl), the bichromanol 3 (2 hydroxyls) exhibited a significantly higher radical scavenging activity (P \leq 0.001). In agreement with the present result, a novel dimeric antioxidant containing two hydroxyl groups in fused double chromanol rings was reported to possess better DPPH radical scavenging activity and provide double reducing power compared to \( \alpha \)-tocopherol, [24, 25].
It is well established that phenolic compounds scavenged radicals by proton donation [6]. Consequently, the present results are consistent with this observation because higher number of hydroxyl group resulted in higher capacity for proton donation. Additionally, this test established that the presence of a second methoxy substituent in ortho position of the hydroxyl group significantly increases the antioxidant effectiveness in radical scavenging. Thus, when comparing activities for compounds 2a with 2b where the only structural difference is in the number of methoxy groups on the ring, 1 vs 2 methoxy groups, respectively, the radical scavenging potency of 2b (50.1%) was higher than 2a (37.1%). These results are in agreement with the observation that an electron donating substituent in ortho position to the hydroxyl group increases the radical scavenging activity [12, 26]. Kikuzaki et al [26] reported a higher radical scavenging activity for sinapic acid compared to ferulic acid. Comparing to the previously published results by the same authors, antioxidants 2a, 2b, and 2c are better scavengers than the trolox hydroxybenzoates (Fig. 2) [11]. Thus, as expected, the presence of an alkyl chain between the ester group and the phenolic rings significantly increases the radical scavenging activity of the antioxidants.

ORAC assay

The antioxidant capacities of 2a, 2b, 2c and 3 have also been evaluated by the ORAC assay. The linearity between the AUC_{net} and the antioxidant (AH) concentration has been checked for each synthesized phenolic compound, and a regression coefficient above 99% has been observed (Results not included). Antioxidant capacities of each compound, expressed in trolox equivalent (TEq), are given on Table 1. According to these results, all of the synthesized phenolic compounds exhibited a higher antioxidant capacity than trolox. An improved antioxidant capacity was reported for a synthesized compound containing hydroxytyrosol attached to the chromanol ring, which inhibited the formation of malondialdehyde in rat liver microsomal membrane when oxidation was induced by AAPH [27]. The data obtained for caffeic acid (4.46 TEq) was similar to the value published by Gomez-Ruiz et al (4.52 TEq) [28]. Antioxidant 2c (5.41 TEq) was the most effective radical scavenger among the studied compounds whereas compound 3 (1.48 TEq) was the least active. Similarly to the results reported in previous investigations [11, 29], no correlations between ORAC and DPPH assays have been observed. Lack of similarities in trends between these two antioxidative tests is related to the different radicals used and the possible interaction between radicals and antioxidants. Additionally, for both tests different
mechanism of antioxidant radical interaction is involved and with it different response to the radicals scavenging activity [30-32]. For instance, compound 3 (1.48 TEq) is less effective than 2a (3.30 TEq) in the ORAC assay whereas the opposite has been observed in the DPPH test (Table 1). Furthermore, no trend has been observed regarding the nature and position of the substituent on the ring, eg para position to the hydroxyl group, when the radical scavenging activity was measured by ORAC.

Accelerated storage stability (Schaal Oven Test)

Radicals scavenging activity is not directly indicative of antioxidant effectiveness in a food system, we tested the novel compounds effectiveness in the food applications [32]. The ability of the new compounds 2a, 2b, 2c and 3 to protect CTG from oxidation has been determined under Schaal Oven test (SOT) conditions. The results of the SOT indicated that the new antioxidants better protected CTG, compared to α-tocopherol (Fig. 3). Indeed, the amounts of hydroperoxides formed after five days of storage were significantly lower for CTG fortified with 2a (39.9 meq/kg), 2b (39.3 meq/kg), 2c (15.9 meq/kg) or 3 (14.0 meq/kg) compared to unprotected CTG (110.8 meq/kg). Although no significant difference was observed even at P ≤ 0.12 in the protective capacities of novel antioxidant 2a, 2b and α-tocopherol, whereas antioxidants 2c and 3 were significantly more efficient (P ≤ 0.001) than α-tocopherol. Compound 3 was the most effective antioxidant among all tested compounds. These results indicate lack of direct correlation between the radical scavenging activity measured by DPPH and ORAC assays and the antioxidant activity measured in the food system. Similar lack of correlation have been reported in the literature, ascribing it to the nature of the radicals involved and the difference in the scavenging mechanism between DPPH and ORAC [30-32].

Protection during frying

Many compounds are known for their antioxidant activities at ambient temperature. The extreme conditions used during frying such as elevated temperature, prolonged exposure to oxygen, adding special limitations for antioxidants. Consequently, the antioxidant for institutional and industrial frying should not only be effective at frying conditions but also need to be: thermally stable and of low volatility to prevent evaporation [33]. As an example, the effectiveness of BHT under frying conditions is compromised by its evaporative losses at elevated temperature [34]. The capacities of the new antioxidants to protect CTG from
degradation during frying have been determined using a frying test developed in our laboratory. Their efficiency has been assessed by measuring the TPC after different frying times. Results are presented in Figure 4. This study has demonstrated that antioxidants 2a, 2b, 2c and 3 much better protected CTG during frying, compared to α-tocopherol. Indeed, at the end of the frying period, the TPC amounts were: 18.3%, 17.8%, 15.8%, and 15.4% for 2a, 2b, 2c and 3, respectively (Fig 4). These values are significantly lower (P ≤ 0.001) from unprotected CTG (25.7%). Besides, the new antioxidants were significantly more efficient (P ≤ 0.004) than α-tocopherol (20.4%). Among the synthesized antioxidant, compound 3 was the most efficient. Compared to previously published results, trends related to the difference in chemical structure have not been observed [11].

Antioxidant stability

In order to investigate the stability of 2a, 2b, 2c and 3, the amounts of antioxidant remaining at different stages during storage and frying have been measured (Figures 5 and 6). All the novel antioxidants exhibited higher stability than α-tocopherol. Indeed, under accelerated storage at the end of storage time the following amounts of antioxidant have been retained: 75.5%, 69.5%, 64.5%, and 68.7% for 2a, 2b, 2c and 3, respectively while only 35.2% of α-tocopherol was observed (Fig 5). The same superior stability was observed during frying test (Fig. 6). After 60 min of frying test, significantly higher amounts (P ≤ 0.001) of antioxidants 2a (56.3%), 2b (48.1%), 2c (42.1%) and 3 (20.5%) were retained when compared to 12.1% of α-tocopherol. Consequently, the higher amounts of antioxidant will be carried over to the fried foods when frying oil containing one of the novel antioxidants. Correspondingly, foods fried in such oil will have better storage stability than foods fried in oils containing α-tocopherol. Additionally, fried foods containing higher amounts of antioxidants can offer better nutritional value. It is worth mentioning that the new antioxidant 3 was depleted faster than the compounds 2a, 2b and 2c. This might be explained by the higher reactivity of the chromanol ring [6].

Conclusions

Developed novel antioxidants have been prepared in four steps from trolox. A convenient method for the synthesis of the compound 3 has also been developed. DPPH and ORAC assays demonstrated that the novel antioxidants offered higher radical scavenging activities
than trolox and α-tocopherol. It appears that the presence of an alkyl chain between the ester groups and/or the aromatic ring increases the radical scavenging activity of the compound. Moreover, a higher number of methoxy substituents in the ortho position to the hydroxyl group increased the efficiency of an antioxidant. Results from accelerated storage demonstrated that the compound 2c and 3 offered better protection for CTG than α-tocopherol. Furthermore, each of the synthesized compounds was more efficient than α-tocopherol when applied during frying. The developed novel antioxidants, particularly compounds 2c and 3, when used in food applications may extend storage stability and improve fry-life of oil. The remarkably higher thermal stability of these novel antioxidants makes them very valuable for high temperature applications such as frying.

Further investigations are underway to develop better understanding of the relationship between structure and antioxidant activity, the nature of the novel antioxidants degradation products formed, and to address any safety issues related to these antioxidants and their degradation products. This study was designed to prepare novel antioxidants and assess their activity in an oil system, however their potential application in various foods and regulatory acceptance will be a matter of further development.

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References

Table 1. Radical scavenging activity of the novel and reference antioxidants

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>DPPH Test</th>
<th>ORAC Test</th>
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<tbody>
<tr>
<td></td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; (mM)</td>
<td>Inhibition (%)</td>
</tr>
<tr>
<td>2a</td>
<td>1.52 ± 0.01</td>
<td>37.1 ± 2.0</td>
</tr>
<tr>
<td>2b</td>
<td>1.05 ± 0.04</td>
<td>50.1 ± 1.8</td>
</tr>
<tr>
<td>2c</td>
<td>0.76 ± 0.01</td>
<td>71.0 ± 2.4</td>
</tr>
<tr>
<td>3</td>
<td>1.15 ± 0.01</td>
<td>51.4 ± 1.9</td>
</tr>
<tr>
<td>Trolox&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.75 ± 0.02</td>
<td>31.7 ± 1.9</td>
</tr>
<tr>
<td>α-Tocopherol&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.61 ± 0.02</td>
<td>34.6 ± 1.7</td>
</tr>
<tr>
<td>Caffeic acid&lt;sup&gt;b&lt;/sup&gt;</td>
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* Compounds 2a–2c, and 3 developed the novel antioxidants. For their structures and properties see Fig 1 and the text.

<sup>b</sup>Reference antioxidants
Figure 1. Chemical structure of the novel antioxidants 2a, 2b, 2c and 3.
Figure 2. Structures of the intermediate phenolic compounds used for the synthesis of antioxidants. For details see the text.
Scheme 1. Reagents and conditions used for the synthesis of cinnamic acid derivatives. (i) BnBr, K₂CO₃, DMF; (ii) KOH, H₂O/EtOH, reflux. For details see the text.
Scheme 2. Reagents and conditions used for the synthesis of antioxidants 2a–2c. (i) BnBr, K₂CO₃, DMF; (ii) LiAlH₄, THF, 0 °C; (iii) 6a-6c, DCC, DMAP, DCM; (iv) H₂, Pd/Charcoal 10%, THF. For details see the text.
Scheme 3. Reagents and conditions for the synthesis of antioxidant 3: (i) KOH, H₂O/EtOH, reflux; (ii) 8, DCC, DMAP, DCM; (iii) H₂, Pd/Charcoal 10%, THF. For details see the text.
Changes in hydroperoxides during the storage of canola triacylglycerols with different antioxidants added at 350 µg/g. CTG - canola oil triacylglycerides. For details see the text.
Figure 4. Formation of polar components during test frying of canola triacylglycerols with added α-tocopherol and the novel antioxidants 2a–2c and 3 at 500 µg/g. CTG - canola triacylglycerides. For details see text.
Figure 5. Percentage of remaining antioxidants during the accelerated storage of canola triacylglycerides fortified with α-tocopherol and the novel antioxidants 2a–2c, and 3 added at 350 µg/g. For details see the text.
Figure 6. Percentage of remaining antioxidants during test frying canola triacylglycerides fortified with α-tocopherol and the novel antioxidants 2a – 2c, and 3 added at 500 µg/g. For details see the text.
Novel caffeic acid amide antioxidants: Synthesis, radical scavenging activity and performance under storage and frying conditions

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ABSTRACT

Twelve novel dihydro-caffeic acid amides were synthesized in good yields and fully characterized by $^1$H NMR, $^{13}$C NMR, and MS. Their radical scavenging activities were assessed by DPPH assay. Additionally, their abilities to protect polyunsaturated oils under accelerated storage and frying conditions were evaluated. All the new compounds possessed significantly higher radical scavenging activities than α-tocopherol and BHT. The radical scavenging activity of N-decyl-N-(3,5-dimethoxy-4-hydroxybenzyl)-3-(3,4-dihydroxyphenyl) propanamide was 1.7 and 4 times higher than α-tocopherol and BHT, respectively. At the end of storage period, the respective amounts of hydroperoxides in canola oil triacylglycerols (CTAG) fortified with α-tocopherol and BHT was 6.1 and 1.4 times higher than CTAG containing the amide. Frying test exhibited that CTAG containing N-decyl-N-benzyl-3-(3,4-dihydroxybenzyl) propanamide was 1.3, 1.4, and 1.6 times more stable compared to oil fortified with dihydro-caffeic acid, α-tocopherol and BHT, respectively, as assessed by the amounts of total polar compounds. Moreover, these compounds were remarkably thermally stable, making them suitable for frying applications.

1. Introduction

Recent global awareness campaigns for the health benefits of essential fatty acids have led to considerable increase in human consumption of polyunsaturated oils. However, these oils are susceptible to oxidative degradation leading to generation of free radicals and other toxic products. Free radicals have been implicated in the pathogenesis of inflammatory and cardiovascular diseases, cancer and aging (Reuter, Gupta, Chaturvedi, & Aggarwal, 2010). Under storage and frying conditions, polyunsaturated oils are fast degrading, negatively impacting the physical, flavor, nutritional and functional qualities of the oils and the foods containing them. It is imperative, therefore, that these oils are protected against oxidative and thermal degradation. One of such measures is application of antioxidants. Phenolic antioxidants are particularly potent and many of them occur naturally in food. They slow the degradation of food lipids by inhibiting the propagation of oxidation, and many of them are known to possess variety of interesting biological activities (Jiang, Lau, Hon, Mak, Woo & Fung 2005; Ou & Kwok, 2004; Priscilla & Prince, 2009). On the other hand synthetic antioxidants such as BHT, BHA and TBHQ offer good protection during storage, however their use has been limited because of the potential detrimental effects on the human health (Frankel, 2007). As a
consequence, there is a growing interest in the development of new antioxidants exhibiting low toxicity, high antioxidant capacity, good thermal stability and prepared from natural precursors.

Recently, Rajan, Vedernikova, Cos, Berghe, Augustyns & Haemers (2001) described the synthesis of new caffeic acid amides exhibiting good antioxidant activities. In their study, the amide group was chosen for enhanced metabolic stability. Jung et al (2002) also reported the synthesis of 4-hydroxyphenylacetic acid amide possessing potent analgesic activity. The synthesis of caffeic acid amides possessing antimicrobial activities was recently reported by Fu, Cheng, Zhang, Fang, & Zhu (2010). The amide derivative of N-acetylcysteine (NACA), a commercially available pharmaceutical drug and nutritional supplement, was found to permeate more efficiently cell membranes than the parent form (Ates, Abraham, & Ercal, 2008). Several hydroxycinnamic acid amides occur in different parts of plants and a number of them possessing interesting physiological activities (Chen, Chang, Yen, & Wu, 1998; Dalby-Brown, Barsett, Landbo, Meyer, & Mølgaard, 2005; Fagerlund, Sunnerheim, & Dimberg, 2009).

In this paper, we synthesized a variety of new N-alkyl-N-aryl-3-(3,4-dihydroxyphenyl)propane-amides and assessed their radical scavenging activities using the DPPH assay. Recognizing that there is a lack of direct relation between the radical scavenging activity measurements by DPPH or ORAC assays and the real antioxidant activity in a food system (Alamed, Chaiyasit, Mc Clements, & Decker, 2009), we assessed the effectiveness of the novel antioxidants using standard accelerated storage and frying tests. Canola oil triacylglycerols, typical polyunsaturated oil, were used as the substrate. We chose to prepare derivatives of dihydro-caffeic acid due to its higher radical scavenging and antioxidant activity compared to caffeic acid (Nenadis, Boyle, Bakalbassis, & Tsimidou, 2003).

2. Materials and Methods

2.1. Reagents

Benzaldehydes; 4-hydroxybenzaldehyde; vanillin (3-methoxy-4-hydroxybenzaldehyde); syringaldehyde (3,5-dimethoxy-4-hydroxybenzaldehyde); propylamine; hexylamine; decylamine; dihydro-caffeic acid (DCA); 2,2-diphenyl-1-picryl-hydrazyl (DPPH) and silica gel were purchased from Sigma-Aldrich (St. Louis, MO, USA). Benzotriazole-1-yl-oxy-tris(dimethylamino) phosphonium hexafluorophosphate (BOP), butylated hydroxytoluene (BHT) and α-tocopherol were from Calbiochem-Novabiochem (San Diego, CA, USA). Dry dichloromethane (DCM) was purified using a MBraun Solvent Purification System (M. Braun Incorporated, Stratham, NH). All other solvents were HPLC grade.
2.2. Synthesis

2.2.1. General procedure for the synthesis of amines 2a to 2L

The aldehyde (10 mmol) and the amine (15 mmol) were dissolved in methanol (10 mL) and the mixture was stirred for 3 hrs, then NaBH₄ (10 mmol) was added in small portions. The mixture again was stirred for 3 hrs following by solvent removal under reduced pressure using a rotary evaporator. Then distilled water (10 mL) was added and the solution extracted three times with ethyl acetate (10 mL each). The organic layers were combined and dried on magnesium sulfate followed by solvent removal under reduced pressure in a rotary evaporator and the desired crude product was obtained.

Propylbenzylamine (2a); 4-[(propylamino) methyl] phenol (2d) and 2-methoxy-4-[(propylamino) methyl] phenol (2g) were used in the next step without purification. The crude hexylbenzylamine (2b) and decylbenzylamine (2c) were purified by a flash chromatography using ethyl acetate as eluant. 4-[(decylamino) methyl] phenol (2f); 2-methoxy-4-[(hexylamino) methyl] phenol (2h) and 2-methoxy-4-[(decylamino) methyl] phenol (2i) were similarly purified using ethyl acetate/methanol (9:1) as eluant. The crude 4-[(hexylamino) methyl] phenol (2e) was purified in the same manner using an ethyl acetate/methanol (9.5:0.5). Whereas 2,6-dimethoxy-4-[(propylamino)methyl]phenol (2j); 2,6-dimethoxy-4-[(hexylamino) methyl] phenol (2k) and 2,6-dimethoxy-4-[(decylamino) methyl] phenol (2L) were purified by recrystallization in hexane.

2.2.2. General procedure for the synthesis of new DCA amides (3a-3L)

DCA (200 mg, 1.1 mmol) and triethylamine (1.1 mmol) were added to dimethylformamide (2 mL) and the mixture was stirred at 0°C for 30 min. Then an amine (2.2 mmol) dissolved in a minimum of DCM and BOP (486 mg, 1.1 mmol) dissolved in 2 mL of DCM were added and stirred for 2 hrs at room temperature. The solution was concentrated under reduced pressure using a rotary evaporator. To the residue 10 mL of water was added and the solution extracted three times with 10 mL of ethyl acetate. The organic layers were combined and dried on magnesium sulfate and solvent removed under reduced pressure using a rotary evaporator.

Utilizing an ethyl acetate/hexane mixture (3:2) as eluant, the crude N-propyl-N-benzyl-3-(3,4-dihydroxyphenyl) propanamide (3a); N-decyl-N-benzyl-3-(3,4 dihydroxyphenyl) propanamide (3c); N-hexyl-N-(4-hydroxybenzyl)-3-(3,4-dihydroxyphenyl) propanamide (3e) and N-decyl-N-(4-hydroxybenzyl)-3-(3,4-dihydroxyphenyl) propanamide (3f) were purified by a flash chromatography. The crude N-hexyl-N-benzyl-3-(3,4-dihydroxyphenyl) propanamide (3b) was purified in the same manner using an ethyl acetate/hexane mixture (1:1). Crude N-propyl-N-
(4-hydroxybenzyl)-3-(3,4-dihydroxyphenyl) propanamide (3d); N-propyl-N-(3-methoxy-4-hydroxybenzyl)-3-(3,4-dihydroxyphenyl) propanamide (3g); N-hexyl-N-(3-methoxy-4-hydroxybenzyl)-3-(3,4-dihydroxyphenyl) propanamide (3h); N-decyl-N-(3-methoxy-4-hydroxybenzyl)-3-(3,4-dihydroxyphenyl) propanamide (3i); N-propyl-N-(3,5-dimethoxy-4-hydroxybenzyl)-3-(3,4-dihydroxyphenyl) propanamide (3j); N-hexyl-N-(3,5-dimethoxy-4-hydroxybenzyl)-3-(3,4-dihydroxyphenyl) propanamide (3k) and N-decyl-N-(3,5-dimethoxy-4-hydroxybenzyl)-3-(3,4-dihydroxyphenyl) propanamide (3L) were purified by a flash chromatography using an ethyl acetate/hexane mixture (7:3).

2.3. Instruments

$^1$H NMR and $^{13}$C NMR were recorded on a 300 MHz Bruker Avance II spectrometer (Bruker Daltonics, Billerica, MA) with TMS as internal standard. HPLC analyses for residual antioxidants were performed on a Finnigan Surveyor LC system (Thermo Electron, Waltham, MA, USA) with a Finnigan Surveyor Autosampler Plus, Finnigan Surveyor FL Plus fluorescence detector and a Finnigan Surveyor photodiode array detector (PDA). The column was a normal phase Diol column (5µm; 250 × 4.6 mm; Monochrom, Varian, CA). Column chromatography with EMD silica gel Si 60 (40-63 µm) was used for separation and purification. Melting points (Mp) were measured with an Electrothermal MEL-TEMP 3.0 (Barnstead, Dubuque, IA, USA). A Beckman DU-65 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA) was used in the DPPH assay as well as for the determination of PV. High-resolution mass spectra (HRMS) were obtained with a QSTAR Elite mass spectrometer (AB SCIEX, Concord, ON, Canada) equipped with an electrospray operated in positive ion mode.

2.4. DPPH radical scavenging assay

The DPPH assay was performed according to a method described by Nenadis et al (2003). Briefly, an ethanolic DPPH solution (0.1 mM; 2960 µL) was added to the 40 µL of antioxidant solution in ethanol at the following concentrations: 0.74 mM, 1.11 mM, 1.85 mM, 3.7 mM, providing the ratios of the molar amounts of antioxidant to DPPH at 0.10, 0.15, 0.25 and 0.5, respectively. The decrease in absorbance at 516 nm was measured at 25 °C after 20 min of reaction. The blank solution contained the same amount of DPPH reagent and 40 µL of ethanol and each test was performed in triplicate. The results were expressed as the percentage of DPPH inhibition calculated as follows:
\[ \% \text{DPPH} = \frac{(A_{\text{control}} - A_{\text{test}}) \times 100}{A_{\text{control}}} \]

Where: \( A_{\text{control}} \) and \( A_{\text{test}} \) are the absorbances of the control and test samples, respectively. All standard deviations (SD) for DPPH tests were below 3.0%. BHT, \( \alpha \)-tocopherol and DCA were used as references. Calculated IC\text{50} represents the concentration of antioxidant required to decrease the DPPH amount by 50%.

2.5. Antioxidant activity under storage and frying conditions

2.5.1. Purification of canola oil

Canola oil was stripped of its endogenous minor components including antioxidants via adsorption chromatography, following the method described by Lampi & Kamal-Eldin (1998).

2.5.2. Accelerated storage - Schaal oven test

The ability of the novel antioxidants 3a to 3L to protect oil from oxidative degradation was determined using Schaal oven test. Canola triacylglycerols (1.0 g), fortified with 350 µg/g of the tested antioxidant, were introduced in the vials (National Scientific Target DP Vials; 2 mL, 12 × 32 mm). The ratio of surface area to volume was at 0.78. The uncapped vials were stored in dark at 60 °C for up to seven days. Samples were examined at 24 hrs intervals by collecting individual vials at particular period. The oxidative stability of the samples was evaluated by peroxide value (PV). The effectiveness of the new compounds was compared with \( \alpha \)-tocopherol as natural antioxidant and butylated hydroxytoluene (BHT) as synthetic antioxidant. Experiments were set up in two repetitions for each tested antioxidant, and samples from each repetition were analyzed in triplicate.

2.5.3. Determination of peroxide value

PV was assessed according to procedure described by Arkadiusz, Roszko, Sosińska, Derewiaka, & Lewicki (2010). Briefly, 200 mg of oil was dissolved in 5 mL of hexane. Two hundred µL of the solution was mixed with 5 mL of methanol/chloroform/HCl solution (1:1:0.012, v/v). Thereafter, 100 µL of FeCl\text{2} (0.4% water solution) and 100 µL of NH\text{4}SCN (30% water solution) were added. The reaction was run at room temperature for 5 minutes, and the absorbance measured at 480 nm using all reagents as blank sample.
2.5.4. Frying test

The effectiveness of the developed antioxidants to protect CTAG’s under frying conditions was assessed using a frying test developed in our laboratory (Aladedunye & Przybylski, 2011). Briefly, 12 g of CTAG’s fortified with 500 µg/g of the tested antioxidant, was weighed into a glass beaker (Pyrex, USA). Octagonal stir bar (ThermoFischer Scientific, USA) was placed into the vessel, forming the final surface to volume ratio of 0.42. Then vessel with the content was heated at 185 ± 5 °C for 10 minutes and 1.2 g of formulated food containing a mixture of gelatinized potato starch, glucose and silica gel (4:1:1w/w) was added. The heating was continued for another 20 minutes and the mixture stirred at 500 rpm. Heating and stirring were maintained for another 90 minutes. About 0.5 g of fat sample was withdrawn at the 60th, 80th, 100th and 120th minutes of heating. Selected sampling points are equivalent to 1, 3, 5 and 7 days of actual frying time using institutional fryer (General Electric Company, NY, USA). At selected intervals oil accumulated similar amounts of polar components compared to actual frying. Frying performance of oils was assessed by measuring an amount of the total polar components (TPC) and by assessing the amount of retaining antioxidant in the oil. BHT, α-tocopherol and DCA were used as references. Samples from two repetitions of frying test were analyzed in duplicate for TPC and residual antioxidant.

2.5.5. Total polar compound

TPC were determined by gravimetric method following AOAC Method 982.27 (AOAC, 1990) with Schulte modification (2004). Briefly, oils were separated into non-polar and polar fractions using a hydrated silica gel in a column chromatography.

2.5.6. Analyses of residual antioxidants

Tocopherol was analyzed according to AOCS Official Method Ce 8–89 (AOCS, 1999). Briefly, 50 mg of oil samples were weighed directly into the autosampler vials and dissolved in 1 mL hexane. The mobile phase consisted of 7% methyl–tert–butyl–ether in hexane with a flow rate of 0.6 mL/min and the fluorescence detector set for excitation at 292 nm and emission at 325 nm were used. For BHT, DCA and the novel antioxidants the mobile phase was changed to 50% methyl-tert-butyl-ether in hexane and a flow rate to 0.3 mL/min using PDA at 281 nm for quantification, injecting 10 µL of each sample.
2.6. Statistical Analysis

Data were analyzed by single factor analysis of variance (ANOVA) and regression using Minitab 2000 statistical software (Minitab Inc., PA, ver. 13.2). Statistically significant differences between means were determined by Duncan’s multiple range tests for P < 0.05.

3. Results and discussion

3.1. Synthesis

New antioxidants 3a to 3L were prepared in two steps synthesis from the corresponding aldehydes 1a to 1d (Fig. 1). Amines 2a to 2L were obtained by reductive amination. Reaction of aldehydes 1a to 1d with an excess of alkylamine in methanol, yielded the expected imines. The imines were subsequently reduced with sodium borohydride and the desired amines were isolated in moderate to good yields (Table 1). The physical and spectroscopic characteristics of the amines are presented in Tables 1 and 2, respectively. The spectral results for compounds 2a, 2b and 2c were in agreement with the data reported in literature (Guino, Brule, & de-Miguel, 2003; Sato, Sakamoto, Miyakawa, & Kikukawa, 2004; Lai, Lee, & Liu, 2008). Antioxidants 3a to 3L were prepared from DCA and the corresponding amines 2a to 2L using BPO as coupling reagent (Rajan et al., 2001). After purification by a flash chromatography, compounds 3a-3L were obtained in moderate to good yields, each as a mixture of the two rotamers (Table 1).

The structure of the novel antioxidants were confirmed by $^1$H NMR, $^{13}$C NMR and HRMS (Tables 1 and 3). As an example, the $^1$H NMR spectrum of antioxidant 3a is reported in Fig 2. The $^1$H NMR spectrum is characterized by a methyl group at 0.85 and 0.87 ppm (2t, one for each rotamer), five methylene groups at 1.55 ppm (sx), 2.63 and 2.71 ppm (2t, one for each rotamer), 2.87 and 2.93 ppm (2t), 3.12 and 3.32 ppm (2t) and at 4.45 and 4.61 ppm (2t). The protons of the aromatic rings are identified by two doublets at 6.52 and 6.53 ppm as well as by three multiplets at 6.75 - 6.83 ppm, 7.06 - 7.15 ppm and 7.24 - 7.39 ppm. The calculated molecular mass agreed very well with the mass established by mass spectrometry (Table 1).

3.2. DPPH radical scavenging assay

Results indicate that all the developed compounds displayed a considerable concentration dependent radical scavenging activity (Fig. 3, data for 1.11mM concentration are presented). At the tested concentrations, all the novel antioxidants exhibited significantly better (p < 0.05) radical scavenging capacity than α-tocopherol (Results not included). At concentration of 1.11
mM, 3L, the most active of the new antioxidants was four times more effective than BHT (Fig. 3). Interestingly, no significant differences were observed in the radical scavenging capacity between DCA and the following new antioxidants: 3a, 3b, 3c, 3d, 3e and 3f, indicating that the dihydro-caffeoyl group is mainly responsible for their radical scavenging activities. However, the presence of one or more methoxy groups in the ortho position to the hydroxyl group seemed to make the benzyl amine moiety more efficient in radicals scavenging. This is consistent with the general knowledge that an electron donating group in the ortho or para position increases the radical scavenging activity of some compounds (Fagerlund, Sunnerheim, & Dimberg, 2009). Consequently, antioxidants 3g, 3h, 3i, 3j, 3k and 3L displayed radical scavenging activity significantly higher than DCA (Fig. 3). The superior radical scavenging activity of the new antioxidants was also confirmed by assessing their IC50, demonstrating significantly lower values than: α-tocopherol (1.75 mM); BHT (2.55 mM); DCA (1.31 mM) (Fig. 3).

Furthermore, results implied that the length of alkyl chain did not have an effect on the radical scavenging activities and no significant differences were observed for the following compounds (chain length): 3a (n = 2), 3b (n = 5) and 3c (n = 9). Our results are in agreement with data published by Silva et al (2000) and Hsu et al (2009).

3.3. Antioxidant activity under accelerated storage

Results indicated that all new antioxidants outstandingly protected CTAG’s from oxidative degradation and the formation of rancidity (Fig. 4). At the end of the accelerated storage time the amount of hydroperoxides formed in unprotected CTAG’s was 10 times higher than in samples fortified with the novel antioxidants. Compared to α-tocopherol, a natural lipophilic antioxidant, CTAG’s fortified with the new antioxidants were significantly more stable (p < 0.05). The amounts of hydroperoxides formed in CTAG’s containing 350 µg/g of compound 3L was 6.1 times lower compared to CTAG’s containing the same amount of α-tocopherol (Fig 4). BHT and the novel antioxidants: 3a, 3b, 3c, 3d, 3e and 3f protected CTAG’s with the same efficiency during accelerated storage (Fig 4). However antioxidants 3g, 3h, 3i, 3j, 3k, and 3L offered much better protection than BHT. These results are consistent with observations from DPPH assay, suggesting that more hydroxyl and methoxy groups activate the benzyl amine moiety and improves antioxidant efficiency. Consistently, as was observed within each group, the antioxidant activity decreases when the alkyl chain length was shorter in the following order n = 9 < n = 5 < n = 2, however, this trend was statistically not significant at p < 0.05.
3.4. Protection under frying conditions

The effectiveness of the novel antioxidants to protect CTAG’s from oxidative degradation under frying conditions was determined using a frying test developed in our laboratory (Catel, Aladedunye, & Przybylski, 2010). CTAG’s fortified with 500 µg/g of antioxidant were tested under frying conditions and performance assessed by the measurement of an amount of the total polar components (TPC) formed as a function of frying time. The effectiveness of the new antioxidants was compared to BHT, α-tocopherol and DCA. Results showed that all novel antioxidants (3a to 3L) were more efficient in protecting CTAG’s during frying. At the end of the frying time, the amounts of TPC formed in CTAGs fortified with the novel antioxidants were significantly lower (p < 0.05) compared to CTAG alone (Fig 5). Further, the novel antioxidants were more efficient than α-tocopherol and BHT (Fig 5). Among the synthesized antioxidants, compound 3c was the most efficient and better protected oil than the parent DCA.

Interestingly, the group of antioxidants in which the benzyl amine moiety was not hydroxylated, compounds 3a, 3b, and 3c, were the most effective in CTAG’s protection during frying. This observation is contrary to the results from a radical scavenging and an accelerated storage stability tests, suggesting that the nature of the reactions under frying conditions are different from those happening during low temperature applications (Frankel 2007). The increase in alkyl chain length presumably enhanced the lipophilicity of the antioxidants, however, trend was observed but without statistical significance in their performance (Fig 5).

3.5. Residual antioxidants

In addition to being effective, an antioxidant should also be thermally stable so that it can provide lasting protection to food and biological systems at ambient and frying temperatures. This is particularly important during frying where temperature of 185°C is generally utilized. In the present study, the stability of the new antioxidants under both accelerated storage and frying were investigated and the amounts of residual antioxidants remaining at storage and frying in Fig 6 and 7 are presented, respectively. Evidently, the novel antioxidants were significantly more stable than α-tocopherol, BHT and DCA when tested with CTAG’s during accelerated storage at 60°C (Fig 6). At the fifth day of storage, the remaining amounts of α-tocopherol, BHT and DCA were at 39, 65, and 54%, respectively, compared to a minimum of 75% for the novel antioxidants. The same superior stability was observed during frying (Fig 7). At all stages of frying the significantly higher amounts of the novel antioxidants were present in the oil compared to α-tocopherol, BHT and DCA (Fig 7).
4. Conclusion

The novel antioxidants 3a to 3L have been conveniently prepared in good yield from DCA. DPPH assays demonstrated their significantly higher radical scavenging activities than α-tocopherol, BHT and DCA. Furthermore, when compared to α-tocopherol and BHT, the new compounds offered better protection to polyunsaturated oil both under storage and frying conditions. Beside their superior antioxidant activities, the higher lipophilicity and thermal stability make them more desirable than the precursor phenolic acid.

Further investigations are underway to develop better understanding of the relationship between structure and antioxidant activity, the nature of the novel antioxidants degradation and products formed. This study was designed to prepare novel antioxidants and assess their activity in an oil system; however their potential application in various foods and regulatory acceptance will be a matter of further development.
References


Guino, M., Brule, E., & de-Miguel, Y. R. (2003). Recycling and reuse of a polymer-supported


Figure 1. Reactions and structures of the novel antioxidants 3a – 3L. For details see the text.
Figure 2. $^1$H-NMR spectrum of the novel antioxidant 3a. For details see the text.
Figure 3. DPPH radicals scavenging and IC$_{50}$ for $\alpha$-tocopherol, BHT, DCA and the novel antioxidants. All antioxidants were tested at concentration of 1.11 mM. $\alpha$T - $\alpha$-tocopherol; BHT - butylated hydroxytoluene; DCA – dihydro-caffeic acid. For details see the text.
Figure 4. Changes in peroxide value during accelerated storage of canola triacylglycerols fortified with α-tocopherol, BHT, and the novel antioxidant 3a-3L at 350 µg/g. CTAG – canola triacylglycerols; DCA – dihydro-caffeic acid; BHT – butylated hydroxytoluene; αT–α-tocopherol.
Figure 5. Formation of polar components during frying test of canola triacylglycerides with added α-tocopherol, BHT, DCA and the novel antioxidants 3a – 3L at 500 µg/g. CTAG – canola triacylglycerols; αT – α-tocopherol. For details see the text.
Figure 6. Changes of the antioxidants during accelerated storage of canola triacylglycerols fortified with BHT, α-tocopherol and the novel antioxidants 3a-3L added at 350 µg/g. For details see the text.
Figure 7. Changes of antioxidants during frying tests of canola triacylglycerols fortified with BHT, α-tocopherol, DCA and the novel antioxidants 3a-3L added at 500 µg/g. For details see the text.
Table 1. Characteristics of synthesized compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Description</th>
<th>Mp (°C)</th>
<th>Yield (%)</th>
<th>[M + H]^+ Calculated</th>
<th>[M + H]^+ Found</th>
<th>Rotamers ratio</th>
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</thead>
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<tr>
<td>2d</td>
<td>light yellow, viscous oil</td>
<td>NA</td>
<td>98</td>
<td>ND</td>
<td>ND</td>
<td>NA</td>
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<tr>
<td>2e</td>
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<td>61 – 62</td>
<td>89</td>
<td>ND</td>
<td>ND</td>
<td>NA</td>
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<tr>
<td>2f</td>
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<td>61 – 63</td>
<td>85</td>
<td>ND</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>2g</td>
<td>light orange solids</td>
<td>56 – 58</td>
<td>98</td>
<td>ND</td>
<td>ND</td>
<td>NA</td>
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<tr>
<td>2h</td>
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<td>86</td>
<td>ND</td>
<td>ND</td>
<td>NA</td>
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<td>2i</td>
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<td>80</td>
<td>ND</td>
<td>ND</td>
<td>NA</td>
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NA = Not Applicable; ND = Not determined; Compounds 2d to 2L are intermediates used in synthesis; Mp = Melting point
Table 2. $^1$H and $^{13}$C NMR of the amine precursors synthesized in the study

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<tr>
<th>Hydrogen</th>
<th>2d*</th>
<th>2e</th>
<th>2f</th>
<th>2g*</th>
<th>2h</th>
<th>2i</th>
<th>2j*</th>
<th>2k</th>
<th>2L</th>
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<tbody>
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<td>0.90, J=6.9</td>
<td>0.94, J=6.9</td>
<td>0.89, J=6.9</td>
<td>0.88, J=6.9</td>
<td>0.88, J=7.2</td>
<td>0.88, J=6.9</td>
<td>0.86, J=6.9</td>
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<td>CH$_2$(m)</td>
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<td>1.22-1.38</td>
<td>1.22-1.38</td>
<td>1.20-1.38</td>
<td>1.24-1.38</td>
<td>1.14-1.38</td>
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<td></td>
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<tr>
<td>CH$_2$CH$_2$N</td>
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<td>1.50-1.61(m)</td>
<td>1.49-1.62(m)</td>
<td>1.56(sx), J=7.5</td>
<td>1.45-1.57(m)</td>
<td>1.43-1.58(m)</td>
<td>1.43(sx), J=7.2</td>
<td>1.46-1.58(m)</td>
<td>1.42-1.58(m)</td>
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<tr>
<td>CH$_2$CH$_2$N (t)</td>
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<td>2.70, J=7.5</td>
<td>2.69, J=7.5</td>
<td>2.63, J=7.2</td>
<td>2.62, J=7.5</td>
<td>2.62, J=7.5</td>
<td>2.44, J=7.2</td>
<td>2.63, J=7.2</td>
<td>2.62, J=7.2</td>
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<td>6.59(d), J=8.4</td>
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*Solvent = DMSO-d6; s, singlet; d, doublet; t, triplet; sx, sextet; m, multiplet. J, coupling constant in Hertz. For details see the text.
Table 3. $^1$H and $^{13}$C NMR of the novel antioxidants 3a – 3L

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*Solvent = DMSO-d$_6$; s, singlet; d, doublet; t, triplet; sx, sextet; m, multiplet; J, coupling constant in Hertz. For details see the text.