Endogenous Phenolics from Expeller-pressed Canola Oil Refining Byproducts: Evaluation of Antioxidant Activities in Cell Culture and Deep-fat Frying Models

by

Yougui Chen

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Department of Human Nutritional Sciences

University of Manitoba

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ABSTRACT

Sinapic acid derivatives and tocopherols in refining byproducts of commercially produced expeller-pressed canola oils were characterized and isolated. Additionally, the antioxidant activities of the phenolics were examined by three systems including an in vitro non-biological related assay, a cellular assay and a deep-fat frying model. Sinapic acid (SA： 42.9 μg/g), Sinapine (SP: 199 μg/g), and Canolol (CAN: 344 μg/g) were found in different byproducts of canola oil refining, namely, soapstock, spent bleaching clay, and wash-water, respectively. Tocopherols (3.75 mg/g) and other non-identified phenolic compounds (2.7 mg /g) were found in deodistillates (DDL). CAN and DDL revealed significant protection effect ($p<0.05$) against hydrogen peroxide induced oxidation in two mammalian cell lines. The results of deep-fat frying studies indicated positive effects of CAN and DDL in preventing lipid oxidation. The canola oils fortified with DDL and CAN showed a considerable reduction ($p < 0.05$) in oxidation products of lipid after frying.

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FOREWORD

This thesis was prepared in a manuscript style format and it is composed of four manuscripts following the General Introduction and Literature Review. These chapters were standardized for presentation in the thesis format.

Chapter 4 was published with authorship by Chen, Y., Thiyam-Hollander, U., Barthet, V.J., & Aachary, A.A. in 2014 as "Value Added Potential of Expeller-Pressed Canola Oil Refining: Characterization of Sinapic Acid Derivatives and Tocopherols from By-Products" in the Journal of Agricultural and Food Chemistry 62 (40), 9800–9807.

Chapter 5 has been submitted for peer review for the purpose of publication to the Journal of Functional Foods with authorship by Chen, Y., Thiyam-Hollander, U., Eskin, N.A.M., Eck, P. entitled: "Phenolics from Canola Crude Extracts Protect Cells from Oxidative Stress".

Chapter 6 was published with authorship by Aachary, A.A., Chen, Y., Eskin, N.A.M., & Thiyam-Hollander, U. in 2014 as "Crude canolol and canola distillate extracts improve the stability of refined canola oil during deep-fat frying" in the European Journal of Lipid Science and Technology 116, 1467-1476. The author's contributions in this paper include conducting the experiment, analyzing data and drafting the manuscript together with Aachary, AA.

Chapter 7 was published with authorship by Matthäus, B., Pudel, F., Chen, Y., Achary, A.A., Thiyam-Holländer, U. in 2014 as "Impact of Canolol-Enriched Extract from Heat-Treated Canola Meal to Enhance Oil Quality Parameters in Deep-Frying: a Comparison with Rosemary Extract and TBHQ-Fortified Oil Systems" in the Journal of American Oil Chemists' Society 91, 2065-2076. The author designed the study, conducted the frying experiment and analyzed the color, tocopherol and canolol content of oils, and was involved in the data analysis and manuscript writing.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AnV	*p*-anisidine value
AOCS	American Oil Chemists' Society
ASE	Accelerated Solvent Extraction
AV	Acid value
BHT	Butylatedhydroxytoluene
Caco-2	Human colon adenocarcinoma cells
CAN	Canolol
CD	Conjugated diene
CHO	Chinese hamster ovary cell
CNT	Control treatment
CT	Conjugated triene
DDL	Deodistillates
DGF	German Society for Fat Science
DPPH	2,2-diphenyl-1-picrylhydrazyl radical
DPTG	Di- and polymer triacylglycerols
FRAP	Ferric Reducing Antioxidant Power
IC_{50}	Concentration to give 50% inhibition
IV	Iodine value
PV	Peroxide value
RM	Rosemary extract
ROS	Reactive oxygen species
TBHQ	*tert*-Butylhydroquinone
TOTOX	Total Oxidation

CHAPTER 1

1. GENERAL INTRODUCTION

1.1 Introduction

Antioxidants function by delaying or inhibiting food oxidation as well as reducing oxidative stress on the human body (Velioglu *et al.*, 1998). The demand for natural antioxidants has increased in recent decades with the search for novel and low cost sources of antioxidants occupying the attention of both researchers and food industries. Among all the natural antioxidants, phenolic compounds are the most abundant, being present in plants and exhibiting significant antioxidant potential due to their redox properties.

Canola (*Brassica napus*), the major oilseed crop in Canada, has a seed that is a rich source of phenolics such as sinapic acid, sinapine, and tocopherols. It is reported that expeller pressing of canola seeds produces oils with a higher content of heat-induced phenolics. The crude expeller-pressed canola oil contains significant amounts of canolol, a decaboxylated derivative of sinapic acid. Canolol has been of particular interest in recent times because of its highly potent antioxidative nature; several studies have suggested that it is highly active in both food and biological systems. However, commercially refined canola oil has a lower content of phenolics with poorer antioxidant activities compared to those of the crude oils (Zacchi & Eggers, 2008; Ghazani & Marangoni, 2013). It has been suggested that both chemical and physical refining processes could result in a significant loss of phenolics and tocopherols from the oils. However, refining is essential for the production of most vegetable oils due to the high content of minor undesirable non-triglyceride components. These components include phospholipids, free fatty acids, sterols, coloring pigments, proteins and oxidation products in crude oils which impart an undesirable flavor and color, and shorten the shelf life of oil. The refining process is designed to remove these substances from oil to achieve the desired quality.

Recently, a number of attempts were made to reduce the losses of phenolics during oil refining by modifying refining conditions or methods, but these attempts appeared to be ineffective (Ghazani *et al.*, 2013). However, evaluation of the occurrence of phenolics in the oil refining byproducts is lacking. The bioactive antioxidants of the edible oils may not only be degraded during the high temperature exposure, but also leach into the byproducts during refining (Nogala−Kalucka *et al.*, 2004; Cvengros, 1995). Some of the antioxidants found in canola oils are phenolics with a hydrophilic nature (Thiyam *et al.*, 2006 a), and, may thus leach into wash-water, soap-stock or allied mixtures used during the refining process. There are only a few studies reporting the antioxidant composition in refining byproducts (Ortega-García *et al.*, 2006; Nogala−Kalucka *et al.*, 2004; Harbaum-Piayda *et al.*, 2010; Ghazani & Marangoni, 2010). If selectively recovered, these phenolic compounds might have a high added value as natural antioxidants for potential use in cosmetic, food and pharmaceutical applications.

Since the phenolic profiles of different canola oil processing and refining byproducts may be different, evaluation of the antioxidant potential of the recovered phenolics from different byproducts is needed to confirm their utilization feasibility. A number of *in vitro* assays, such as radical scavenging, metal reducing power, metal chelating, oxygen radical absorbing capacity (ORAC) assays etc., have been used for screening plant phenolic extracts by determining their antioxidant activities. Antioxidant activities of different types and forms of phenolic compounds from canola have been intensively studied (Amarowicz *et al.*, 2000; Vuorela *et al.*, 2004; Terpinc *et al.*, 2011; Sørensen *et al.*, 2013). However, the antioxidant activity varies due to the fundamental differences of media used in the antioxidant assays. It has been reported that sinapic acid and sinapine exhibited stronger antioxidant activities in homogeneous polar media (such as aqueous medium) than canola tannins and canolol (Terpinc *et al.*, 2011; Sørensen *et al.*, 2013). In contrast, Galano *et al.* (2011) predicted that

sinapic acid would be less reactive as an antioxidant in lipid media than in aqueous solution using the Density Functional Theory. Moreover, similar findings were reported by Terpinc *et al.* (2011) who found that canolol exhibited higher antioxidant activity than sinapic acid in emulsion medium as determined by a β-carotene bleaching assay. This phenomenon could be explained by the different structural characteristics and partitioning properties in different medium and intermolecular hydrogen bond interactions. Thus canolol could be a promising antioxidant since it might be more reactive in lipid and emulsion systems, making it a better antioxidant in lipid containing food and biological systems.

In addition to natural antioxidants, varieties of synthetic antioxidants, such as butylatedhydroxytoluene (BHT), butylatedhydroxyanisole (BHA) and *tert*-butylhydroquinone (TBHQ) have been commonly used as food additives to slow down the oxidation rate of lipid over the past 50 years. However, the safety of some of these synthetic antioxidants has been questioned due to their toxic and carcinogenic effects in animals and humans. Therefore, there is an increasing trend towards adding natural phenolic extracts to foods to improve their oxidative stability. The effects of natural antioxidants extracted from different plants on food oxidative stability have been intensively studied in recent decades, with some of the extracts found to exhibit effects similar to synthetic antioxidants (Wanasundara & Shahidi, 1994; Man & Tan, 1999). In the published literature a high antioxidant activity is described for canola phenolics in different food systems such as bulk oil (Thiyam *et al.*, 2006 a, b; Wanasundara & Shahidi, 1994), oil-in-water emulsion (Sørensen *et al.*, 2013) and meat (Salminen *et al.*, 2006), but the antioxidant performance in deep-fat frying has not yet been studied. Deep-fat frying is commonly used both at home and commercially to enhance and create unique food sensory properties. However, frying oil can deteriorate through various complex physical and chemical reactions, with lipid oxidation being the most important. Thus, the endogenous phenolics recovered from canola oil refining byproduct might have a similar effect on

improving oil stability during deep-fat frying.

Canola phenolics also exhibit promising antioxidant activities in biological systems, such as mammalian cells and animals. Recent studies confirmed that both sinapic acid and canolol have protective effects against oxidative stress-induced cell death and neuronal protection against the peroxynitrite ($ONOO^-$) associated diseases in animals (Kim *et al.*, 2010; Kuwahara *et al.*, 2004; Dong *et al.*, 2010). In addition to the antioxidant activities, other functional properties have been studied by canola phenolics. Sinapic acid and canolol were also found to possess anti-carcinogenic, anti-mutagenic and cyto-protective effects (Cao *et al.*, 2008; Kuwahara, 2004). However, only one type of phenolics, either sinapic acid or canolol was examined in the above studies. The bioactivities of the different fractions or forms of canola phenolics have not been compared through these studies.

In summary, data on key issues including biologically and food relevant antioxidant activities of the endogenous phenolics from different canola byproducts needs to be established.

1.2 Objectives

The long term objective of this thesis is to evaluate the value-added potential of expeller-pressed canola oil refining byproducts by recovery of the endogenous phenolic compounds including SADs and tocopherols, and to assess the beneficial effects of these phenolics for both foods and human health. The specific objectives of the research are indicated below:

1. To recover and characterize phenolics and tocopherols from refining byproducts of expeller-pressed canola oil;

2. To evaluate the antioxidant activities of phenolics from different canola byproducts using different *in vitro* antioxidant assays;

3. To evaluate the cytoprotective effect of canola phenolics against H_2O_2-induced

oxidation;

4. To study the effect of canolol and deodistillate phenolic extracts on improving the canola oil stability in a deep-fat frying model.

1.3 Thesis overview

The present thesis is divided into four main parts: a general introduction, a literature review, research manuscripts, and a general conclusion and future perspectives.

PART 1: *General introduction*

Chapter 1: This chapter consists of the introduction, research objectives and thesis overview, describing the background information, long-term and specific objectives, and an overview of the major works completed in the thesis.

PART2: *Literature review*

Chapter 2: This chapter summarizes recent literature regarding lipid oxidation, vegetable oil refining and the byproducts, extraction techniques of phenolics from canola byproducts, and antioxidative and bioactivities of canola phenolics. Moreover, it covers the research hypotheses that were established based on the literature review.

PART 3: C*onsists of four research manuscripts for achieving the research objectives. Each manuscript includes a specific introduction, research approach, results and discussion, and the specific conclusion obtained from each study.*

Chapter 3: In order to achieve objective 1, various rapid methods were used to recover and characterize the SADs and tocopherols from four byproducts from refining of expeller-pressed canola oil: soap-stock, wash-water, spent bleaching clay, and deodistillates (Chapter 3).

Chapter 4: To assess the antioxidant activity of the canola phenolics (objective 2 and 3), different extracts of canola byproducts were examined for their antioxidant capacities by using three “test tube” assays and a cellular assay.

Chapter 5: In order to examine the antioxidant potential of canola phenolics in a deep-fat frying system (objective 4), a preliminary study was conducted to evaluate the effect of crude canolol and deodistillates extracts at a concentration level of 200 ppm on frying performance by assessing the primary and secondary oxidation products and color of frying oil.

Chapter 6: To further study the concentration effect of canolol (objective 4), the second frying study was carried out to assess the effect of canolol at treatment concentrations from 200 to 750 mg/kg on frying stability of high-oleic canola oil by assessing several important oil quality parameters (di-and polymer triacylglycerols content, total polar compounds content, anisidine value, iodine value and color) and tocopherol content.

PART 4: *General conclusions and future perspectives*

Chapter 7: General conclusions based on the results obtained from the four studies are drawn and future research perspectives are discussed in this chapter.

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CHAPTER 2

LITERATURE REVIEW

2.1 Lipid oxidation and antioxidants

Lipid is one of the major bulk constituents present in food and biological systems (Jadhav *et al.* 1996). Many foods derived from plants and animals contain a high amount of unsaturated fatty acids which make lipids more susceptible to oxygen attack or lipid oxidation. This process leads to a complex chemical change in food, ultimately causing the development of rancidity and off-flavor. The mechanism of lipid oxidation and its role in food quality degradation have been known for more than 60 years. Antioxidants are considered to be important inhibitors of the process of oxidation (Kumar, 2011).

2.1.1 Mechanism of lipid oxidation

It is well known that lipid oxidation is one of the most important causes of food quality deterioration. It results in both physical and nutritional degradations of food including nutritional losses, off-flavor development, texture and color degradation, and increasing toxicity (Finley & Given, 1986). Since the 1940s, the mechanism of lipid oxidation has been found to involve free radicals and reactive oxygen species (Shahidi & Zhong, 2005).

The free radical chain reaction typically unfolds according to the following three steps: initiation, propagation and termination (Figure 2.1). The reaction starts with the initiation stage in which free radicals (L•) are produced from the lipid molecule (LH) by their interaction with catalysts, atmospheric singlet oxygen or energy sources such as radiation. The energy barrier required to initiate lipid radicals is lower than those required to initiate oxidation of proteins, carbohydrate, or nucleic acids (Kolakowska & Bartosz, 2013). The

initiation stage can be accelerated by many environmental conditions including heat, ultraviolet (UV), visible light, and metal catalysts.

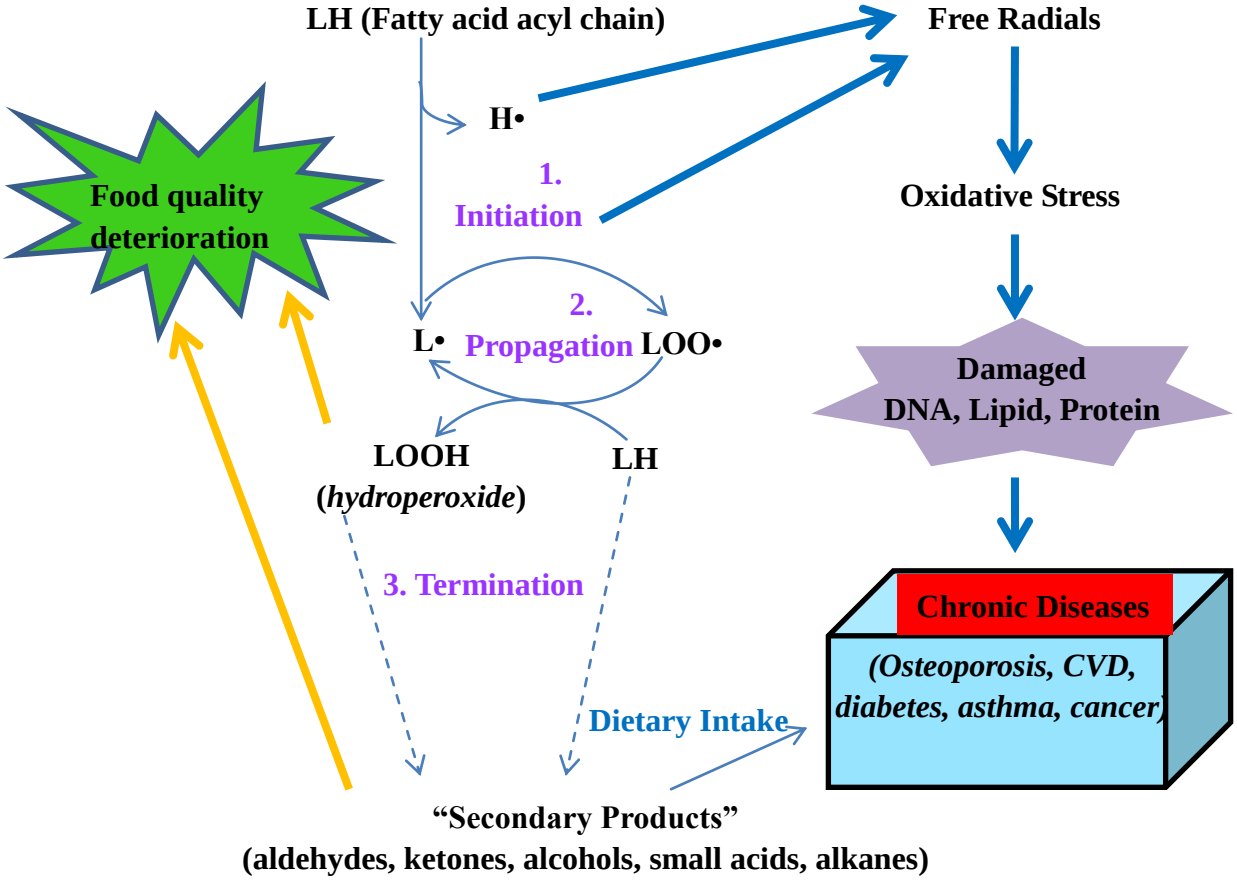


Figure 2.1 Stages of lipid oxidation and their effect on food quality deterioration and human health (Adapted from Frankel, 1984)

The free lipid radical is unstable, thus the propagation process occurs once the free radical is formed. During the propagation stage, two important reactions occur to transfer the unpaired electron on a radical (such as L•) to other molecules. Firstly, the alkyl radical reacts with triplet oxygen to form peroxyl radicals (LOO•). Then the peroxyl radical is able to abstract proton (H•) from another lipid molecule such as neighboring fatty acids and form lipid hydroperoxides at the same time. In addition, lipid hydroperoxides (LOOH) can also easily break down to form peroxyl radicals which would again accelerate the production of

free lipid radicals (L•). Thus, an autocatalytic chain reaction occurs in this stage until the termination stage which results in larger amounts of radical (L• and LOO•) produced. The peroxyl radicals of polyunsaturated fatty acids (PUFAs) can be more easily transformed into cyclic peroxides or even cyclic endoperoxides which may further accelerate the lipid oxidation rate (Kolakowska & Bartosz, 2013).

The termination stage is a process in which two radicals (L• and LOO•) combine together to form a non-radical compound. The resultant products include dimers, alcohols and peroxides which could cause food rancidity. When protein is present in food, the radicals can also combine with protein forming more complex oxidation products (Kumar, 2011).

2.1.2 Antioxidants

Since free radicals are responsible for lipid oxidation, abundant natural or synthetic antioxidants have been studied. These substances, defined as antioxidants, prevent or slow down the chemical reaction of various food constituents or molecules with oxygen and reduce oxidative damage (Flora, 2009; Shahidi & Zhong, 2005). In food application, antioxidants have been accepted as free radical scavengers, inactivators of peroxides, reactive oxygen species (ROS), chelators of metals, and quenchers of secondary lipid oxidation products that produce rancid odors (Shahidi & Zhong, 2005). In health related areas, antioxidants have also been widely used due to their ability to protect the body against damage caused by ROS as well as reactive nitrogen species (RNS) as well as those of reactive chlorine species (RCS) (Shahidi, 1997).

Different antioxidants can inhibit the oxidation process by several route such as chain -breaking reactions (e.g. tocopherols); reducing concentration of reactive oxygen species (e.g.

glutathione); scavenging initiating radicals (e.g. superoxide dismutase); and chelating transition metal catalyst (e.g. lactoferrin) (Kumar, 2011; Shahidi & Zhong, 2005). Antioxidants can be classified into different categories according to different scenarios. According to mechanisms of performance, they can be classified as primary and secondary antioxidants as shown in Figure 2.2 (Gordon, 1990). Common antioxidants being used in food systems are also indicated in the Figure 2.2. From a pharmaceutical perspective, Venkat Ratnam *et al.* (2006) classified antioxidants into enzymatic and non-enzymatic. The non-enzymatic antioxidants include minerals, vitamins, carotenoids, organosulfur compounds, low molecular weight antioxidants, antioxidant cofactors, and polyphenols. Since there is an abundance of antioxidants and the classification above is complex, antioxidants can also be classified as either natural or synthetic.

2.1.2.1 Synthetic antioxidants

Since the development of modern antioxidant technology, hundreds of synthetic compounds have been evaluated for their antioxidant activities for food. Many of the synthetic antioxidants have better antioxidant activities than natural antioxidants and can be used in various food products more easily. However, the safety issues of these synthetic compounds become a significant concern due to their possible toxic and carcinogenic effects on human health (Karovicova & Simko, 2000).

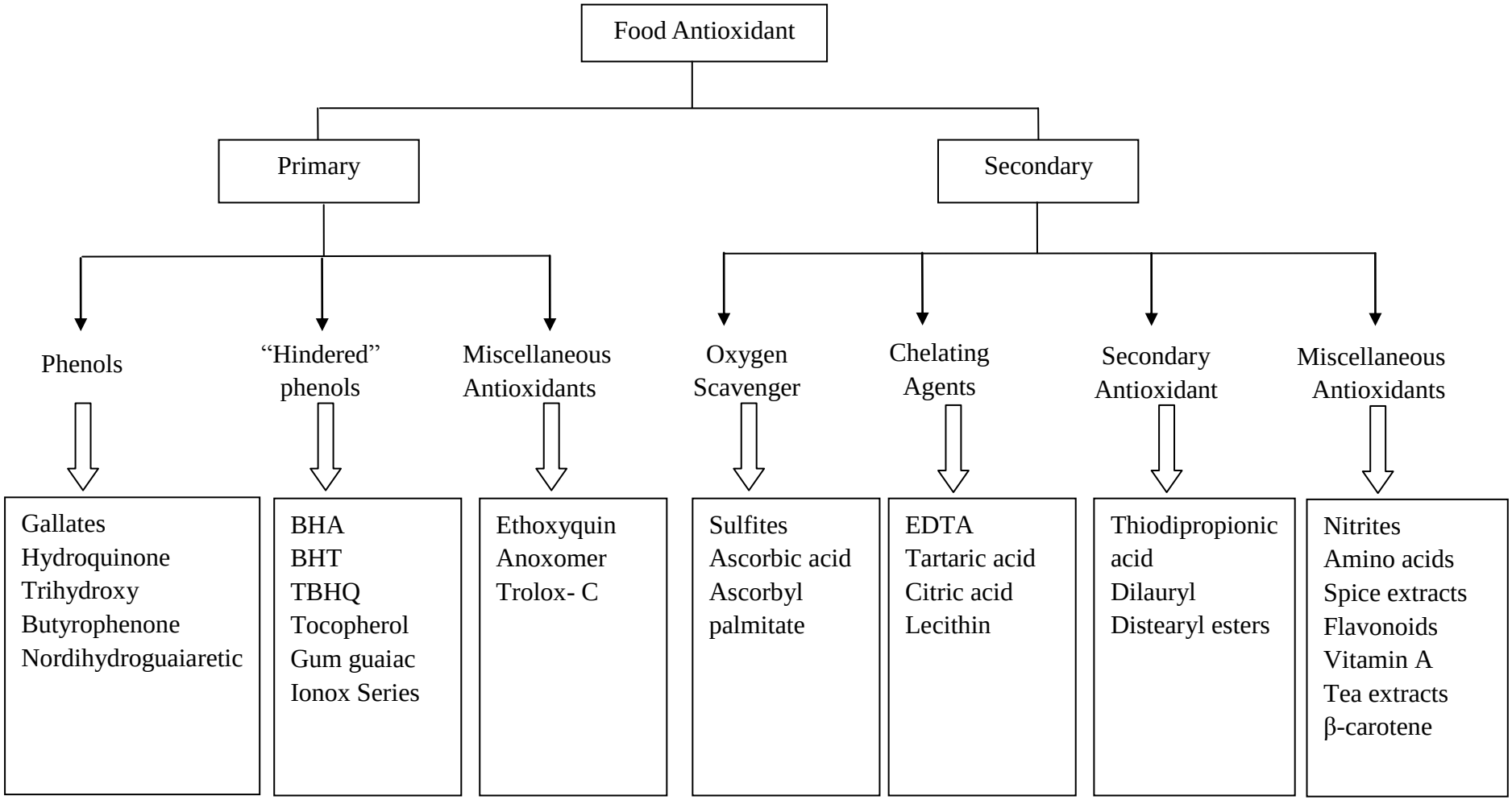


Figure 2.2 Classification of Antioxidants and examples (Adapted from Gordon, 1990)

Most countries have regulations for controlling the use of these synthetic antioxidants. In this context, of all the synthetic antioxidants, only four are widely used in food systems, namely, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and *tert*-butylhydroquinone (TBHQ). These showed very high antioxidant activities due to their structural properties as phenols (Saad *et al.*, 2007). Besides their application in the food industry, they are also commonly used as standard antioxidants in research.

The use of synthetic antioxidants has, however, decreased due to their potential adverse effects on the kidneys and livers, as well as carcinogenic effects, in animals (Farag *et al.*, 2003; Eriksson & Siman, 1996; Lanigan & Yamarik, 2002). BHT and BHA are legally used, within limited levels, in most countries including USA, Canada, Japan, Australia and Europe. TBHQ is not allowed to be used as a food additive in most European countries and PG is not allowed to be used in combination with TBHQ in USA (Shahidi & Zhong, 2005). According to the existing food additive regulations established by the US Food and Drug Administration (FDA), the maximum level of use of these four antioxidants is 0.02% or 200 ppm based on lipid content of food products.

2.1.2.2 Natural antioxidants

Due to the restrictions on the use of synthetic antioxidants, the current trend is focused on identifying and developing new natural sources of antioxidants. Natural antioxidants constitute a broad range of compounds including phenolic compounds, carotenoids, ascorbic acid etc. (Loliger, 1991; Valenzuela *et al.*, 2003). Phenolic compounds have been seen as important compounds in inhibiting lipid oxidation. Their structures allow them to form low-energy radicals which will not further propagate the oxidation of fats or oils (Karovicova & Simko, 2000). The mechanism

of the action of phenolic antioxidants is shown in Figure 2.3. Phenolic antioxidants (PhH) can react with peroxyl radicals (ROO•) and produce phenoxyl radical (Ph•) which is low-energy and relatively unreactive. Lastly, this radical can undergo the termination reactions with peroxyl radicals to produce non-radical products (ROO-Ph) which break the free radical chain reaction.

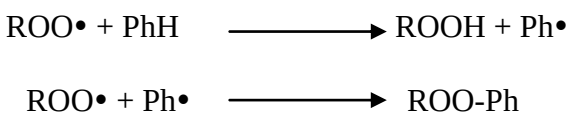


Figure 2.3 Mechanism of action of phenolic compounds as antioxidants

In addition, phenolic compounds can be found in a variety of plant sources and vegetable extracts. Many of them are recognized as useful phytochemicals in our diets (Dimitrios, 2006). There are more than 8000 known phenolic structures which range from simple molecules such as phenolic acid to highly polymerized compounds such as tannins. The major sources of plant phenolic antioxidants are fruits and vegetables. In these plant sources, the main phenolics include phenolic acids, flavonoids and tannins (Dai *et al*., 2010).

Table 2.1 lists the major plant sources of phenolic antioxidants and the classes of phenols they contain. Flavonoids are the most abundant phenolics present in fruits and vegetables. Phenolic acids can be found in many fruits, herbs and spices. Herbs and spices are rich sources of tannins. The phenolic extracts from various plants (such as rosemary, sage, green tea, grape and berries etc.) have been found to demonstrate high antioxidant actives (Rababah *et al.*, 2004; Seeram *et al.*, 2006).

Table 2.1 Major plant sources of phenolic antioxidants (Adapted from Dimtrios, 2006).

	Plants	Phenolic classes	Major phenolic antioxidant
Fruits	Berries	Phenolic acid, flavonoids	Flavanols hydroxycinammic, hydroxybenzoic acids, anthocyanins
	Cherries	Phenolic acid, flavonoids	Hydroxycunnamicacids Anthocyanins
	Black grapes	Phenolic acid, flavonoids	Anthocyanins, flavonols
	Citrus	Phenolic acid, flavonoids	Phenolic acids, flavonones, flavonols
	Plums	Phenolic acid, flavonoids	Hydroxycinnamic acids, catechins
Vegetables	Aubergin	Phenolic acid, flavonoids	Anthocyanins, Hydroxycinnamic acids
	Chicory	Phenolic acid	Hydroxycinnamic acids
	Parsley	Flavonoids	Flavonones
	Rhubarb	Flavonoids	Anthocyanins
	Sweet potato leaves	Flavonoids	Flavonols, Flavones
	Yellow onion	Flavonoids	Flavonols
	Beans	Flavonoids	Flavonols
	Spinach	Phenolic acids, flavonoids	Flavonoids, *p*-coumaric acid
Flours	Oats, wheat , rice	Phenolic acids	Caffeic and ferulic acids
Drinks	Teas	Flavonoids	Flava-3-ols, flavonols
	Red wine	Flavonoids	Flava-3-ols, flavonols, anthocyanins
	Orange juice	Flavonoids	Flavonols
	Coffee	Phenolic acids	Hydroxycinnamic acids
Herbs and spices	Rosemary, sage	Phenolic acids, Tannins	Carnosic acid, carnosol, rosemarinic acid and rosemanol
	Oreganol	Phenolic acids, flavonoids, Tannins	Rosemarinic acid, phenolic acids, flavonoids
	Ginger	Flavonoids and other minor phenolics	Gingerol and related phenolics

Oilseeds have also been found to be an important source of antioxidants in recent decades. Olive oil contains a large amount of polyphenols ranging from 100-300 mg/kg (Dimitrios, 2006). Visioli *et al.* (2004) reported that significant amounts of polar phenolic compounds were present in olive oil which accounted for oil stability and its biological properties. Other oilseeds products such as canola, sesame and mustard have also been reported to have significant phenolic content with antioxidant

activity (Wanasundara *et al.*, 1994; Shahidi *et al.*, 2006; Amarowicz *et al.*, 1996).

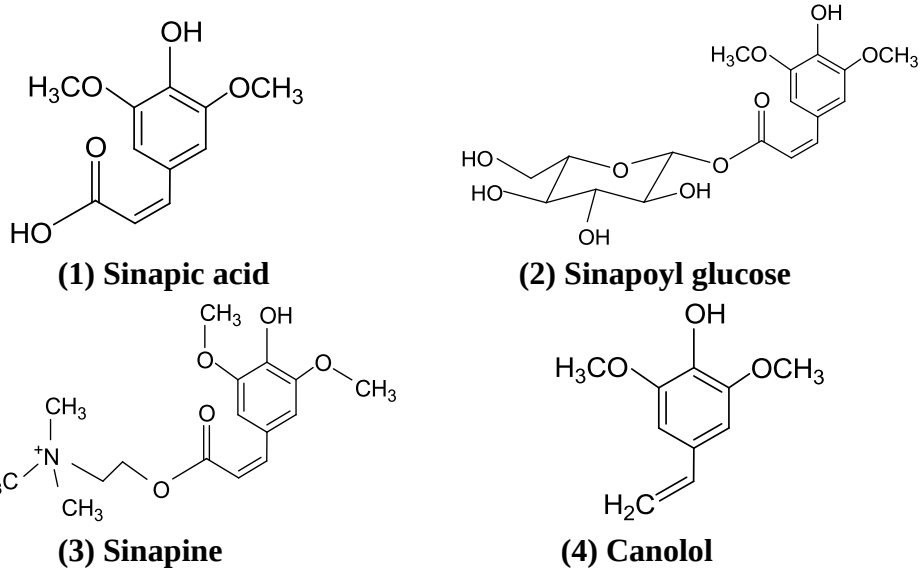
Besides the natural occurring phenolics, the most important commercial natural antioxidants are tocopherols and ascorbic acids. Tocopherols are lipophilic monophenolic compounds and can be found in plant tissues. The common sources of tocopherols include palm, rice bran oils, cereals and legumes (Reische *et al.*, 1998).

2.1.3 Canola antioxidants

Canola seed is a variety of rapeseed which is one of the most valuable crops in Canada and contributes $15.4 billion to the Canadian economy each year (Canola Council of Canada, 2001). It is used to produce high quality edible oil and its meal products are used as animal feed. The content of phenolic compounds in canola products is much higher than other oil seeds (Shahidi & Naczk, 1992). These phenolic compounds were once seen as unfavorable substances in canola meal due to their undesirable effect on the sensory and nutritional values of canola meal as a protein source (Shahidi & Naczk, 1992). However, Wanasundara *et al.* (1994) isolated and identified the phenolic components from canola meal and reported that these compounds were very effective in preventing oxidation of β-carotene. Canola phenolic antioxidants have been studied by many researchers (Amarowicz *et al.*, 2000; Cai & Arntfield, 2001; Khattab *et al.*, 2010) and the structures and antioxidant activities of these compounds have been identified.

The major phenolic compounds present in canola seed are sinapic acid derivatives (SAD) and tocopherols. Sinapic acid derivatives include sinapic acid, sinapine, and glucopyranosyl sinapate. Their chemical structures are shown in Figure 2.4. Kozkowska *et al.* (1991) reported that the amount of sinapic acid derivatives in rapeseed/canola meals varied from 6.39 to 18.37 mg/g depending on the variety of oilseed and types of oil processing method. Sinapic acid is the major phenolic acid

present in canola seed, accounting for 70.2-85.4% of the total phenolic acids in the whole seed (Aachary & Thiyam-Holländer, 2013). In raw canola seed and canola meals, the major phenolic is an esterified form of sinapic acid which is called sinapine. Glucopyranosyl sinapate (also called sinapoyl glucose) is a phenolic glucoside, which accounts for a small amount of phenolic compounds in canola seed. Thiyam-Holländer & Schwarz (2013) reported that the percent contribution of sinapic acid derivatives in canola meal extracts was 61-70%, 14-27%, 7-10% for sinapine, sinapoyl glucose and sinapic acid, respectively.



(1) Sinapic acid **(2) Sinapoyl glucose**

(3) Sinapine **(4) Canolol**

Figure 2.4 Chemical structures of sinapic acid derivatives (SADs)

After oil extraction, phenolic compounds are retained in meal in significant amounts, while some of the phenolics are also found in crude canola oil. Zacchi & Eggers (2008) reported that about 1.40 mg sinapic acid derivatives were present in one gram of crude canola oil. However, significant amounts (approximately 200 mg/kg) of canolol (4-vinylsyringol) were also present in crude canola oil, although this compound is not a naturally occurring phenol in canola plants. Canolol was reported to be a highly active antioxidant and a potent lipid peroxyl radical scavenger in many studies (Koski *et al.*, 2003; Wakamatsu *et al.*, 2005). The formation of

canolol in canola oil may be due to the decarboxylation of sinapic acid during the thermal treatment in oil processing. Wakamatsu *et al.* (2005) reported that the canolol content was significantly increased in the crude canola oil when the seed was subjected to roasting treatment. Similar results were reported in rapeseed (Spielmeyer *et al.*, 2009) and mustard seed (Shrestha *et al.*, 2012).

Tocopherols are significant antioxidants found in canola oil. They occur as four derivatives, namely alpha-, beta-, gamma- and delta-tocopherols. The total tocopherol content ranged from 312 to 928 mg/kg in crude high-erucic oil and 424 to 1054 mg/kg in crude low-erucic canola oil, respectively (Thiyam-Hollände & Schwarz, 2013). Gama-tocopherol is the major form of tocopherol in canola oil and accounts for 64% of the total tocopherol, followed by alpha-tocopherol (35%) (Goffman & Becker, 1999). Delta-tocopherol only accounted for a very small amount in canola oil (<1%) while belta-tocopherol was almost absent.

2.2 Vegetable oil refining and associated byproducts

Vegetable oil refining is a complex process to remove the undesirable non-triacyglycerol components from the oils by the most commonly used refining methods in the oil industry - physical and chemical refining. The four major stages in chemical refining include degumming, neutralization, bleaching and deodorization. However, neutralization is not included in the physical refining process where removal of free fatty acid is completed by deodorization. Figure 2.5 shows the processing stages of vegetable oil using chemical and physical refining and the different byproducts produced in each stage. The characteristics of different refining byproducts including soap stocks, wastewater, spent bleaching clay, and deodorization distillates are summarized in Table 2.2.

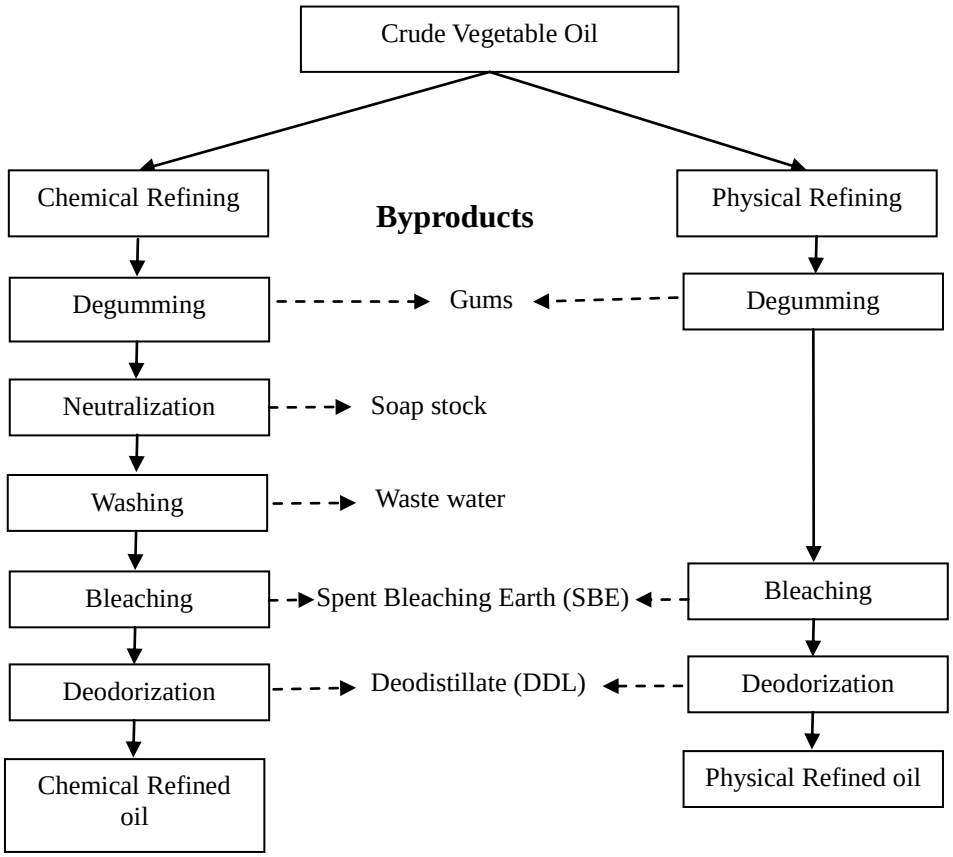


Figure 2.5 Vegetable Oil Refining Process and the Byproducts

2.2.1 Degumming

Degumming is the first stage in the oil refining process and removes gums, phospholipids, proteins, carbohydrate and colloidal components (Bockisch, 1993). The mechanism of degumming entails the use of water, acids or enzymes to remove the lipophilic character from phospholipids by hydration. A moderate temperature of 60-85 ℃ is usually applied in the degumming process to evaporate the water (Bockisch, 1993). However, the thermal treatment might result in the degradation of some heat-sensitive antioxidants.

Many studies have been conducted to determine antioxidant losses during the oil refining process. Most of the studies showed that the reduction of polyphenol and other minor components in degumming is limited in vegetable oils (Zacchi & Eggers, 2008; Ferrari *et al.*, 1996; Garcia *et al.*, 2006). Zacchi & Eggers (2008) indicated that

both super degumming and water degumming may slightly decrease the polyphenol content in rapeseed oil, but not significantly. Ferrari *et al.* (1996) also reported an approximately 6% loss of tocopherol in the sunflower oil degumming process. Similar results were found with soybean and sunflower oils, where only 4-5% tocopherols were lost after degumming (Garcia *et al.*, 2006).

The gums (byproducts produced during oil degumming) contain large concentrations of different phospholipids and some researchers have isolated lecithin from them (Ceci *et al.*, 2008; Cabezas *et al.*, 2013). Lecithin, a good source of choline or phosphatidylcholine for our body (Canty & Zeise, 1994), is also an excellent emulsifier for the production of oil-in-water emulsions and may prevent lipid oxidation (Pan *et al.*, 2013).

2.2.2 Neutralization

Oil neutralization is the process which removes free fatty acids by chemical reaction or physical methods. Alkali neutralization using caustic soda is the most common method in the oil industry. During this process, soaps (R-COONa) and water are formed from the free fatty acids by the following chemical reaction:

$$\text{R-COOH} + \text{NaOH} \longrightarrow \text{R-COONa} + H_2O$$

It has been shown that significant amounts of polyphenols and tocopherols were decreased after oil neutralization in different oils. Kraljic *et al.* (2012) reported that 63% of polyphenolic compounds and 80% of canolol was lost after rapeseed oil neutralization. Another study on olive oil refining (Garcia *et al.*, 2006) indicated that most polyphenols, especially o-diphenols, were lost in significant amounts during alkali neutralization stage. Tocopherols may also be oxidized under alkaline conditions during neutralization (Ghazani & Marangoni, 2012). Other researchers reported that the amount of tocopherol reduced during the neutralization process was

24%, 9-11% and 16.5% in rapeseed (Zacchi & Eggers, 2008), corn (Malaughlin & Anderson, 1977) and sunflower (Naz *et al.*, 2011) oils, respectively.

Soapstock is the major byproduct produced during chemical neutralization, and is usually present as a wet lipidic mixture. In South America, soybean oil refining produces approximately 100 million pounds of soapstock per year which retails at 1/10th the cost of the refined vegetable oil. The major components in soapstock are water and fatty acids (more than 80%) plus other substances including organic phosphates, monoglycerides, diglycerides, triglycerides, sterols, polyalcohols, and carbohydrates (Dowd, 1996). Due to the hydrophilic properties of most plant polyphenols, considerable amounts of phenolic compounds are reported in the soapstocks of several vegetable oils such as olive, rice bran and canola oils (Garc á *et al.*, 2006; Seetharamaiah *et al.*, 1986). Gossypol is a polyphenolic compound found in cotton seed soapstocks. Although gossypol is toxic, it exhibited strong anti-tumor (Xu *et al.*, 2005) and anti-viral properties (Keshmiri-Neghab & Goliaei) and increased people's interest in recovering this substance from soapstock. Thus, in addition to the traditional application of oil refining soapstock as animal feed additives, the recovery and isolation of polyphenols may prove to be another value-added potential of soapstocks.

Table 2.2 Characteristics of vegetable oil refining byproducts

Byproducts	Nature and Sources/generation pathway	Major components	Conventional utilization	Alternative utilization
Soap-stocks	Lipidic mixtures produced during the chemical neutralization	Fatty acids, organic phosphates, monoglycerides, diglycerides, triglycerides, sterols, polyalcohols, carbohydrates, Polyphenols	Animal feed additives	Sources of polyphenols and other bioactive compounds after extraction and isolation; Biodiesel application
Wastewater (acid oil water)	Wastewater generated from oil extraction and washing processes	Fats, organic acids, lipids, alcohols and polyphenols	No traditional application Usually directly dispose to environment	Used as organic fertilizer; Composting; Recovery of polyphenol
Spent bleaching earth (SBE)	Used bleaching earth come from bleaching of vegetable oils	Oil, chlorophyll, gossypol, carotenoids and their derivatives, polyphenols	Animal feed additives Landfill disposal	Regeneration; Recovery of oil; Biogas utilization; Soil improvement; Wastewater treatment; Recovery of bioactive compounds
Deodorization distillates (DDL)	Complex mixture come from deodorization of vegetable oils	Fatty acid, Phytosterol, tocopherol, polyphenol	Extraction and isolation of phytosterols	New sources of tocopherol and polyphenols

After neutralization, the oil is washed to remove any remaining soap-stock residues. In addition, the wastewater from the oil extraction is usually poured together with water from refining stages. Consequently, a large amount of wastewater is produced by the edible oil industry, especially the olive mill industry. It has been reported that the wastewater is the main pollutant of the oil processing industry. The oil processing wastewaters (OPWWs) usually contain complex organic compounds such as organic acids, lipids, alcohols and polyphenols (Roig *et al.*, 2006; Takaç & Karakaya, 2009). If OPWWs are not managed correctly, the complex organic substances always turn them into phytotoxic materials and lead to great environmental problems. On the other hand, OPWW is also a valuable source of organic matter and nutrients, which can be utilized as fertilizers while the polyphenols can be used as valuable antioxidants.

2.2.3 Bleaching

Oil bleaching is a process of removing color pigments such as chlorophyll, gossypol and carotenoids from neutralized oils using bleaching earth (Chazani & Marangoni, 2012). Bleaching earth is a clay material with high absorptive activities which can readily absorb the color pigments from vegetable oils (Ghazani *et al.*, 2013). During this process, activated bleaching earths can absorb both primary and secondary oxidation products from the oil. Several conditions, including the type of bleaching earth, temperature, and moisture content can affect the efficiency of bleaching (Bockisch, 1993). High temperatures, between 90 and 110 ℃, are usually employed in the bleaching process. A certain humidity, which should not exceed 0.1%, can increase the activity of bleaching earth and fats and may not be easily hydrolyzed under these conditions (Bockisch, 1993).

Due to the high absorptive properties of bleaching earth, the spent bleaching earth (SBEs) has a complex composition which may include a significant amount of oil (20-40%), chlorophyll, gossypol, carotenoids and their derivatives, and polyphenols and their oxidation

products (Pollard *et al.*, 1993; Tsai *et al.*, 2002). In integrated crushing/refining plants, SBE is usually brought back into the meal. Bleaching earth originating from pure refining plants or hardening plants may contain nickel. This is excluded from recycling into the feed materials and disposed outside the feed sector. In addition to the animal feed application and landfill disposal of SBEs, several alternative uses of SBEs, including regeneration, recovery of oil, biogas utilization, soil improvement, and wastewater treatment (Werner, 1994; Tsai *et al.*, 2002), have been reported in recent years. Few studies have focused on characterization and isolation of bioactive compounds in SBEs because of the chemical complexity of these materials. Several isolation techniques were developed for regeneration of oils from SBEs and they may be precursors of further recovery of bioactive compounds from SBEs.

2.2.4 Deodorization

Deodorization is the final stage of refining and is used to remove flavour and odour components from the oils to extend shelf-life. It is a steam distillation process using high vapour pressure that strips the odour components and other undesirable constituents such as oxidation products, phytosterols and pesticides because of their volatility (Ghazani & Marangoni, 2012). High temperatures around 240 to 260 ℃ are usually applied in this stage.

Kraljic *et al.* (2012) reported the loss of polyphenolic compounds was highest (67%) during the deodorization processing of rapeseed oil. Garcia *et al.* (2006) also reported that the polyphenols were almost completely lost in the deodorized oil. However, significant amounts of minor components such as phytosterols, tocopherols and polyphenols were observed in the byproducts of deodorization called deodorization distillates (DDL) (Lin & Koseoglu, 2003; Nogala-Kalucka, 2004; Harbaum-Piayda *et al.*, 2010). A recent study by Harbaum-Piayda *et al*. (2010) reported the presence of a newly identified dimer of canolol in significant amounts (3.50g/kg) in rapeseed deodistillates, followed by sinapic acids (500 mg/kg) and canolol (200 mg/kg). In addition to the high polyphenol content of DDL, the tocopherol content of

commercial deodistillates from different vegetable oils, such as rapeseed, soybean and sunflower, is much higher than the content in crude vegetable oils which ranged from 175.4-277.1 g/kg (Nogala-Kalucka *et al.*, 2004; Shimada *et al.*, 2000).

2.3 Extraction of phenolics from canola byproducts

It has been shown that a large amount of antioxidants are present in oil byproducts including meals and refining byproducts. Many people have recognized that these byproducts are valuable starting materials for food and pharmaceutical industries (Obied *et al.*, 2005). Techniques to extract antioxidants from the byproducts, which have been highly developed in recent decades, have been considered a way of using these valuable materials. In addition to the conventional solvent extraction of phenolics from bio-materials, other novel and efficient techniques such as accelerated solvent extraction (ASE), supercritical fluid extraction (SFE), membrane separation, and resin absorbent extraction have also been developed (Sørensen *et al.*, 2013; Pereira *et al.*, 2010; Takaç & Karakaya, 2009).

2.3.1 Solvent Extraction

Solvent extraction is the most widely used technique to extract antioxidants from various bio-materials, including both original plant materials and byproducts. Conventional solvent extraction was commonly used as a convenient method for the extraction of phenolic compounds from canola meal or hulls in the early days (Wanasundara, 1994; Amarowicz, 2000). The extraction yield was affected by various factors including type of solvents, extraction time, temperature, sample-to-solvent ratio and properties of extracted materials.

Solvent selection is the most important factor affecting the amount and rate of antioxidant extracted in solvent extraction. The selection of solvent should be based on the natural properties of antioxidants and starting materials (Pokorný & Korczak, 2001). For example, the polarity of the phenol molecule structure is very high, therefore a solvent with high polarity should be used to obtain higher extraction yield (Szydłowska-Czerniak *et al.*,

2010; Dai & Mumper, 2010). Various solvents, such as methanol, ethanol, acetone, and ethyl acetate, with different proportions of water have been used to extract phenolic compounds from canola products (Wanasundara *et al.*, 1994; Szydłowska-Czerniak *et al.*, 2010; Embaby *et al.*, 2010).

Hassas-Roudsari *et al.* (2009) reported that reflux extraction with 95% ethanol was efficient in obtaining high amounts of phenolics from canola meal with a yield of 10 mg sinapic acid equivalent per gram meal. Khattab *et al.* (2010) recommended that 70% methanol was the optimal solvent for canola meal extraction when ultra-sonication extraction was applied. Methanol was found to be the optimal solvent for extraction of sinapoylglucose in this study. Methanol and ethanol are both considered as safe solvents because of their high efficiency for low molecule weight polyphenols.

Solvent extraction is not only applicable to solid materials but is also efficient for liquid materials such as oil (Koski *et al.*, 2003) and wastewater (Tornberg & Galanakis，2008). Solvent extraction has been used to recover phenolics from olive oil refining wastewater by several researches (Obied *et al.*, 2005; Tornberg & Galanakis，2008). Obied *et al.* (2005) evaluated the extraction efficiency of 6 polar solvents from olive mill waste and reported that 60% methanol obtained maximum amount of polyphenols. A patent filed by Tornberg & Galanakis (2008) described procedures for isolating polyphenols and fibers from olive mill waste using different solvents. In their patent, ethanol was a good solvent for polyphenol extraction.

2.3.2 Accelerated Solvent Extraction (ASE)

Conventional solvent extraction is convenient; however, it results in undesirable effects on the environment and food components (Hassas-Roudsari, 2009). Many novel techniques such as supercritical fluid extraction (SFE) and accelerated solvent extraction (ASE) have been developed to reduce the amount of solvent used in the extraction.

Accelerated solvent extraction is a liquid solvent extraction technique that uses high pressure and temperatures (above boiling point), and is suitable for solid or semi-solid samples. Although it was initially invented for environmental analysis, it has proven useful for laboratory extractions in the food and pharmaceutical industries (Luthria *et al.*, 2004). ASE holds the following advantages over traditional solvent extraction: 1) reducing the amount of solvents; 2) shortening the extraction time; and 3) being more economical.

ASE is usually performed under nitrogen atmosphere with samples in sealed vials. It can be used for antioxidant extraction to avoid contact with oxygen (Luthria *et al.*, 2004). ASE may be ineffective for extracting total phenolics, but it is effective for extracting some specific phenolics. Bonoli *et al.* (2004) reported that ASE is a reliable method for extracting free phenols from barley flour. Similar studies were also conducted to extract specific target compounds from spices using ASE (Okuda *et al.*, 2009; Siriwong *et al.*, 2009). There is some evidence that thermal treatment can increase canolol content in canola meal extracts and suggesting ASE as a potential technique for canolol production. Sørensen *et al.* (2013) applied ASE at 185 °C for extracting canolol and other phenolics from defatted canola meal. Hossain *et al.* (2011) optimized the ASE conditions to maximize the antioxidant yield from rosemary, oregano and marjoram and suggested that 129 °C was the optimum temperature while solvent concentration varied between the different plant sources.

2.4 Antioxidant and bioactivities of canola phenolics

The antioxidant activities of canola phenolics such as sinapic acid, sinapoyl glucose, sinapine and canolol have been intensively studied over the past decades (Kuwahara *et al.*, 2004; Vuorela *et al.*, 2004; Thiyam *et al.*, 2006 a; b). Recent research interest in utilizing canola phenolics has been focused on two aspects: 1) addition of the phenolics in foods to enhance the oxidative stability and nutritional qualities; and (2) evaluation of the potential of

phenolics in nutraceutical or pharmaceutical applications to improve human health.

2.4.1 *In vitro* antioxidant activities of canola phenolics

A number of *in vitro* antioxidant activity assays (eg. radical scavenging, metal reducing power, metal chelating, oxygen radical absorbing capacity (ORAC) assays etc.) have been commonly used to assess the antioxidant capacity of food phenolics as they have the advantages of simple operation, time saving and effectiveness (Badarinath *et al.*, 2010). Antioxidant activity involves several mechanisms so that none of the assays above reflect all their different modes of action (Badarinath *et al.*, 2010). Consequently, the antioxidant activities determined by various assays may have large discrepancies so that it is recommended that more than one type of antioxidant assays should be used at the same time in order to obtain more reliable results of antioxidant activity (Frankel & Meyer, 2000).

Canola/rapeseed phenolics include tannins, sinapic acid, sinapine, and canolol (Kozkowska *et al.*, 1991; Amarowicz *et al.*, 2000; Vuorela *et al.*, 2005; Aachary & Thiyam-Holländer, 2013). The antioxidant activities of these bioactive phenolics have been well-documented (Table 2.3). One of the most popular methods used to assess the antioxidant capacity of food or phenolic extract is 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, because of its simplicity and effectiveness. Sinapine, sinapic acid and the canola extracts rich in these two compounds always exhibited higher DPPH radical scavenging activities than the tannins or canolol rich extracts (Table 2.3). Similarly, the metal reducing powers, superoxide anion radical scavenging activities, and low-density lipoprotein (LDL) antioxidant activities of sinapine and sinapic acid were stronger than canolol. The above results indicated that the antioxidant activities of canolol were lower in the homogeneous polar mediums than sinapic acid and sinapine. Interestingly, Terpinc *et al.* (2011) reported that the antioxidant activity of canolol in β-carotene-linoleate emulsion system was significantly higher than its corresponding hydroxycinnamic acid, sinapic acid.

The authors related this difference to the partitioning phenomena of phenolic compounds between the lipid and aqueous phase. The partition coefficient of canolol is higher than that of sinapic acid, suggesting that canolol may be more successfully inserted into the lipid bilayer and may exhibit higher antioxidant activity in the emulsion systems (Terpinc *et al.*, 2011). This suggests the potential of canolol as a promising antioxidant in emulsion or other lipid systems to prevent lipid oxidation as it does not exhibit a high antioxidant capacity in homogeneous polar mediums.

2.4.2 Antioxidant efficiency of canola phenolics in food systems

Antioxidants vary in their ability to slow down lipid oxidation in different conditions of food processing. The rapeseed/canola phenolics have been extensively studied over the last 20 years for their potential to prevent lipid oxidation in different food systems (bulk oil, oil-in-water emulsion, deep-fat frying, and meat) (Table 2.4). Different fractions or purified canola phenolic compounds including crude canola extract, sinapic acid, sinapine and canolol have all been shown to significantly improve lipid/oil stability in bulk oil, oil-in-water emulsion and meat systems and some of the studies have indicated that they are superior to synthetic antioxidants (BHA, BHT) (Thiyam *et al.*, 2006 a, b; Wanasundara & Shahidi, 1994; Wijesundera *et al.*, 2008; Sørensen *et al.*, 2013; Salminen *et al.*, 2006; Vuorela *et al.*, 2005). Some of the studies, however, have suggested the effectiveness of these phenolic compounds may vary (Thiyam *et al.*, 2006 a, b; Sørensen *et al.*, 2013).

In bulk oils, both crude and fraction of phenolic extracts of canola/rapeseed were better at inhibiting lipid oxidation than α-tocopherol (Thiyam *et al.*, 2006 a, b). Wanasundara & Shahidi (1994) reported that a higher concentration of canola meal ethanolic extracts (500 and 1000 ppm) was more effective in improving bulk oil stability than the commonly used antioxidants (BHA and BHT). The oxidative stabilities of crude canola and mustards oils were significantly improved by increasing the canolol content. Although the effects of canolol

in bulk oils have not yet been compared with sinapic acid or sinapine, it was expected to exhibit stronger antioxidant activities in lipid systems since the solubility of phenolic antioxidants is crucial to their antioxidant function (Decker & Xu, 1998) and canolol has been shown to be a high lipophilic antioxidant (Khattab *et al.*, 2014). This assumption was confirmed in a study using a canola oil-in-water emulsion system (Sørensen *et al.*, 2013). The authors found that canolol exhibited a significantly stronger effect than other sinapic acid derivatives in inhibiting lipid oxidation in oil-in-water emulsion at a concentration of 350 µM. A lower concentration (100 µM) of canolol was not that effective, suggesting the effect of canolol in emulsions was concentration-dependent.

Table 2.3 In vitro antioxidant activities of canola/rapeseed phenolics

Extracts	Bioactive phenolics	DPPH (%)	β-carotene-linoleate activity	Metal reducing power	LDL model (%)	Superoxide anion radical scavenging activities (%)
Crude tannin extract of canola hull	Tannins	44.5-50.5[1]	0.3-0.4[a1]	0.7-1.1[b1]	N/A	N/A
Crude tannin extract of rapeseed hull	Tannins	35.2-40.7[1]	0.15-0.21[a1]	0.3-0.4[b1]	N/A	N/A
Rapeseed meal phenolics	Sinapine*, sinapic acid	35-50[2]	N/A	N/A	89.4-90.3[2]	N/A
Rapeseed meal supercritical CO_2 extract	Canolol*, sinapic acid	N/A	N/A	N/A	32.0-35.0[2]	N/A
Sinapic acid	Sinapic acid	90.8[2] 55[3] 79.6[4]	70[d3]	17[c3] 0.21[e4]	95.3[3]	35[3]
Sinapine	N/A	31.5[4]	N/A	0.18[e4]	N/A	N/A
Canolol	N/A	25[3] 21.1[4]	82[d3]	5[c3] 0.10[e4]	N/A	25[3]

* Major phenolic compound in the extract

[a] absorbance values at 470 nm; [b] absorbance values at 700 nm; [c] the slope coefficient representing the dependence of absorbance on the concentration of the phenolic compound/extracts; [d] Percent of inhibition of lipid oxidation in aqueous emulsion system of linoleic acid and β-carotene; [e] absorbance values at 700 nm of 100 μM phenolic solution/extract;

[1] Amarowicz et al. (2000); [2] Vuorela et al. (2004); [3] Terpinc et al. (2011); [4] Sørensen et al. (2013)

Table 2.3 Effect of rapeseed/canola and rosemary phenolic extract on inhibition of lipid oxidation in different food systems

Food systems	Testing antioxidants, concentrations	Oil, Storage/processing conditions	Results	References
Bulk oil	Rapeseed crude and fractionated phenolic extract, 500 μM/kg	Stripped rapeseed oil, accelerate storage (40 ℃, dark)	The addition of rapeseed phenolics caused inhibition of peroxide formation. The effectiveness: Crude canola extract >sinapic acid>sinapine>α-Tocopherol>control	Thiyam *et al.* (2006 a,b)
	Ethanolic extract of canola meal, 100, 200, 500, 1000 ppm	Refined bleached canola oil, Schaal oven test conditions at 65 ℃	Canola extracts at 500 and 1000 ppm were more active than BHA, BHT and BHA/BHT/MGC and less effective than TBHQ at a level of 200 ppm.	Wanasundara & Shahidi (1994)
	Canolol produced from canola and mustard seed roasting	Canola and mustard oil accelerate storage at 60 ℃	The oxidative stability of both canola and mustard crude oils can be improved dramatically by roasting the seeds (improved canolol concentration by 7-20 times)	Wijesundera *et al.* (2008)
Oil-in-water emulsion	Fractionated extracts rich in sinapic acid, sinapine, or canolol and a non-fractionated extract, 100 and 350 μM	Canola oil-in water emulsion stored at 30 ℃	At 100 μM the effectiveness of the extracts was as follows: sinapine>whole extract> sinapic acid>canolol>Control At 350 μM the ranking was as follows: canolol>sinapine>whole extract>sinapic acid>Control.	Sørensen *et al.* (2013)
Frying	Commercial rosemary extract, 400 mg/kg	Blend of cottonseed (80%), palm (10%), and sunflower seed oil (10%), frying of potato chips at 185 ℃	The oil containing the rosemary extract showed greater antioxidant activity, and reduced darkening and rancidity more than the control oil.	Lalas & Dourtoglou (2003)
	Oleoresin rosemary extract, sage extract, BHT, BHA, 200 ppm	Refined, bleached, and deodorized (RBD) palm olein, frying of potato chips at 180 ℃	The order of effectiveness ($P < 0.05$) in inhibiting oil oxidation in RBD palm olein was oleoresin rosemary > BHA > sage extract > BHT> control.	Man & Tan (1999)
Meat	Rapeseed, camelina, soy meal, and rosemary extract, 0.3, 0.5, 0.7 g/100 g meat	Cooked pork meat oxidized for 10 days at 5 ℃	Rapeseed meal (0.5 and 0.7 g/100 g meat) and camelina meal (0.7 g/100 g meat) and their combination (addition of 0.5 g/100 g) with rosemary extract (0.04 g/100 g) were effective antioxidants toward both protein and lipid oxidation	Salminen *et al.* (2006)
	Rapeseed and pine bark phenolics extracts, 4.7-282.4 mg/100 g meat	Pork meat patties oxidized for 9 days at 5 ℃ under light	Rapeseed and pine bark were excellent antioxidants toward protein oxidation (inhibition between 42 and 64%).	Vuorela *et al*. (2005)
	Rosemary extracts (OxiKan-WS4 (4.0% rosemarinic acid), OxiKan-S10 (10% carnosic acid) and OxiKan-R8 (8% carnosic acid)), 10 mg/g meat	Cooked ground chicken patties oxidized for 21 days at 4 ℃	Lipid oxidation was minimized in meat with added extracts, as indicated by lower ($P < 0.05$) thiobarbituric acid-reactive substances, peroxide value and free fatty acid.	Maheswarappa *et al.* (2013)

Compared to bulk oil and emulsion lipid systems, lipid oxidation in other food systems such as deep-fat frying and meat is much more complicated, due to the presence of more food constitutes in the systems. The major reactions of oil deep-fat frying and their products have been previously reported (Stevenson *et al.*, 1984) and are illustrated in Figure 2.6. Evaluation of oil quality during the deep-fat frying process required the examination of the products in which the above reactions occurred, including conjugated dienes, anisidine values, di- and polymerized compounds etc. In order to improve the oil stability during deep-fat frying, antioxidants are normally added to reduce the lipid oxidation. Many antioxidants have been studied during frying (Aladedunye & Przybylski, 2009; 2012; Sharayei *et al.*, 2011; Ardabili *et al.*, 2010). Among them, TBHQ and rosemary (Rosmarinus *officinalis* L.) extracts appeared to be the most effective for deep-fat frying applications (Man & Tan, 1999; Lalas & Dourtoglou, 2003). However, the use of synthetic antioxidants including BHA, BHT and TBHQ in food is of concern to consumers as their safety has been questioned due to their health hazards in animals and humans (Deshpande *et al.*, 1996). As a result, they have been increasingly contested or even banned in a number of countries. In this context, natural antioxidants such as rosemary extracts have become more popular. Currently, rosemary extracts are used as food additives and nutraceuticals and are commercially available around the world. The price of rosemary extracts for food applications is high since tons of rosemary herbs are required to get the precious extracts. The food industry therefore needs to explore cheaper alternative sources of antioxidants.

The fact that large amounts of phenolics can be recovered from canola meal and the byproducts of canola oil processing makes the canola phenolics more competitive than other natural antioxidants for food and nutraceutical application. However, to our knowledge, no data is available on the effect of canola phenolics on vegetable oil deep-fat frying.

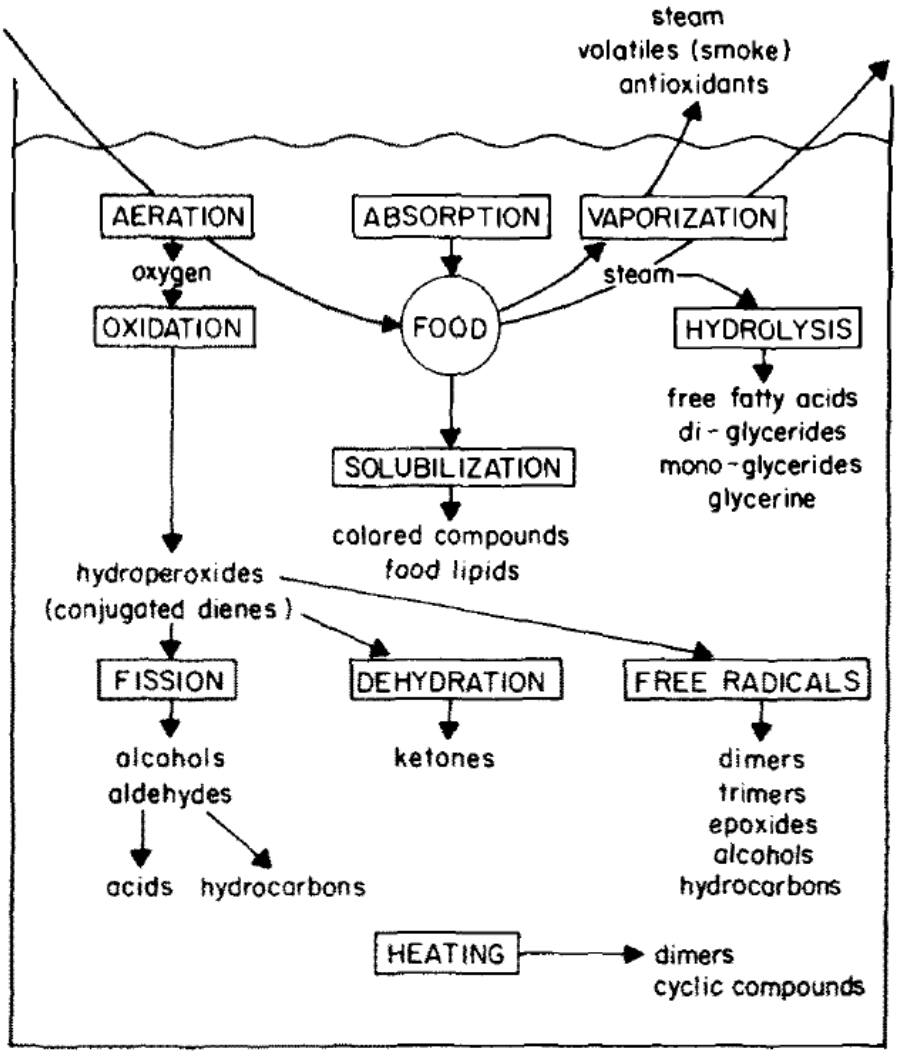


Figure 2.6 Oil changes occur in deep-fat frying process (Stevenson *et al.* 1984; Permission taken from JAOCS)

2.4.3 Bioactivities of canola phenolics

It is widely accepted that free radical formation and production of ROS in our bodies can trigger oxidative stress and affect lipid cell membranes, which are linked to a variety of chronic diseases in the human body (Shahidi, 1997). Phenolics may inactivate ROS involved in the initiation and progression of cell damages. A number of studies have reported the biological activities of canola antioxidants including sinapic acid, sinapine, and canolol (Kim *et al.*, 2010; Wakamatsu *et al.*, 2005; Cao *et al.*, 2008).

Sinapic acid and sinapine have been consumed for generations as a part of our diet,

especially through the consumption of Brassica vegetables. Several positive health promoting effects are attributed to the consumption of these dietary phenolics. Sinapine and sinapic acid are both known to have wide biological activities and effects. Sinapine has been used as a component of Chinese medicine for treating articulation and neuropathic pain, in particular chronic bronchitis disease, since ancient times (Liu *et al.*, 2006). Vuorela *et al.* (2005) reported that rapeseed meal phenolics enhanced the permeability of verapamil and ketoprofen, indicating that they may have an impact on drugs and other components being actively transported across the cell membrane. Studies also indicate that sinapic acid might play a crucial role in the neuronal protection against the peroxynitrite ($ONOO^-$) associated diseases (Kim *et al.*, 2010). In addition, they also reported that sinapic acid exerts an anxiolytic effect in mice and has peroxynitrite scavenging activity in a neurodegenerative disease model in mice.

Since the first report of the antioxidant activity of canolol by Koski *et al.* (2003), studies have expanded on the beneficial effect of canolol in biological systems. Canolol has been reported in several studies to present higher antioxidant activity and potent lipid peroxyl radical scavenger activity than sinapic acid (Kuwahara *et al.*, 2004; Wakamatsu *et al.*, 2005). Canolol isolated from crude rapeseed oil has since been shown to exhibit antioxidative, anti-inflammatory and strong scavenging capacity against the endogenous mutagen, peroxynitrite and suppression of bacterial mutation, consistent with the earlier observed protection from DNA damage, and prevention of oxidation of lipids and proteins (Koski *et al.*, 2003; Kuwahara *et al.*, 2004). Wakamatsu *et al.* (2005) subsequently proposed that canolol was more efficient as an alkyl peroxyl radical scavenger than α-tocopherol, vitamin C, β-carotene, rutin, and quercetin. Taking into account that the reactivity of peroxyl radicals is significantly lower than that of other reactive oxygen species (ROS), canolol seems to be a promising chemical agent for preventing and combating oxidative stress. Eid (2010) reported that

canolol markedly reduced the markers of lipid peroxidation and oxidative stress and maintained a fairly high level of α-tocopherol concentrations in the liver and muscles. Dong *et al.* (2010) reported that canolol exhibited a protective effect against oxidative stress-induced cell death in ARPE-19 cell. This is a very desirable property of canolol because oxidative stress has been associated with the development of a large number of chronic health disorders, such as cancer, cardiovascular disorders, atherosclerosis, and Alzheimer's disease. In addition to the antioxidant properties, canolol has also been shown to possess anti-carcinogenic, anti-mutagenic and cyto-protective properties (Cao *et al.*, 2008; Kuwahara, 2004).

2.5 Research hypotheses

Earlier studies showed that chemical refining resulted in a significant loss of antioxidants in the vegetable oil (McGinely, 1991; Ortega-Garcá *et al.*, 2006; Tasan, 2005; Ferrari *et al.*, 1996; Van Hoed *et al.*, 2006; McLaughlin & Weihrauch, 1979). In contrast, phenolics are more stable and may be retained in the byproducts of the refining (McGinely, 1991; Harbaum-Piayda *et al.*, 2010; Nogala-Kakucka *et al.*, 2004; Ghazani & Marangoni, 2013). Additional studies also suggested that canola phenolics exhibited antioxidative and bioactive properties (Koski *et al.*, 2003; Kuwahara *et al.*, 2004; Wakamatsu *el al.*, 2005). Based on the literature reviewed, the following hypotheses were developed:

(1) Endogenous phenolics are retained in the refining byproducts of post-expelled canola oil and could be recovered;

(2) Endogeneous phenolics from canola can improve the frying stability of canola oil;

(3) Endogenous phenolics from canola possess antioxidative properties in cell culture model.

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CHAPTER 3

MANUSCRIPT 1

VALUE ADDED POTENTIAL OF EXPELLER-PRESSED CANOLA OIL REFINING: CHARACTERIZATION OF SINAPIC ACID DERIVATIVES AND TOCOPHEROLS FROM BYPRODUCTS

3.1 Abstract

Valuable phenolic antioxidants are lost during oil refining, but the evaluation of their occurrence in the byproducts is lacking. Sinapic acid derivatives and tocopherols are present in rapeseed and canola oil. The retention and loss of sinapic acid derivatives and tocopherols in commercially produced expeller-pressed canola oils subjected to various refining steps and the respective byproducts was investigated. Loss of canolol and tocopherols were observed during bleaching (84.9%) and deodorization (26.1%), respectively. Additionally, sinapic acid (42.9 μg/g), sinapine (199 μg/g), and canolol (344 μg/g) were found in the refining byproducts namely soapstock, spent bleaching clay, and wash-water for the first time. Tocopherols (3.75 mg/g) and other non-identified phenolic compounds (2.7 mg sinapic acid equivalent/g) were found in deodistillates, a by-product of deodorization. DPPH radical scavenging confirmed the antioxidant potential of the byproducts. This study suggests value added potential of byproducts of refining as sources of endogenous phenolics.

Keywords: expeller-pressed canola oil, refining byproducts, phenolics, tocopherol, antioxidant activity

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3.2 Introduction

Expeller-pressed canola oil is obtained from canola seed by a combination of heat and friction. The pressing process is an environmentally-friendly and solvent-free technique that has special appeal to consumers. Expeller-pressing removes around 75% of the total oil from the oilseeds, and the oils may contain heat- and friction-induced bioactive phenolics that could enhance the oxidative stability of oils (Subramanian *et al.*, 1998; Wakamatsu *et al.*, 2005). Post-expelled and solvent extracted crude canola oils contain a high amount of polyphenols and tocopherols (Wakamatsu *et al.*, 2005; Vuorela *et al*., 2003). Hydrophilic and hydrophobic sinapic acid derivatives, namely sinapine (the choline ester of sinapic acid), free sinapic acid, and canolol (a decarboxylated product of sinapic acid), are the major phenolics found in post-expelled crude canola or rapeseed oils (Wakamatsu *et al.*, 2005; Vuorela *et al.*, 2003). Although sinapic acid and sinapine occur in canola seed, only a small portion are transferred to the canola oils (sinapic acid: ~16 μg/g, sinapine: ~19 μg/g) (Vuorela *et al.*, 2003). Canolol, found at a concentration of about 200 μg/g in degummed canola oil, is primarily responsible for the antioxidant activity of crude canola oils (Wakamatsu *et al.*, 2005). Our recent study (Aachary *et al*., 2014) showed that canolol retarded darkening (Y value) of refined canola oil during deep frying. Canolol also exhibited promising bioactivities such as anti-inflammatory, anti-mutagenicity, and DNA protection activities (Kuwahara *et al.*, 2004; Cao *et al.*, 2008).

However, in addition to canolol, crude canola oil contains other constituents such as phospholipids, free fatty acids, pigments, proteins, sterols and degradation products, which may adversely influence the quality of the final oil (Koski *et al.*, 2003). Refining removes these undesirable components from the oils, improving oil quality. Unfortunately, some beneficial, antioxidative components in the oils are also removed, compromising stability (McGinely, 1991). Indeed, considerable losses of antioxidants during refining in most

vegetable oils (including sunflower (Ortega-Garcia *et al.*, 2006; Tasan *et al.*, 2005), soybean (Ferrari *et al.*, 1996), rice bran (Van Hoed *et al.*, 2006), corn (Ferrari *et al.*, 1996; McLaughlin *et al.*, 1979) and rapeseed (Koski *et al.*, 2003; Zacchi & Egger, 2008) have been reported. Koski *et al.* (2003) observed that both sinapic acid and canolol in rapeseed oil were significantly reduced by the super degumming process and removed completely by the deodorization. Koski *et al.* (2003) and Zacchi & Eggers (2008) showed a 30-80% tocopherol reduction in rapeseed oil during the chemical refining processes.

These refining processes generate a number of byproducts (Figure 3.1). Soapstock, the by-product of the neutralization step, is produced by the reaction of free fatty acids and alkali (Zeldenrust, 2012). Recently soapstocks have been widely utilized for agricultural and industrial applications such as substrates for growth of industrially relevant microorganisms, various chemical processes (e.g. free fatty acid transesterification), fertilizer ingredient, raw material for animal feed and/or a novel source for biodiesel production (Luxem & Troy, 2004). The utilization of soapstock to recover other bioactive compounds is an emerging area to add value to the edible oil processing and refining sector. After neutralization, water is commonly added to the oil to reduce the soap content. This soapy water containing the neutralized lipids is a commercial by-product and is referred to in the present study as wash-water. After these refining steps, bleaching and deodorization follow. Spent bleaching clay is generated as a by-product during bleaching. Finally, edible oil deodorization is conducted to remove off flavors utilizing steam distillation under elevated temperature and vacuum conditions (Dudrow, 1983). This results in the production of another by-product, deodistillates or deodorization distillates, which are a rich source of many bioactive compounds including tocochromanols, phytosterols and polyphenols. For instance, a novel canolol dimer, in addition to sinapic acid and canolol, was detected in significant amounts (3500 mg/kg) (Harbaum-Piayda *et al.*, 2010). Thus, the economic values of the oil refining

byproducts can be increased by recovering these valuable bioactive components such as tocopherols and the sinapic acid derivatives (Harbaum-Piayda *et al.*, 2010; Nogala-Kakucka *et al.*, 2004).

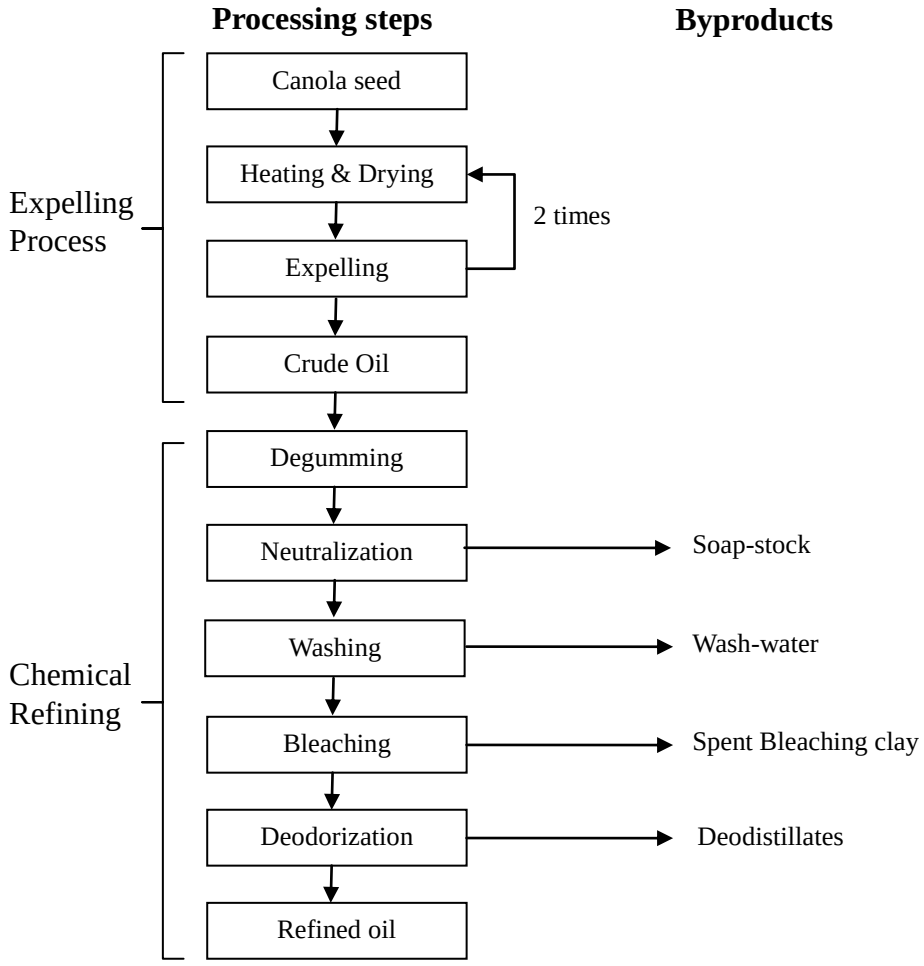

Figure 3.1 Expeller-pressing process for canola oil and the corresponding chemical refining and residual byproducts.

Another implication of the refining processes is that bioactive antioxidants of the edible oils are lost, possibly degraded or even leached during the high-temperature processing of refining (Harbaum-Piayda *et al.*, 2010; Cvengros, 1995). The majority of the antioxidants found in canola are polyphenols with a hydrophilic nature (Thiyam *et al.*, 2006), and thus, may leach into wash-water or soapstock. There are only a few studies associating the antioxidant compositions and their oil refining byproducts (McGinely, 1991; Harbaum-

Piayda *et al.*, 2010; Nogala-Kakucka *et al.*, 2004; Ghazani & Marangoni, 2013). Ortega-Garc á *et al.* (2006) reported many polyphenols in the olive oil refining byproducts (819.9 mg/kg of soapstock and 134.4 mg/kg of wash-water samples). The reports that these endogenous antioxidants are being removed by the adsorbent during the bleaching process make the corresponding by-product, the spent bleaching clay, a candidate for a further recovery study (Ghazani & Marangoni, 2013).

These polyphenols and tocopherols have added value by being both abundant and naturally occurring for potential use in cosmetic, food and pharmaceutical applications (Wanasundara & Shahidi, 1994; Vuorela *et al.*, 2005; Jiang *et al.*, 2013). Although several studies have been conducted to evaluate the loss of these antioxidants from edible oil refining (McGinely, 1991; Ortega-Garc á et al., 2006; Tasan, 2005; Ferrari et al., 1996; Van Hoed *et al.*, 2006; McLaughlin & Weihrauch, 1979), only a few of these studies explored the possibility of recovery from the corresponding refining byproducts. Thus, this paper is the first ever attempt to characterize and quantitate the retention and loss of sinapic acid derivatives and tocopherols in the corresponding byproducts associated with the refining processes. In addition, characterization and quantification of antioxidant phenolics of expeller-pressed canola oils at different stages of refining were also studied. Furthermore, the rapid extraction techniques of these bioactive phenolics were also examined to highlight the value addition potential of byproducts of refining.

3.3 Materials and Methods

3.3.1 Industrial samples and chemicals

Canola oils from different refining stages (crude, degummed, neutralized, bleached, and deodorized oils), and corresponding byproducts including soapstock, wash-water, spent bleaching clay and deodistillates were supplied by Viterra Canola Processing Inc. (Winnipeg, MB, Canada). All samples were collected from the same batch of processed oil and stored at -

20 ℃ before analysis. All chemicals used in this study are purchased from Sigma-Aldrich and of analytical grade.

3.3.2 Extraction of sinapic acid derivatives

Canola oils: Canola oils were extracted with aqueous methanol (70%) as previously described (Koski *et al.*, 2003). Briefly, two grams of each sample were dissolved in 5 mL extraction solvent, vortexed for 3 min followed by centrifugation at 5000 x *g* for 5 min in refrigerated conditions. The supernatant was collected, and the residue was re-extracted following the same procedure one more time. The supernatants were pooled, and the mixture was then made up to 10 mL using 70% aqueous methanol.

Refining byproducts: Wash-water and deodistillates was extracted using the method described above to produce "Extract-1." Additionally, they were also extracted with 50% aqueous methanol to produce "Extract 2-5" as previously described (Nogala-Kakucka *et al.*, 2004). Briefly, samples (2 g) were solubilized with 10 mL hexane (1:5 w/v), followed by extraction using 50% methanol (3 mL) three times. Instead of hexane, other non-polar solvents such as chloroform, cyclohexane, and toluene were used for solubilization prior to extraction. The methanolic extracts were pooled and made up to 10 mL using 50% methanol.

Spent bleaching clay was defatted using a Soxtec 2050 (Foss-Tecator, Foss North America, MN, USA) and the oil was extracted as described elsewhere. The defatted spent bleaching clay was extracted following the procedure described by Khattab *et al.* (2010). Briefly, spent bleaching clay sample (1 g) was extracted with 9 mL 70% methanol with ultrasound treatment for 1 minute twice. The filtrates were pooled and made up to 30 mL.

The soap-stock samples (pH 12) were adjusted to two different pH levels (7 and 2) using 2M HCl and extracted with 10 mL of ethyl acetate thrice. The extracts were pooled, concentrated in a rotary evaporator at 50 ℃ to dryness and then re-dissolved in 5 mL 70% methanol.

3.3.3 Extraction of tocopherols

The oils, wash-water or deodistillate samples were extracted directly with absolute methanol and methanol/isopropanol (1:1, v/v) mixture for several times (Tasioula-Margari & Okogeri, 2001). Briefly, 2 grams of oil or by-product sample were first extracted with 10 mL of absolute methanol for 3 min. The residue was extracted with 10 ml of methanol/isopropanol mixture (1:1, v/v) thrice. The supernatants were pooled and made up to 40 mL, filtered using a Whatman No 1 filter paper, evaporated to dryness under N_2 and re-dissolved in 5 ml of the methanol/isopropanol mixture.

3.3.4 Synthesis of canolol

The synthesis of canolol was carried following a previously reported method (Sinha *et al.*, 2007) with slight modifications. Syringaldehyde and malonic acid were mixed and microwave irradiated for 5 min with piperidine-acetic acid mixture as condensing agent using a domestic microwave oven. The highly viscous reaction mixture was diluted with 70% methanol and filtered using syringe filters (0.45 μm) prior to HPLC quantification. The purity of the obtained canolol was approximately 82%.

3.3.5 HPLC analyses of sinapic acid derivatives and tocopherols

Sinapic acid derivatives were quantified using a reversed-phase HPLC-DAD (Dionex, Sunnyvale, USA), as described previously (Khattab *et al.*, 2010), with an 80 mm × 4.0 mm i.d., 4 μm, Synergi Fusion-RP column (Phenomenex, Torrance, Canada). Standards of sinapine, sinapic acid, and synthetic canolol were used to authenticate the retention time and UV absorption spectra. The contents of all sinapic acid derivatives were expressed as sinapic acid equivalents (SAE) in μg/g of sample, wherein sinapine and sinapic acid was detected at 330 nm and canolol was detected at 270 nm. Standard of sinapic acid was prepared in the concentrations between 50 to 500 μg/mL.

The chromatographic separation of tocopherols was performed on a 150 mm x 4.6 mm

i.d. 5µm, C18 Prodigy ODS-2 column (Phenomenex, Torrance, Canada) as described previously (Tasioula-Margari & Okogeri, 2001), with slight modifications. The elution solvents used were (A) 1% acetic acid/water (1:99) and (B) 1% acetic acid/methanol (1:99). An isocratic elution was performed with 1% A and 99% B for 30 min. The column was maintained at 25 ℃ with a flow rate of 1.0 ml/min. The injection volume was 20 µL. Chromatograms were acquired at 294 nm; identification of tocopherols was achieved by comparing the relative retention times and spectrum with the standards of α-, β and γ-tocopherols. Triplicate samples were analyzed with duplicate injections of each sample for statistical validation of results.

3.3.6 Estimation of total phenolic content

Total phenolic contents in the extracts of oil and their corresponding refining byproducts were estimated using the Folin–Ciocalteu reagent as described (Thiyam *et al.*, 2006; Swain & Hillis, 1959), using a DU 800 UV/Vis Spectrophotometer (Beckman Coulter Inc., Mississauga, ON, Canada). An authentic standard of sinapic acid was used for calibration purpose, and the results of duplicate analyses were expressed as µg SAE/g of sample.

3.3.7 Estimation of antioxidant activity

The antioxidant activity of the phenolic extract was determined by the free radical scavenging activity using DPPH (2,2-diphenyl-1-picrylhydrazyl) according to the method proposed by Brand-Williams *et al.* (1995) with slight modifications. Briefly, 1 ml of extract was added to 2.9 ml of 0.1 mM DPPH ethanolic solution and reacted for 10 min, and the absorbance was measured at 516 nm. The linear range of the calibration curve of sinapic acid standard was prepared from 10 to 500 µM, and the antioxidant activity was expressed as µM SAE/g of sample.

3.3.8 Statistical Analyses

All extractions were carried out in triplicate with duplicate analysis of samples and results were presented as mean ± SD (Microsoft Excel, Version 2010). One way analysis of variance (ANOVA) was done using SAS 9.4 software (SAS Institute Inc., USA). Statistical significance was determined using least significant difference *t*-test. Statistical significance was accepted at $p<0.05$. Regression analysis was carried out using Microsoft Excel (Version 2010).

3.4 Results and Discussion

3.4.1 Effect of refining on sinapic acid derivatives and tocopherol contents of expeller-pressed canola oil

The contents of endogenous phenolics of the various canola oils are indicated in Table 3.1. With respect to sinapic acid derivatives, crude expeller-pressed canola oil retained canolol (696.6 μg SAE/g), sinapic acid (30.5 μg/g oil) and sinapine (beyond detection limit) in agreement with the levels observed in Koski et al. (2003). The amount of canolol in canola oil was slightly decreased during the degumming process, while the concentration of sinapic acid was not significantly affected. Sinapic acid decreased to an undetectable level in the canola oils during neutralization while the concentration of canolol was slightly increased. In the industrial oil refining practices, neutralization of oil is commonly conducted by converting free fatty acid to soaps by adding caustic soda solutions and the neutralized oil is normally obtained after soapstock separation, oil washing and drying. The increase of canolol content in neutralized oil might be attributed partially due to the conversion of sinapic acid and other precursors, accelerated by higher temperatures during the drying of washed oil. Thermal decarboxylation and cleavage of sinapic acid has been previous reported in different heat treatments (Wakamatsu *et al.*, 2005; Bernini *et al.*, 2007), but further studies are required to confirm the contribution this increase. This contradicted Zacchi's *et al.* (2008) findings that

polyphenol content was reduced after neutralization. However, the neutralization process in Zacchi's *et al.* (2008) study was simulated in a laboratory condition which may be different from industrial practices. Also, different conditions of neutralization such as the type and concentration of caustic solution and varieties of oilseeds may have significant influence on the content of polyphenols in oils (Ghazani &Marangoni, 2013). A significant amount of canolol was lost during the bleaching process (about >90%), wherein it was further reduced to an undetectable level during deodorization. Substantial reductions in rapeseed oil polyphenols during bleaching and deodorization processes have been previously reported (Zacchi & Eggers, 2008); the authors related this to the high adsorptive activity of the bleaching clay and the elevated temperatures of the deodorization process.

With respect to the total phenolic content, solvent-extracted crude canola oils contain about 125 ppm (as gallic acid equivalents) total phenolics, and this included sinapine, sinapic acid and 4-vinyl-2,6-dimethoxyphenol (canolol) (Ghazani & Marangoni, 2013). In the current study, total phenolic contents of expeller-pressed canola oil extracts were assessed using HPLC-DAD and Folin-Ciocalteu method. The phenolic contents estimated by these two different methods, HPLC and Folin-Ciocalteu were compared to determine the correlation. The HPLC-DAD based total phenolic content was the highest in the crude oil and decreased slightly during degumming and neutralization (Table 3.1). Bleaching and deodorization further reduced the total phenolic content to a negligible quantity. The total phenolics quantified by Folin-Ciocalteu method indicated a similar trend, while the values were much lower than the total phenolics detected through the DAD-HPLC method. Folin-Ciocalteu assay is a well-known method used to determine total phenolic content; however, it is not efficient to measure the decarboxylation products of the hydroxycinnamates (Terpinc *et al.*, 2011). Previous studies were in agreement that the Folin-Ciocalteu method could underestimate canolol.

With respect to tocopherols, another class of antioxidants, the total tocopherol content in crude expeller-pressed canola oil was estimated to be 374 mg/kg with α- and γ-tocopherol in an approximate ratio of 1:3 and δ-tocopherol was absent. Degumming and neutralization had little or no effect on the tocopherols, but bleaching resulted in a slight reduction of γ-tocopherol in canola oil. This study was consistent with earlier studies on other vegetable oils (Ferrari et al., 1996; Cmolik et al., 2008; Naz et al., 2011), bleaching resulted in a slight reduction (6%) and deodorization led to the most significant loss (about 37.6%) of tocopherols.

Table 3.1 Effect of Chemical Refining Steps on the Phenolic and Tocopherol Compositions of Expeller-Pressed Canola Oils

Antioxidant composition	Crude oil	Degummed oil	Neutralized oil	Bleached oil	Deodorized oil
Sinapic acid (μg/g)	30.5 ±0.5[a]	36.2 ±3.5[a]	N.D.[b]	N.D.[b]	N.D.[b]
Canolol (μg SAE/g)	697 ±17[a]	547 ±11[b]	644 ±91[ab]	26.8 ±0.9[c]	N.D.[d]
Sinapine (μg SAE/g)	T.A.	T.A.	T.A.	N.D.	N.D.
α-Tocopherol (mg/kg)	159 ±3[a]	163 ±13[a]	164 ±7 [a]	177 ±9[a]	113 ±15[b]
γ-Tocopherol (mg/kg)	472 ±3[a]	473 ±30[a]	477 ±12[a]	419 ±15[b]	246 ±9[c]
TPC-HPLC (μg SAE/g) [1]	727 ±172[a]	583 ±110[b]	644 ±91[b]	26.8 ±0.9[c]	ND[d]
TPC-FC(μg SAE/g)[2]	107 ±14[a]	89.1 ±11.1[b]	83.8 ±12.2[b]	2.7 ±0.3[c]	ND[d]
Total Tocopherol (mg/kg)[3]	631 ±6[a]	636 ±43[ab]	641 ±19[a]	596 ±24[b]	359 ±24[c]

All data are expressed as mean ± standard deviation (n=6), mean values in each row share same letters are not significant different (p<0.05)
N.D.-No detected; T.A.-trace amount; SAE-sinapic acid equivalent
1: Total phenolic content calculated by adding the major phenolic peaks in HPLC: sinapic acid + canolol
2: Total phenolic content calculated by Folin-Ciocalteu assay

3.4.2 Sinapic acid derivatives and tocopherol profiles in refining byproducts

3.4.2.1 Soapstock

Sinapic acid was the only phenolic compound detected in the soapstock used in this study (Figure 3.2 B). The amount of sinapic acid in soapstock ranged from 17.1 to 42.9 µg/g depending on the extraction pH. Although many studies have noted that high amounts of polyphenols are extractable from soapstocks of some vegetable oils such as rice bran (Seetharamaiah & Prabhakar, 1986; Rao *et al.*, 2002) and olive (MaGinely, 1991) oils, the potential of obtaining sinapic acid from canola oil soap-stock was not considered. Our results provided the first data to support the recovery of sinapic acid from canola soapstock. Acid treatment increased the extraction efficiency through solubilization of sinapic acid from soap-stocks by 1.8 and 2.5 times at pH values of 7 and 2, when compared to pH 12 (Table 3.3). The total phenolics obtained with the Folin-Ciocalteu assay (TPC-FC) also supported this trend. The result conformed with previous observations (Prapakornwirya & Diosady, 2008), wherein they indicated that the acid treatments of phenolic extracts helped to prevent the oxidation of sinapic acid. The TPC-FC values of soapstock extracts were higher than that determined by HPLC, which may be attributed to the Folin-Cioacalteu reactive phenolics and other interfering compounds.

Sinapic acid present in the crude and degummed oils was lost during neutralization perhaps either leached or adsorbed in soapstock. The storage of soapstocks at room temperature is also of special interest as the alkaline pH might contribute towards unavoidable side reactions thereby affecting the composition of minor components including antioxidants and their stability (Ruenroengklin *et al.*, 2008). This instability could be partially avoided by neutralization or acidification of samples facilitating a rapid extraction and recovery of stable antioxidants.

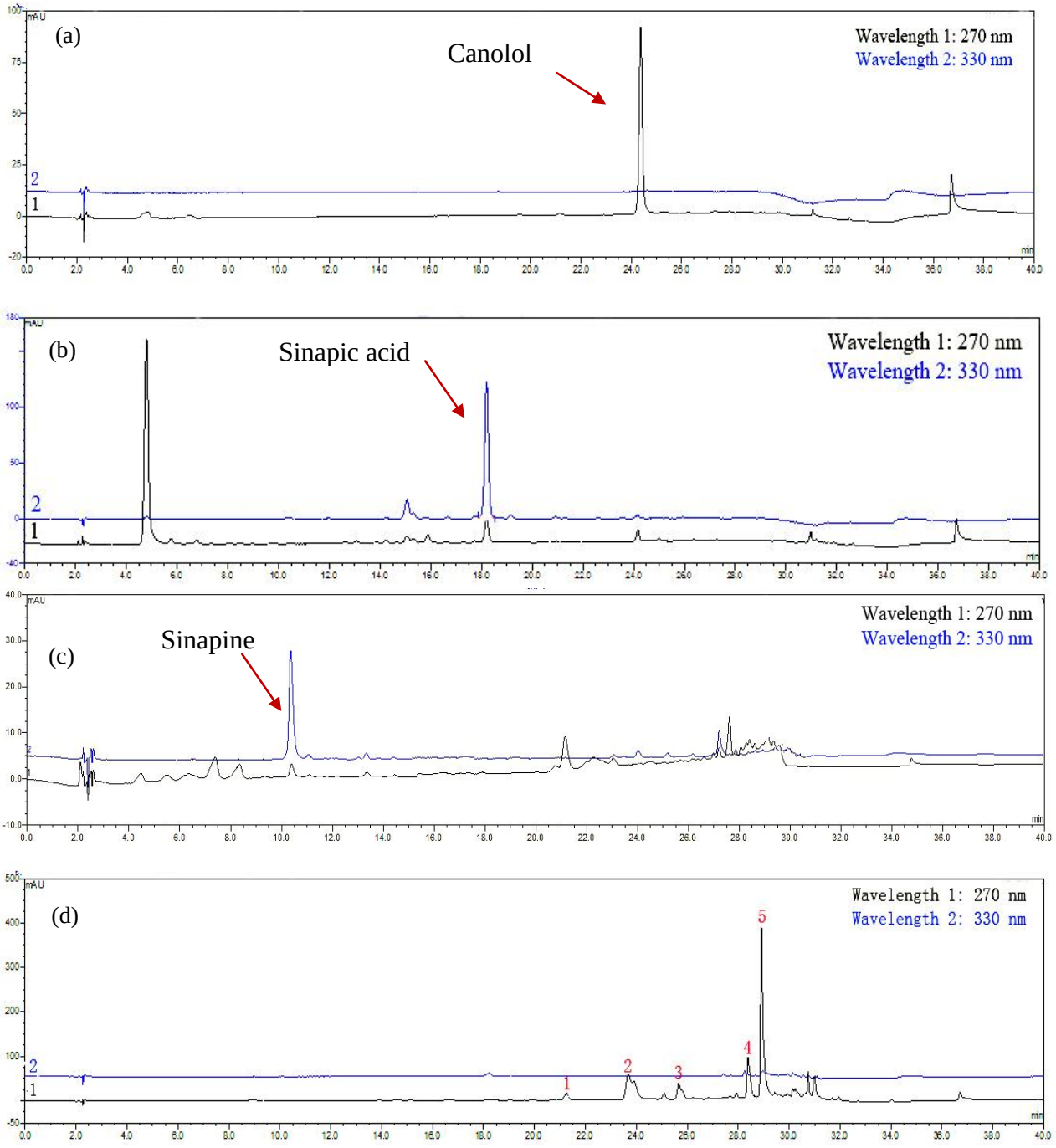

Figure 3.2 HPLC-DAD chromatograms of phenolic extracts obtained from canola oil refining byproducts (a) wash-water, (b) soap-stock, (c) spent bleaching clay and (d) deodistillates.

Table 3.2 Phenolic and Tocopherol Compositions of Expeller-Pressed Canola Oil Refining By-Products

Antioxidant composition	Bleaching Clay	Wash water	Soapstock	Deodistillates
Sinapine (μg SAE/g)	199.3 ±58.5[a]	N.D.[b]	N.D.[b]	N.D.[b]
Sinapic acid (μg/g)	N.D[b]	N.D[b]	42.9 ±2.7[a]	N.D.[b]
Canolol (μg SAE/g)	1.42 ±0.02[b]	365.2 ±20.9[a]	N.D.[c]	N.D.[c]
α-Tocopherol (mg/kg)	9.64 ±0.25[c]	168.6 ±2.1[b]	N.D.[d]	647 ±6[a]
γ-Tocopherol (mg/kg)	14.84 ±0.16[c]	278.4 ±2.0[b]	N.D.[d]	3104 ±16[a]
TPC-HPLC (μg SAE/g) [1]	201 ±58[c]	365.2 ±20.9[b]	42.9 ±2.7[d]	2766 ±2[a]
TPC-FC(μg SAE/g)[2]	677 ±179[a]	167.2 ±10.3[b]	147 ±15[b]	526 ±11[a]
Total Tocopherol (mg/kg)	24.5 ±0.5[c]	446.8 ±2.8[b]	N.D.[d]	3751 ±21[a]
DPPH (μM SAE/g)	140 ±15[bc]	173.5 ±10.7[b]	106.7 ±11.7[c]	350 ±15[a]

All data are expressed as mean ± standard deviation (n=6), mean values in each row share same letters are not significant different (p<0.05)
N.D.-No detected; SAE-sinapic acid equivalent
1: Total phenolic content calculated by adding the major phenolic peaks in HPLC: sinapine + sinapic acid + canolol + unidentified polar compound
2: Total phenolic content calculated by Folin-Ciocalteu assay

Table 3.3 Effect of Different Conditions on the Phenolic Extraction Efficiency and DPPH Radical Scavenging Activity of Canola Oil Refining By-Products

Refining by-products	Extraction conditions	TPC-HPLC (μg SAE/g)	TPC-FC(μg SAE/g)	DPPH (μM SAE/g)
Wash Water	Hexane	448 ±74^{d}	190 ±21^{c}	189 ±15^{c}
	Choloroform	69.8 ±0.1^{f}	29.0 ±3.2^{e}	18.6 ±3.0^{f}
	Cyclohexane	419 ±31^{d}	178 ±5^{c}	168 ±12^{c}
	Toluene	167 ±7^{e}	60.2 ±11.3^{d}	54.2 ±23.3^{e}
	Direct methanol extraction	365 ±21^{d}	167 ±10 ^{c}	174 ±11^{d}
Deodistillates	Hexane	2537 ±28^{a}	525 ±10 ^{a}	336 ±11 ^{a}
	Choloroform	54.1 ±5.0^{f}	79 ±46^{d}	168 ±67^{c}
	Cyclohexane	2065 ±129^{b}	523 ±20 ^{a}	345 ±12^{a}
	Toluene	649 ±114^{c}	354 ±60^{b}	295 ±28^{b}
	Direct methanol extraction	2766 ±2^{a}	542 ±36^{a}	350 ±14^{a}
Soap stock	pH=2	42.9 ±2.7^{f}	147 ±15^{c}	107 ±2^{d}
	pH=7	31.2 ±4.3^{g}	89.4 ±23.6^{d}	96.5 ±17.3^{d}
	pH=12	17.07 ±0.22^{h}	69.2 ±11.9^{d}	22.6 ±6.4^{e}

All data are expressed as mean ± standard deviation (n=6), mean values in each column share same letters are not significant different (p<0.05)

3.4.2.2 Wash-water

Considerable amount of phenolics (365 μg SAE/g) were also observed in the canola oil wash-water in the present study (Table 3.2). Similar results have been reported by many investigators assessing the polyphenol content of wash-water from olive oil refining (Ortega-Garcia *et al.*, 2006; Fki *et al.*, 2005). Canolol was the predominant phenolic compound present in the wash-water sample (69.8 - 448 μg SAE/g) with minor variations based on the solvent used for solubilizing it prior to methanolic extraction (Figure 3.2 A, Table 3.3). For example, solubilization of the wash-water sample with hexane and cyclohexane resulted in higher yield of canolol (448 and 419 μg SAE/g, respectively) compared with chloroform (69.8 μg SAE/g) and toluene (167 μg SAE/g). The presence of canolol in wash-water is because of the high lipid content (>50%) in the sample, associating more of the fat-soluble antioxidants. The results also indicated that canolol was retained during neutralization.

This study demonstrated a rapid method to extract and identify sinapic acid derivatives in edible oil refining wash-water. Results supported that the wash-water retains canolol. Additionally, the tocopherol composition of these samples was assessed, and the result showed that it was similar to that of degummed or neutralized oils. The concentrations of α- and γ-tocopherol in wash-water were 169 and 278 mg/kg, respectively (Table 3.2). The retention of canolol and tocopherols in the wash water could be attributed to the minor amount of neutralized oil that was carried through in the wash-water. Neutralized oils retained canolol and tocopherols as discussed above.

3.4.2.3 Spent Bleaching Clay

Total phenolic content (Folin-Ciocalteu assay) of spent bleaching clay was determined to be 677.9 μg SAE /g and the tocopherol compositions are summarized in Table 3.2. A substantial amount of sinapine (199 μg SAE/g) was present in the spent bleaching clay. It might be due to the occurrence of sinapine in neutralized oil, and it is carried to the bleaching

clay. Sinapine might be concentrated in the bleaching clay due to the high adsorptive activities of bleaching clay. Bleaching clay has been primarily used to remove color pigments such as chlorophyll and carotenoids from vegetable oils (Ghazani & Marangoni, 2013), and high polyphenol adsorption efficiency of has been reported previously (Pollard *et al.*, 1992; Eroglu *et al.*, 2008). The absence of sinapine in bleached and deodorized oils also confirmed this result (Table 3.1). However, phenolics including sinapic acid and canolol were not detected in the bleaching clay. Low amounts of tocopherols were detected in the spent bleaching clay.

It has been suggested by Loh *et al.* (2013) that the residual oil in spent bleaching clay should ideally be extracted and used for industrial applications in order to reduce cost in oil processing. Unlike palm oil refining process, canola oil refining may not generate high residual oil in the bleaching clay but could be used as a novel source for recovering sinapine as demonstrated by our results.

3.4.2.4 Deodistillates

Total phenolic content (Folin-Ciocalteu assay) of deodistillates was 525.7 μg SAE/g, the highest among of all the refining byproducts (Table 3.2). In the extracts of deodistillates, all peaks were identified at 270 nm and the chromatograms are presented in Figure 3C. There were five unidentified peaks eluting between 21 and 29 min, which have not been previously reported. The UV spectra (maximum absorbance) of these peaks are summarized in Table 4. The highest concentrations of the phenolic compounds were observed for the peaks detected at 28 to 29 min (peak 4: 349.7 μg SAE/g and peak 5: 1708.6μg SAE/g). The major peak (Peak 5, retention time: ~28.9 min) exhibited maximum absorbance at 279 nm and did not correspond to any phenolic compounds presented in canola seed or canola oil. The spectrum of the peak 4 had two wavelength maxima at 209 and 281 nm. It is likely that these phenolic compounds are derivatives of canolol or other sinapates. A previous study reported canolol

dimer as the predominant phenolic compound in commercial rapeseed deodistillates based on the dimerization at high temperature or in acid-catalyzed conditions (Dudrow, 1983). The phenolic compounds found in the current study might be oligomers of canolol; however, further studies are required for their structural elucidation and characterization.

Table 3.4 Concentration and UV Spectra Data of Phenolic Peaks of Deodistillates Extracts Detected at 270 nm

Peak number[1]	Retention time (min)	UV maximum absorbance wavelength (nm)	Concentrations (μg SAE/g)
1	21.3	217, 275	52.42 ±2.67
2	23.6	282	180.6 ±3.5
3	25.6	206, 273	182.3 ±23.7
4	28.4	209, 281	349.7 ±20.0
5	28.9	279	1708.6 ±21.6

1: Peak numbers referred to the peaks in Figure 2 D

The polarities of various extraction solvents have a tremendous effect on the solubility and, therefore, extraction efficiency (Chen & Ho, 1995) of phenolics. The efficacy of different non-polar solvents to optimally and rapidly extract deodistillate phenolic compounds was investigated (Table 3.3). The results were similar to that of phenolic extraction in wash-water, where in solubilization with hexane and cyclohexane followed by methanolic extraction produced a higher amount of phenolics (2537 and 2065 μg SAE/g) from deodistillates than pre-dissolution with chloroform and toluene. Direct methanol extraction of deodistillates resulted in the highest yield of phenolics compared to pre-solvent solubilization of deodistillates. However, pre-solvent solubilization showed advantages in improving extraction yield (37% improvement) at large scale extractions as reported in a previous study (Aarchary *et al.*, 2014).

The present study also identified deodistillates as a rich source of tocopherols in addition to other phenolics. Both α- and γ-tocopherols were detected in the deodistillates at 2:1 ratio.

The deodistillate extract contained 647mg of α-tocopherol and 3104 mg of γ-tocopherol per kg of deodistillate, a quantity that is greater than those in any crude vegetable oils. These amounts conformed with the values obtained from rapeseed oil deodistillates (Luxem & Troy, 2004).

The study indicated a two-fold potential of deodistillates. Firstly, deodistillate was the best source for recovery of tocopherols amongst the oil refining byproducts investigated. The tocopherol content of canola oil at various processing stages supported that the maximum tocopherol losses occurred during the deodorization process. Secondly, the extracts of deodistillate showed several phenolic compounds which were not reported until now, requiring further studies for adequate characterization.

3.4.3 Antioxidant activity of phenolic extracts of canola oil and refining byproducts

The antioxidant capacity of expeller-pressed canola oils and refining byproducts were evaluated using the DPPH radical scavenging activities. In the present study, the crude canola oil showed the highest antioxidant activity at a DPPH free radical activity equivalent of 311.9 ± 23.1 μM SAE/g. These values decreased slightly after degumming and neutralization while reduced rapidly to less than 50 μM SAE/g after the bleaching step (Figure 3.3). The decrease was mainly attributed to the loss of endogenous phenolics and tocopherols from expeller-pressed canola oil during refining. Previously, Wakamatsu *et al.* (2005) reported a significant reduction in the anti-ROO · activity of canola oil with each refining stage. The loss of antioxidant activity of oils was translated to a high antioxidant potential of the corresponding refining by-product in the present study. Among various oil refining byproducts, deodistillates exhibited the highest radical scavenging activity (μM SAE/g) of 350.3, followed by wash-water (173.5), bleaching clay (139.7) and soapstock (106.7) (Figure 4). This order corroborated with the concentration of the active phenolic compounds contained in the corresponding refining byproducts.

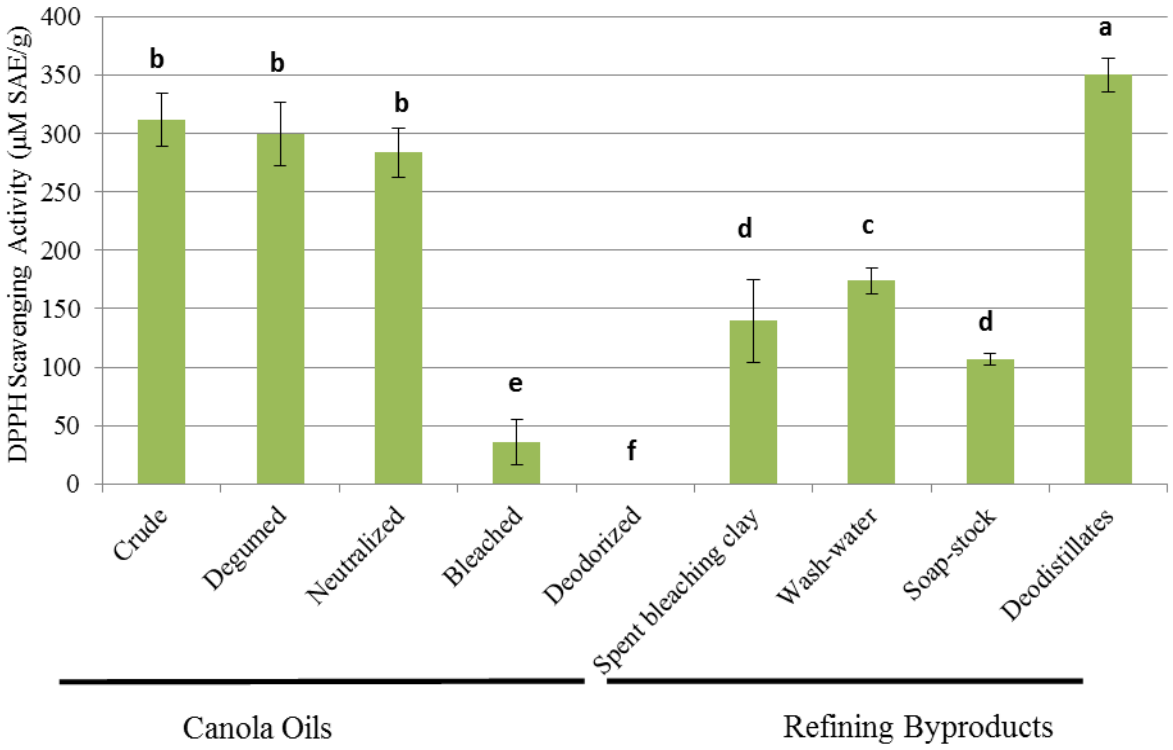

Figure 3.3 DPPH antioxidant activities of expeller canola oils and refining byproducts

In order to further examine the radical scavenging activity of the phenolic compounds recovered from canola oils and the corresponding refining byproducts, the correlation of the sum of phenolic compounds and antioxidant activity was calculated. There were strong correlations between the total phenolic content and radical scavenging activity in all refining byproducts as well as canola oil (R^2 = 0.88-0.99) (Figure 3.4). In addition, the radical scavenging activity of soap-stock phenolic extract increased rapidly (slope: 5.670) as the concentration of phenolics (majorly sinapic acid) increased. While this slope of increase was lower in canola oil (0.414) and wash-water (0.458) extracts, and it was the lowest in deodistillate (0.298) extracts. This result indicated that the DPPH radical scavenging activity of sinapic acid might be more apparent than sinapine, canolol and canolol derivatives at the same concentration. A previous study asserted the 4-vinyl derivatives of hydroxycinnamic acids exhibited lower antioxidant activity than their corresponding phenolic acids (Terpinc *et*

al., 2011). Other studies reported the opposite trend when evaluating the antioxidant potential in different systems such as emulsion (Galano *et al.*, 2011) and cell culture (Cao *et al.*, 2008). Canolol and its polymerized derivatives found in the canola oil refining byproducts could be extracted and potentially used as antioxidants in various food systems.

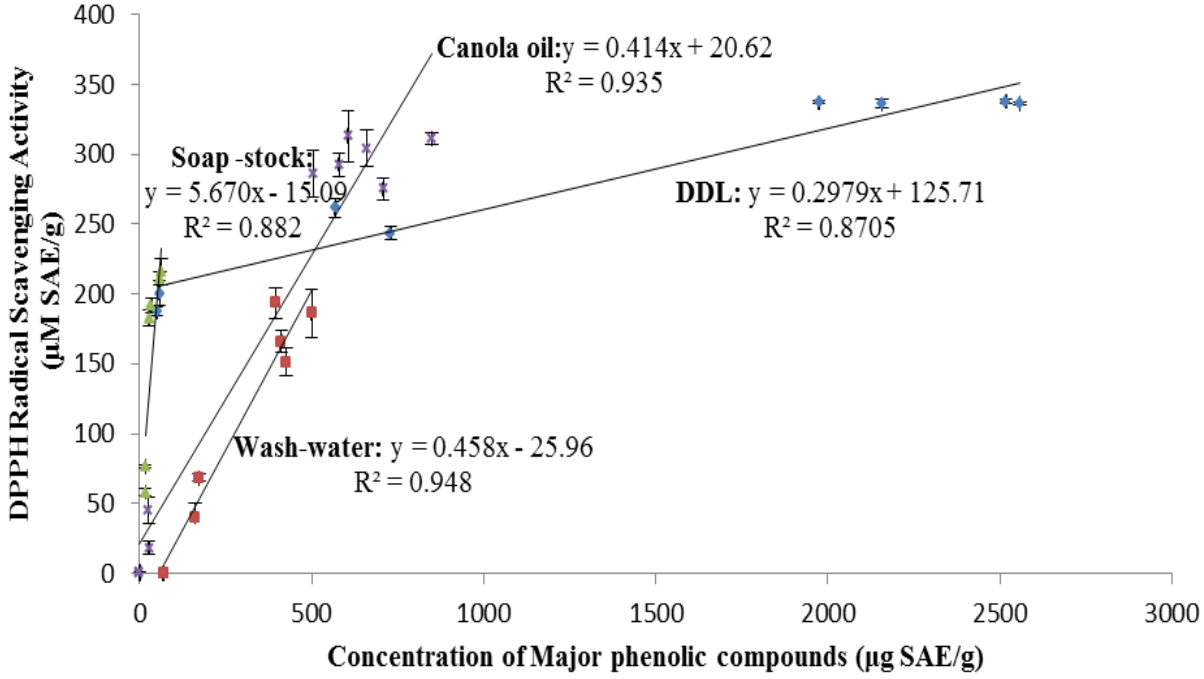

Figure 3.4 Correlation of the concentration of major phenolic compounds in expeller-pressed canola oil and refining byproducts with DPPH radical scavenging activity (all points are expressed as mean ± standard deviation (n=3))

3.5 Conclusion

Renewed interest in utilization of oil processing by-products as sources of various high-value co-products such as phenolics, phytosterol, protein, and lipids persists. Processes have been developed to extract phytosterols and tocopherols from refining by-products of different vegetable oils. The recovery of valuable phenolics with proven bioactivities can improve the economic feasibility of edible oil refining. Given this context, our results provide new knowledge on the by-products of canola oil subjected to different stages of refining. Sinapic acid (42.9 µg/g), sinapine (199 µg/g), and canolol (344 µg/g) were found in the refining by-

products namely soap-stock, spent bleaching clay and wash-water for the first time. Tocopherols (3.75 mg/g) and other non-identified phenolic compounds (2.7 mg SAE/g) were found in deodistillates. In conclusion, this is the first study showing the retention and recovery of the sinapic acid derivatives and tocopherols using by-products generated in a commercial expeller-pressed facility. This approach opens up opportunities for utilization of these by-products as new side streams for commercial utilization in the context of canola and rapeseed oil refining. More studies are recommended to unfold the recovery, extraction and mechanisms of structural changes of these endogenous biophenols using by-products of edible oil refining.

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CHAPTER 4

MANUSCRIPT 2

PHENOLICS FROM CANOLA CRUDE EXTRACTS PROTECT CELLS FROM OXIDATIVE STRESS

4.1 Abstract

Endogenous phenolic compounds obtained from canola oil deodistillates and canola meal using different extraction techniques were identified and examined for their *in vitro* antioxidant activities in "test tube" and cellular assays. Sinapine was the predominant phenolic in the canola meal crude extract, while canolol was the only significant phenol in the accelerated solvent extract of canola meal. The deodistillate did not have canolol or sinapine present, but contained high molecular weight phenols of unknown identity. The "test tube" antioxidant assays indicated that canola meal crude extract and sinapic acid both exhibited stronger antioxidant potentials compared to the other extracts. A dose dependent cyto-protection was observed under oxidative challenge by H_2O_2, when cells were incubated with the canola meal accelerated solvent extract, deodistillate extract and sinapic acid. This study demonstrates that canola by-products can be the sources of health promote phenols, for possible formulation into nutraceuticals.

Keywords: Canola; rosemary; phenolic; antioxidant activity; glucose transport

This chapter is going to be submitted in 2014 to a publisher for publication

4.2 Introduction

Canola (*Brassica napus* L.) is the third largest oilseed crop worldwide with the seeds containing about 42-43% oil (Canola Council of Canada, 2009). The production of canola oil involves crushing the seeds, which separates the crude oil from the meal, followed by oil-refining using neutralization using alkali, bleaching with clay, and deodorizing via steam distillation (Unger *et al.*, 2011). Several by-products are fractionated and used in human nutraceuticals, such as canola meal proteins (Hashmi *et al.*, 2010).

Canola is a member of the *Brassica napus* L. variety which belongs to the mustard family, an ancient food crop plant in many cultures (CFIA, 2012). Due to its origin, the canola seed contains more dietary phenolic compounds than other oilseeds (Naczk *et al.*, 1998), almost all of which remain in the processing by-products (Khattab *et al.*, 2010).

The consumption of dietary phenols is associated with the prevention of some of the most detrimental chronic diseases, such as various cancers, inflammatory and cardiovascular diseases (Stoner & Mukhtar, 1995; Steele *et al.*, 2000; Vita, 2005; Rahman *et al.*, 2006). Although a variety of mechanisms have been reported for the preventative actions of phenolics, significant antioxidative activity has been attributed to dietary phenols (Laranjinha *et al.*, 1994; Škerget *et al.*, 2005). Specifically, some canola derived phenols have been shown to exhibit significant antioxidant activity *in vitro* (Vuorela *et al.*, 2004; Thiyam *et al.*, 2009; Szydłowska-Czerniak *et al.*, 2010) and *in vivo* (Cao *et al.*, 2008; Kim *et al.*, 2010). We therefore hypothesize that canola phenols extracted from processing by-products exhibit antioxidant activity and could protect cells from oxidative damage.

A novel method has been developed for extracting canola phenols from meal and deodorizer distillates. In addition we show significant antioxidant activity for the crude extracts in chemical assays and that they can protect cells from an oxidative challenge.

4.3 Materials and methods

4.3.1 Materials

Canola meal and canola oil deodistillates were provided by Viterra Canola Processing Inc. (Winnipeg, MB, Canada). All chemicals were of analytical grade. Methanol, *n*-hexane, Folin–Ciocalteau phenol reagent, sodium carbonate, iron (III) chloride dihydrate, TPTZ (2,4,6- tripyridy-*s*-triazine, Sigma), D-(+)-Glucose, sodium chloride, potassium chloride, monopotassium phosphate, disodium hydrogen phosphate, magnesium sulfate heptahydrate, calcium chloride dehydrate, sodium dodecyl sulphate, dimethyformamide, glacial acetic acid, and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma Aldrich, Canada. Sinapic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM), Phosphate Buffered Saline (PBS) and antibiotic/antimycotic solution were purchased from Thermal Fisher Scientific (Waltham, MA, USA).

4.3.2 Preparation of crude phenolic extracts from canola by-products

Canola meal was subjected to 2 extraction methods: ultrasonic extraction and accelerated solvent extraction. Canola oil deodistillate was extracted using liquid-liquid solvent extraction.

Ultrasonic extraction of canola meal: Canola meal was defatted using a Soxtec 2050 (Foss-Tecator, Foss North America, MN, USA). The defatted sample was extracted following the procedure described by Khattab *et al.* (2010) to investigate residual phenolics. Defatted sample (1 g) was extracted with 9 mL 70% methanol with ultrasound treatment for 1 minute. The ultrasound treated methanolic mixture was centrifuged at 5000 x *g* for 10 min at refrigerated condition. The supernatant was collected and filtered using Whatman No.1 filter paper. Residue was extracted following the same procedure twice. The filtrates were pooled and made up to 30 mL.

Accelerated solvent extraction of canola meal: Canola meal was heated at 180 ℃ in oven for 15 min (pre-treated) prior to Accelerated Solvent Extraction (ASE) (ASE 300, Dionex). Fifty grams of heated substrates thoroughly were mixed with Ottawa sand in a ratio of 1:1 (w/w) using a spatula. Two filter papers were placed at the bottom of each sample cells followed by completely filling it with canola seeds/ meal. Cell caps were hand tightened securely for both sides and were placed in ASE cell holder and extraction was carried out using *n*-hexane. The ASE extraction was carried under the following conditions: temperature: 190 ℃; static time: 5 minutes; static cycles, 2 cycles; flush volume: 60%; Purge time: 1 min; pressure: 1500 psi; heat time: 5 min. The hexane extract was re-extracted with 70% methanol

Extraction of Phenolics from canola oil deodistillate: The deodistillate sample was extracted with 50% aqueous methanol, as previously described (Harbaum-Piayda *et al.*, 2010). Two grams of deodistillates were solubilized with 10 mL hexane (1:5 w/v), followed by extraction using 50% methanol (3 mL) three times. For production of the extract, the same procedure of extraction was maintained and 400 g of deodistillates was solubilized in 1600 ml of *n*-hexane for 15 minutes, followed by extraction with 400 ml of 50% methanol, five times. The pooled methanolic extracts were evaporated to dryness/ minimum volume in a rotary evaporator (at 50 ^{o}C until methanol evaporates, and at 70 ^{o}C for 30 minutes for the evaporation of water). A minimum quantity (10 mg) of dried DDL extract was dissolved in 5 ml 50% methanol for further quantification.

4.3.3 Quantification and identification of phenolic compounds by RP-HPLC-DAD

Quantification and identification of phenolic compounds in the extracts was established following a RP-HPLC-DAD analysis as described previously (Khattab *et al.*, 2010). An 80 mm × 4.0 mm i.d., 4 µm, Synergi Fusion-RP column (Phenomenex, Torrance, Canada) was used for sinapic acid derivatives separation (UV detection at 270 nm and 330 nm). Standards

of sinapine, sinapic acid, and canolol were used to authenticate the retention time and UV absorption spectra. The contents of all sinapic acid derivatives were expressed as sinapic acid equivalents (SAE) in μg/g of sample, wherein sinapine and sinapic acid was detected at 330nm, canolol was detected at 270 nm.

4.3.4 Preparation of phenolic solutions

All the crude canola phenolic extracts were concentrated using a rotary evaporator at 50 ± 5 ℃ to a minimum volume and re-dissolved in 2 mL methanol prior HPLC quantification. After quantification of phenolic compounds in the canola extracts, they were diluted in (i) HEPES PO_4 treatment buffer (glucose: 5mM, HEPES: 10 mM, NaCl: 147 mM, KCl: 5 mM, KH_2PO_4: 1.9 mM, Na_2HPO_4: 1.1 mM, $MgSO_4$-$7H_2O$: 0.3 mM; MgCl-$6H_2O$: 1 mM, $CaCl_2$-$2H_2O$: 1.5 mM, pH: 7.4) for cell culture experiment or (ii) distilled water for Folin-Ciocalteau and DPPH assays to desired concentrations. Sinapic acid standard was dissolved in dimethyl sulfoxide (DMSO) before further dilution in treatment buffer or distilled water. Final treatment solutions contained <1% solvent, and there was no cytotoxicity to CHO and Caco-2 cells at those concentrations.

4.3.5 Folin-Ciocalteau assay

Folin–Ciocalteu assay was carried out as described (Swain & Hillis, 1959), with slight modifications to be performed in 96-well microplates. Aliquots (20 μL) of phenolic solutions at different concentrations were loaded on a 96-well microplate, followed by addition of 100 μL Folin–Ciocalteau phenol reagent and the microplate was mixed well. After 5 min, 7.5% sodium carbonate (80 μL) was added and the microplate was covered and kept at dark for 2 h. The absorbance was measured at 750 nm using a spectrophotometric microplate reader (BioTek, Winooski, USA). The results were quantitatively expressed as the slope coefficient of the lines representing the dependence of A750 on the concentration of the investigated

phenolic solutions. The slope was obtained by linear regression analysis in a concentration range up to 2 mM.

4.3.6 Ferric ion reducing power (FRAP) assay

The reducing capacity of phenolic solutions was determined by the FRAP assay (Benzie & Strain, 1996) with modifications to be performed in 96-well microplates. Briefly, the FRAP solution was freshly prepared using 10 mL of 0.3 M acetate buffer (pH 3.6), 1 mL of 20 mM $FeCl_3$ solution and 1 mL of 10 mM TPTZ (2,4,6- tripyridy-*s*-triazine) solution in 40 mM HCl and warmed at 37 ℃ before using. Twenty-five μL of phenolic solutions at different concentrations and 175 μL FRAP solution were added on a 96-well microplate and incubated at room temperature for 10 min. The absorbance was measured at 595 nm with a spectrophotometric microplate reader (BioTek, Winooski, USA). The results were quantitatively expressed as the slope coefficient of the lines representing the dependence of A_{595} on the concentration of the investigated phenolic solutions, where higher values of slope indicated higher FRAP antioxidant activity. The slope was obtained by linear regression analysis in a concentration range up to 1 mM.

4.3.6 2,2-diphenyl-1-picrylhydrazyl radical (DPPH●) scavenging activity assay

The DPPH scavenging activity assay was carried out according to the method proposed by Brand-Williams *et al.* (1995) with modifications to be performed in 96-well microplates. Briefly, 25 μL of phenolic solutions at different concentrations were added on a 96-well microplate. Then 175 μL freshly prepared DPPH ethanolic solution (0.1 mM) was added to the sample and the microplate was covered and incubated at room temperature for 10 min. The absorbance was measured at 516 with a spectrophotometric microplate reader (BioTek, Winooski, USA). The dose that inhibited free radicals by 50% (IC_{50}) was calculated linear correlation from the average data.

4.3.7 Cell culture

Human epithelial colorectal adenocarcinoma (Caco-2) and Chinese Hamster Ovary (CHO) cell lines were obtained from ATCC (cat no. HTB-37 and CCL-61) and cultured at 37 ℃ in a humidified 5% CO2 incubator. The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% Fetal Bovine Serum (FBS) and 1% antibiotic. Cells were grown in 25 T-flasks until they reached more than 80% confluence before splitting or seeding. For experiments, the cells were seeded on a 96-well plate in 100 µL of growth medium and incubated for 24 h at 37 ℃.

4.3.8 Cellular protection against H_2O_2 induced oxidative stress

For the oxidative stress induction, hydrogen peroxide (H_2O_2) was prepared in the HEPES PO_4 treatment buffer. Initially, cells in 96-well plate were treated with a range of concentrations of phenolic solutions and incubated at 37 ℃ for 23 h, followed by addition of H_2O_2 (final concentration: 500 µM) for a further 1 h. The wells were washed with HEPES PO_4 treatment buffer and the protection effect of the phenolic solutions on the oxidative induced cells was determined via cell viability using the colorimetric MTT assay. For MTT assay, 100 µL MTT solution (0.5 mg/mL) was added to the buffer washed well and the microplate incubated for 2 h. Further, 100 µL MTT solubilization solution (16% sodium dodecyl sulphate, 4% dimethyformamide, 2% glacial acetic acid, pH: 4.7) was added to the well to dissolve formazan crystals. The plate was then shaken and absorbance read at 570 nm with a spectrophotometric microplate reader (BioTek, Winooski, USA). The final results were expressed as cell viability in percentage of the sham-treated positive control.

4.3.9 Statistical analyses

The results are expressed as mean ± SD of at least three independent experiments. Differences between different treatment groups and the control counterparts were determined using one-way ANOVA with least significant difference *t*-test by using SAS 9.4 software

(SAS Institute Inc., USA). Statistical significance was accepted at $p<0.05$. Regression analysis was carried out using Microsoft Excel 2010.

4.4 Results

4.4.1 Phenols are distinct in canola by-product extracts

The major identifiable phenols in the different canola processing by-products were sinapine and canolol, which are also major phenols of intact canola seeds or crude canola oils (Thiyam et al., 2009; Kuwahara et al., 2004). Sinapine was the predominant component in the canola meal crude extract (CM) (Figure 4.1 A), while canolol was the sole significant phenol in the accelerated solvent extract from canola meal (Figure 4.1 B). The extract from the deodistillate did not contain canolol or sinapine, but higher molecular weight phenols of unknown identity (Figure 4.1 C).

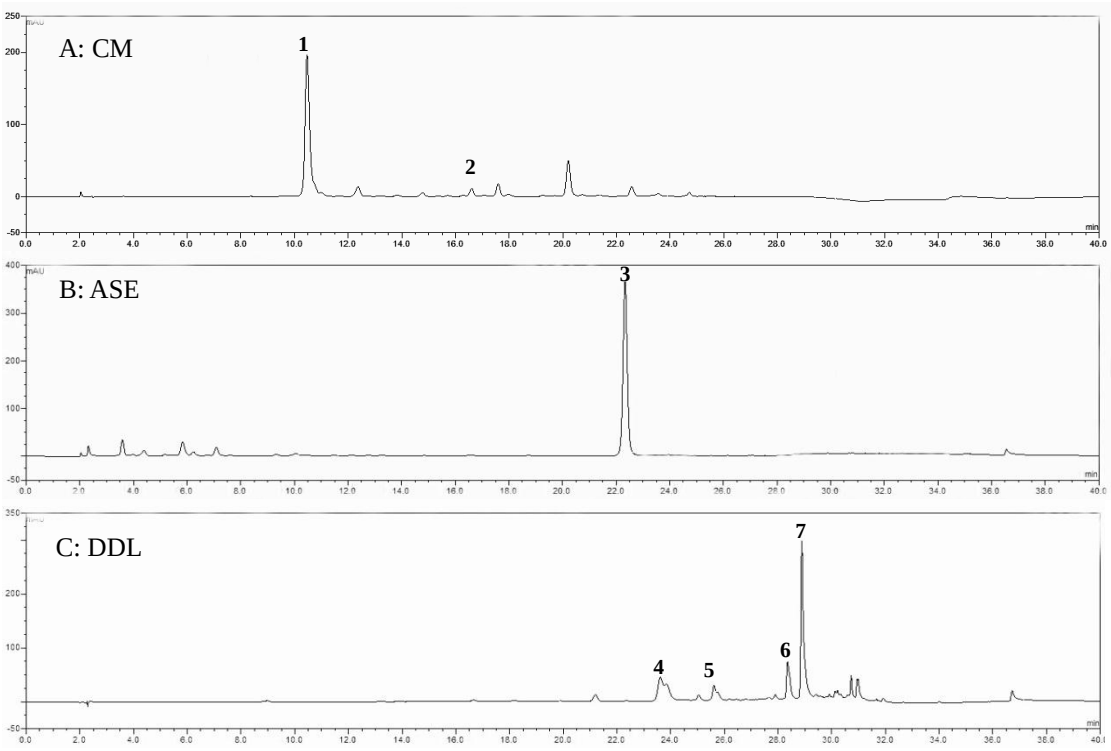

Figure 4.1 HPLC chromatogram of phenolic extracts of canola processing by-products. (A), Canola meal crude extract (CM) predominantly contains sinapine (peak 1) and some other phenols, such as sinapic acid (peak 2). (B), Canola meal extract obtained from Accelerated Solvent Extraction (ASE) exclusively contains canolol (peak 3). (C) The deodistillates extract (DDL) contains higher molecular weight phenols of undetermined identity (peaks 4-7).

4.4.2 Phenolics from canola by-products are antioxidants in artificial chemical test systems

Plants phenolics are increasingly recognized as antioxidants (Dai & Mumper, 2010). We therefore assessed the chemical antioxidant capacity using three chemical methods, Folin–Ciocalteu, FRAP, and DPPH assays, which determine the reducing capacity and scavenging activities to ferric ions and free radicals, respectively. The results for Folin–Ciocalteu assay revealed that crude canola meal extract exhibited the highest antioxidant capacity (slope coefficient: 1.12 ± 0.02 μM^{-1}), followed by sinapic acid (0.50 ± 0.06 μM^{-1}), deodistillates extract (0.38 ± 0.02 μM^{-1}) and canola meal accelerated solvent extract (0.26 ± 0.01 μM^{-1}) (Table 4.1). Crude canola meal and sinapic acid exhibited the highest ability in scavenging ferric ions with FRAP values of 2.64 ± 0.07 and 2.42 ± 0.07 μM^{-1}. Similarly, FRAP values of deodistillate extract (1.40 ± 0.01 μM^{-1}) and canola meal accelerated solvent extract (1.02 ± 0.04 μM^{-1}) were also significantly lower than that of crude canola meal (2.64 ± 0.07 μM^{-1}) and sinapic acid (2.42 ± 0.07 μM^{-1}). The DPPH activity in this study was expressed as the concentration of extract required to inhibit 50% DPPH radicals (IC_{50}). The results showed a similar patent with the other two assays, crude canola meal extract (IC_{50}: 71.2 ± 0.9 μM) and sinapic acid (IC_{50}: 78.1 ± 1.1 μM) exhibited stronger antioxidant activity than that of deodistillates extract and canola meal accelerated solvent extract.

Table 4.1 Antioxidant capacity of phenolic extracts of canola by-products

	Crude canola meal extract	Canola meal accelerated extract	Deodistillate extract	Sinapic acid
FC[a] (mM^{-1})	1.12 ± 0.07^{a}	0.26 ± 0.01^{d}	0.38 ± 0.02^{c}	0.50 ± 0.06^{b}
FRAP[b] (mM^{-1})	2.64 ± 0.07^{a}	1.02 ± 0.04^{d}	1.40 ± 0.01^{c}	2.42 ± 0.07^{b}
DPPH[c](IC_{50} μM)	71.2 ± 0.9^{a}	461.9 ± 5.8^{b}	561.6 ± 72.8^{c}	78.8 ± 1.1^{a}

Data represents the mean ± standard deviation of at least three independent experiments. Values in each row share same letters are not significant different ($p<0.05$).

[a] FC, Reducing capacity measured by Folin-Ciocalteu method; values are expressed as the slope coefficients calculated by linear regression (see material and methods), where higher antioxidant activity is expressed by an increase in the slope.

[b] FRAP, ferric ion reducing antioxidant power, values are slope coefficients calculated by linear regression (see material and methods), where higher antioxidant activity is expressed by an increase in the slope.

[c] DPPH, 2,2-diphenyl-1-picrylhydrazyl free radical scavenging activity, values are the concentration of phenolics needed to inhibit the DPPH● concentration by 50% (IC_{50})

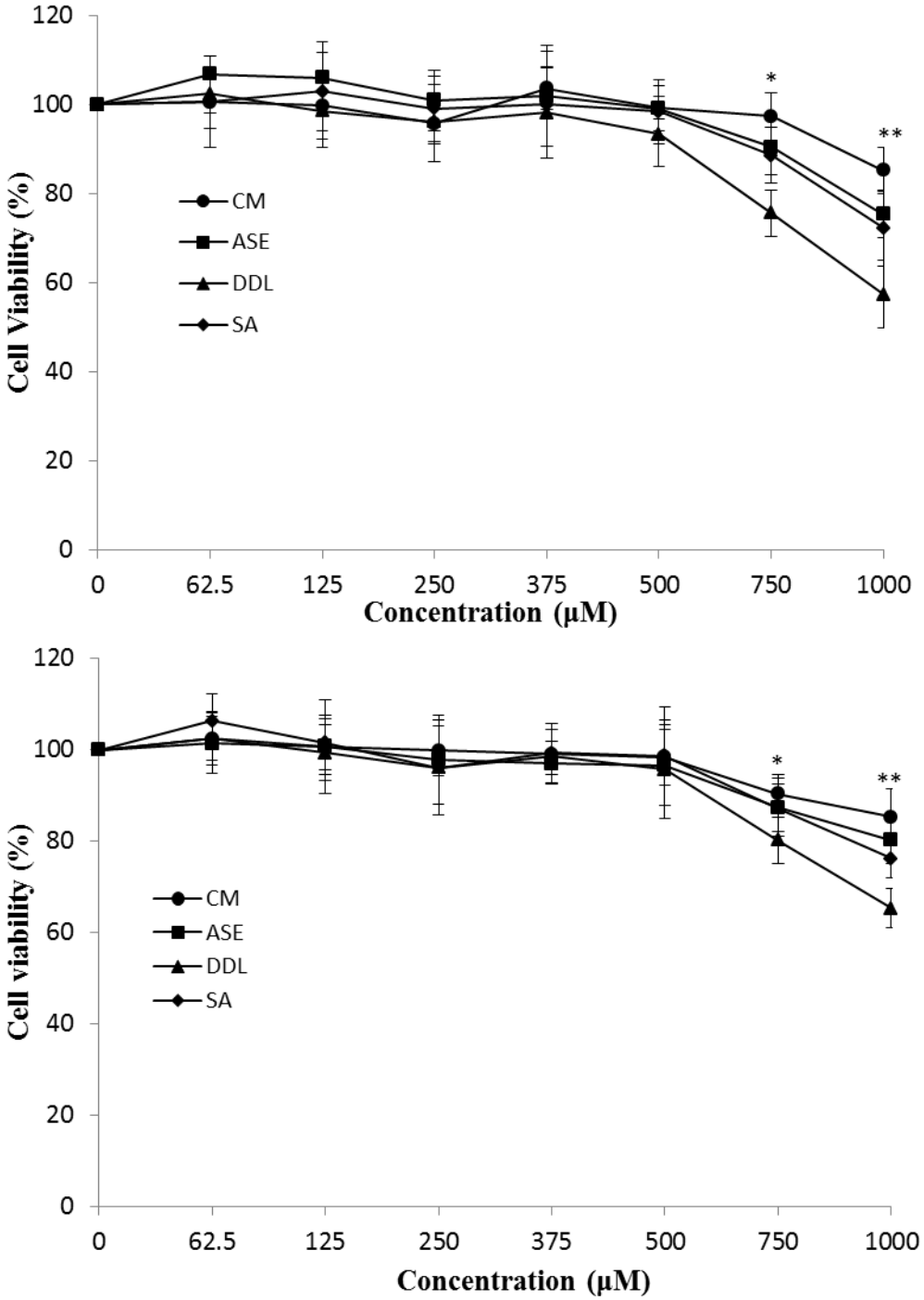

Figure 4.2 Effect of canola by-products extracts on cell viability under different concentrations on CHO (A) and CaCo-2 (B) cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Asterisks indicates significant difference with control (* $p<0.05$, ** $p<0.01$). CM, Canola meal crude extract; ASE, Canola meal accelerated solvent extract; DDL, Deodistillates extract; SA, Sinapic acid.

4.4.3 *Phenolics from canola by-products do not exhibit evident toxicity to CHO and Caco-2 cells*

The cytotoxicity of the phenolics from canola by-products was determined using MTT assay with cells were treated with different concentrations of phenolic solutions from 0 to1000 μM. The data indicated that none of the tested phenolic extracts has obvious cytotoxicity against normal CHO and Caco-2 cells at the concentration below 500 μM (Figure 4.2). All the extracts showed slight or medium cytotoxicity when the concentrations reach 1000 μM. This indicated the concentrations used in the experiment were safe to the cells.

4.4.4 Phenolics from canola by-products protect cells from oxidative damage

Hydrogen peroxide (H_2O_2) is one of the major contributors of oxidative stress and a number of studies have demonstrated that H_2O_2 induces oxidative stress by both acting as a reactive oxygen species (ROS) and a regulator of several redox enzymes (Prasad et al., 1994; Coyle & Kader, 2007). Therefore, we tested whether the antioxidant capacity determined in the chemical assays translated into a protective effect in enterocytes (CaCo-2) and fibroblastic cells (CHO) in culture challenged with H_2O_2.

Of all tested extracts, only the crude canola meal extract did not reduce cell death in both cell lines (Figures 4.3 A, 4.4 A). A dose dependent increase in cell viability was observed, however, when the canola meal accelerated solvent extract (Figures 4.3 B, 4.4 B) and the deodistillate extract (Figures 4.3 C, 4.4 C) were present during the oxidative challenge. Similarly, sinapic acid, a major phenolic in canola, protected the cells in a dose dependent manner (Figures 4.3 D, 4.4 D). We therefore conclude that the canola accelerated solvent extract, with its major component canolol, as well as the deodistillate extract (with some undetermined phenolic compounds), both exhibited an antioxidant effect in living cells.

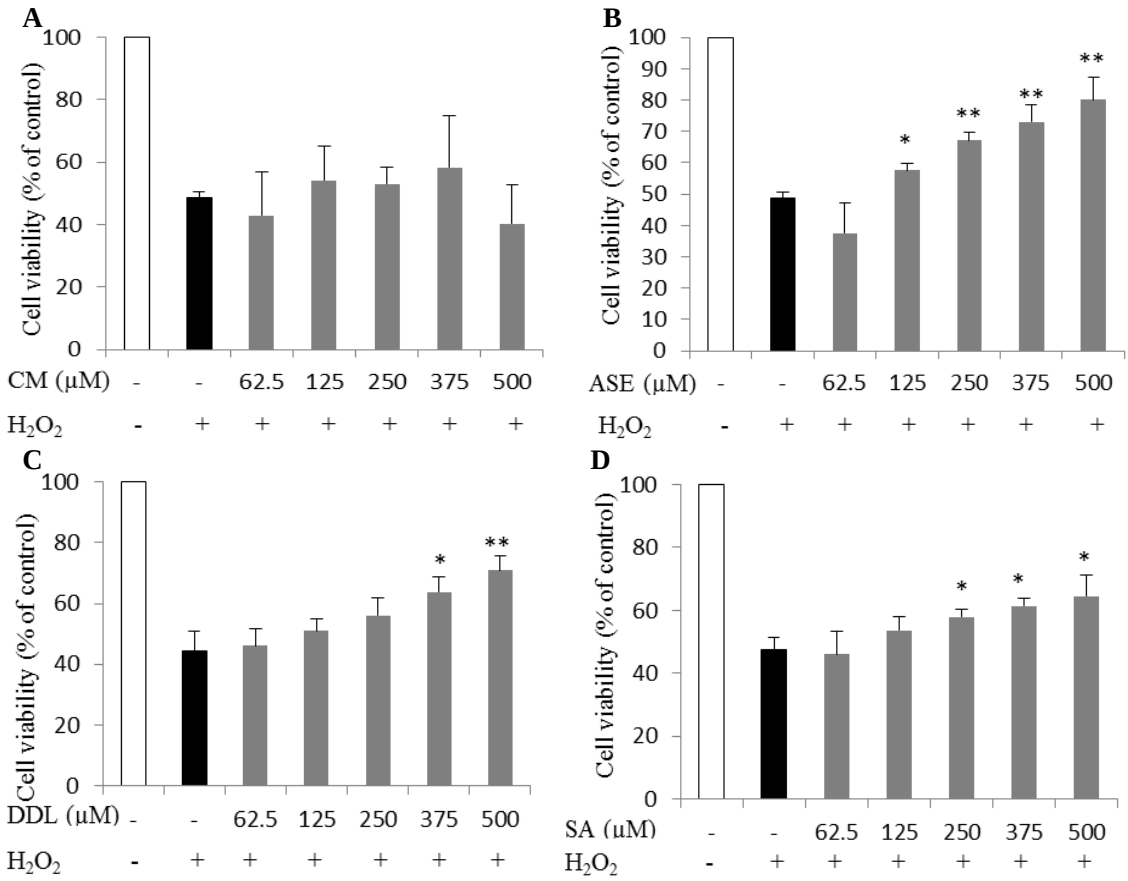

Figure 4.3 Effect of canola by-products extracts on CHO cell treated with H_2O_2-induced cytotoxicity. The data represents the mean ± standard deviation of at least three independent experiments. Asterisks indicates significant difference with H2O2 only treated cells (* p<0.05, ** p<0.01). (A) CM, Canola meal crude extract, (B) ASE, Canola meal accelerated solvent extract, (C) DDL, Deodistillates extract, (D) SA: Sinapic acid.

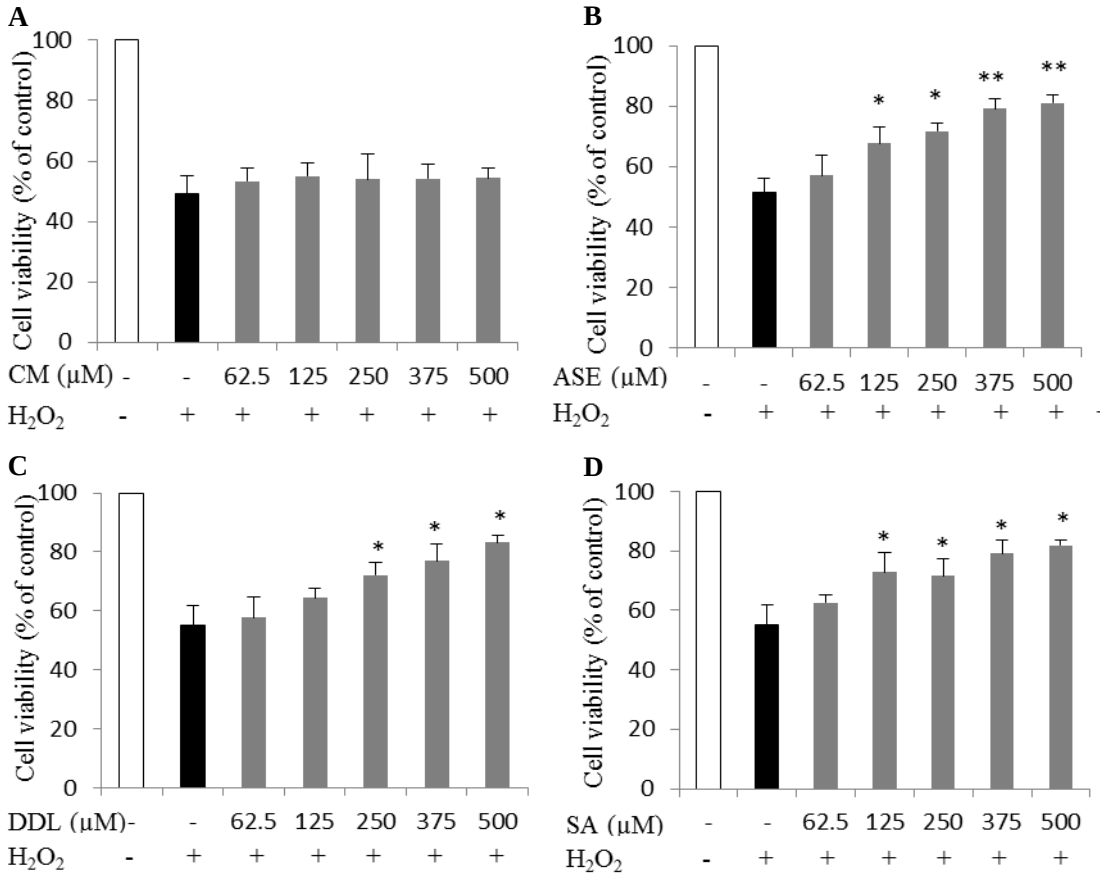

Figure 4.4 Effect of canola by-products extracts on CaCo-2 cell treated with H_2O_2-induced cytotoxicity. The data represents the mean ± standard deviation of at least three independent experiments. Asterisks indicates significant difference with control (* $p<0.05$, ** $p<0.01$). (A) CM, Canola meal crude extract. (B) ASE, Canola meal accelerated solvent extract. (C) DDL, Deodistillates extract. (D) SA, Sinapic acid.

4.5 Discussion

The presented data support both of our hypotheses, that a) canola by-products were a significant source of dietary phenols when extracted properly; and b) that the extracted phenols exhibited antioxidant activity and protected cells from oxidative damage.

The predominant phenols in whole canola seeds are sinapic acid derivatives including sinapine, sinapic acid, and sinapoyl glucose (Thiyam *et al.*, 2009). Canolol does not occur naturally in canola seeds, but is formed in crude canola oils (Kuwahara *et al.*, 2004). In the canola meal crude extract sinapine was the predominant phenol, while in the accelerated solvent extract canolol was almost exclusively present. The presence of canolol in accelerated solvent extract was attributed to the high temperature and pressure which caused decarboxylation of the predominant phenol of canola meal (sinapine). Previous studies have demonstrated that thermal treatment of canola seeds and meals, such as roasting and microwave heating (Spielmeyer *et al.*, 2009; Khattab *et al.*, 2014) and supercritical extraction (Pudel *et al.*, 2014), can produce extracts or oils rich in canolol. Our result suggested that accelerated solvent extraction is a novel technique for production of canolol, and an alternative to previously published methods (Spielmeyer *et al.*, 2009; Khattab *et al.*, 2014; Pudel *et al.*, 2014). Moreover, some unknown phenols of slightly higher molecular weight were found in deodistillate extract.

The phenolic extracts of canola by-products showed significant antioxidant activities in the "test tube" systems. The Folin–Ciocalteu assay is the most commonly used method for measuring the total phenols of foods and nutraceuticals by determining the reducing capacity of the sample (Katsube *et al.*, 2004). Other investigators suggested that the Folin–Ciocalteu assay should be seen as a measure of antioxidant capacity rather than a measure of total phenols (Everette *et al.*, 2010). The other antioxidant assays (FRAP and DPPH) are also based on electron transfer mechanism (Prior *et al.*, 2005). These assays don't measure thiol

antioxidants and are preferred by some researchers as methods for measuring antioxidant activity of foods (Tan *et al.*, 2011). The difference in antioxidant activities in "test tube" systems between the canola by-product samples in our study might be due to the different phenolic compounds in the extracts. The predominant phenolic compound in crude canola meal extract was sinapine which is a hydrophilic phenol. Several earlier studies have demonstrated that hydrophilic phenols can exhibit stronger antioxidant capacities than their lipophilic forms in the homogeneous polar media (Perez-Fons *et al.*, 2009; Jordán *et al.*, 2012). Our results are in agreement with work by Terpinc's *et al.* (2011) who reported that decarboxylation products of hydroxycinnamic acids (e.g. canolol) exhibited poorer reducing capacities when measured by the Folin–Ciocalteu assay than the corresponding phenolic (e.g. sinapic acid). Sørensen *et al.* (2013) also reported that sinapine and sinapic acid exhibit higher antioxidant activity than canolol as determined with FRAP and DPPH assays. However, both Terpinc's *et al.* (2011) and Sørensen *et al.* (2013) suggested that canolol exhibited stronger antioxidant activity in emulsion systems.

Phenols can exhibit antioxidative effects in "test tube" systems relevant to food stability and increased shelf life, however, this might not translate into protective effects in cells. Our data showed a clear dose dependent cyto-protection effect under oxidative challenge by H_2O_2, when the cells were incubated with the canola meal accelerated solvent extract (predominantly containing canolol) and the deodistillate extract (containing unknown high molecular weight phenols). An identical effect was achieved with sinapic acid, a known antioxidant found in canola seeds. The most potent cyto-protective extracts did not exhibit the most potent antioxidant activity when chemically assayed (Folin–Ciocaltau, FRAP, DPPH). We therefore propose that canola derived phenols could have an antioxidative effect *in vivo,* in general tissues or specifically in the intestinal tract. The antioxidant effect exhibited upon cells, which is important for health promoting properties, is different than the antioxidant

activity in solution, which is more relevant for the protection of food (e.g. prolonged shelf-life).

The bioavailability and intracellular concentrations of canola derived phenols, such as canolol, sinapine and sinapic acid, are unknown. Phenolic bioavailability varies greatly although plasma levels are generally not exceeding low micromolar concentrations (Scalbert & Williamson, 2000). All extracts exhibited antioxidative protection from about 100 µM onwards in both cell lines tested, where CHOs represent the parenchymal cells of organs and CaCo2 represent the enterocyte. If phenols are bioavailable, the plasma concentration might reach 100 µM and so that the extracts could exhibit systemic antioxidant effects *in vivo*.

Even if the bioavailability is limited and the phenols not absorbed in the small intestine, they could reach concentrations exceeding 200 µM in the intestinal lumen. Our extracts protected the enterocytes ($CaCo_2$) from oxidative challenge at concentrations of 100 µM and higher, and enterocytes representing the outermost barrier towards the intestinal lumen are likely to experience these levels of phenols. This might be relevant in situations of oxidative challenge in the intestinal tract, such as evident in inflammatory bowel disease.

4.6 Conclusion

This study demonstrated that distinct phenolics can be obtained from different by-products of canola when subjected to different extraction techniques. Although the deodistillate and accelerated solvent extracts of canola showed lower antioxidant activity based on the F-C, FRAP and DPPH assays than crude canola meal extract and sinapic acid, they demonstrated significant antioxidant potentials in reducing H_2O_2 induced cytotoxicity in cells. This study has shown that canola by-products can be the sources of health promoting phenols, with potential as potent nutraceuticals. However, further studies are required to accurately assess the bioavailability of these phenolics.

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CHAPTER 5

MANUSCRIPT 3

CRUDE CANOLOL AND CANOLA DISTILLATE EXTRACTS IMPROVE THE STABILITY OF REFINED CANOLA OIL DURING DEEP-FAT FRYING

5.1 ABSTRACT

Crude canolol (CAN) extracted from canola meals by accelerated solvent extraction and phenolic extracts of canola oil deodistillates (DDL) were assessed for their potential to stabilize canola oil during deep-fat frying. French fries were deep fried in canola oil (3 L) enriched with 200 ppm of BHT, DDL and CAN at 185±5 ^{o}C for five minutes. Twelve batches of fries (30 g each) were fried each day, 30 min apart for a total of 6 h of frying for 5 days. Both CAN and DDL extracts exhibited higher protection against frying oil deterioration than BHT, with a considerable reduction in the hydroperoxides after the first day of frying, indicating their efficacy to reduce primary oxidation (CAN>DDL>BHT>Control). TOTOX and conjugated diene/triene values of oils with DDL and CAN were significantly ($p<0.05$) lower. The color was correlated with AV (R^2=0.822-0.985) in an exponential model. In conclusion, DDL and CAN, based on their antioxidative efficacy, can inhibit lipid oxidation during deep-fat frying.

Keywords: Canolol, deodistillate, canola oil, deep-frying, oxidative stability

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5.2 Introduction

In order to enhance and/or create unique sensory/ organoleptic properties of foods, the deep-fat frying is extensively employed both at home and on a commercial scale (Ahmad & Ismail, 2008). Even though, it has the advantages of operational simplicity, convenience and economic viability, the frying oil can deteriorate and become rancid by complex physical and chemical reactions such as hydrolysis, thermal degradation, oxidation and polymerization (Bensmira *et al.*, 2007; Karoui *et al.*, 2011). Such oxidative degradation in some cases, may also add to potential gastrointestinal disorders and even mutagenesis in the human body (Dana & Saguy, 2001). Hydroperoxides produced from the unsaturated acylglycerols via an autocatalytic reaction make oils rich in unsaturated fatty acids more susceptible to oxidation. Such changes will affect the sensory quality of oil and finished-products by enhancing the production of off-flavors, off-odors, and discoloration (Hraš et al., 2000), leading to consumer rejection.

Synthetic antioxidants, such as butylatedhydroxytoluene (BHT), butylatedhydroxyanisole (BHA) and tert-butylhydroquinone (TBHQ), have been used to extend the induction period of oil oxidation or slow down the oxidation rate (Cuvelier et al., 1994) by providing protection at high temperature (Augustin & Berry, 1983). Refined oils are still being treated with these synthetic antioxidants in many countries as they have been successfully proven to retard oxidation (Lin *et al.*, 1981). However, the use of synthetic antioxidants in food is of concern to consumers as their safety has been questioned due to their health hazards in animals and humans (Sharayei *et al.*, 2011; Deshpande *et al.*, 1996). As a result, they have been increasingly contested or even banned in certain countries. In addition, they have high volatility and are susceptible to rapid decomposition during frying at high temperatures. As a consequence, their use is very much limited to certain products as exemplified by their inefficacy in some common food products such as French fries and they

fail to prevent the development of off flavor in vegetable oils (Chang *et al.*, 1977).

Consequently, there is considerable interest in the use of natural substances or extracts that have antioxidant properties (Gulluce *et al.*, 2003; Berger, 2005). Such naturally derived plant extracts are rich source of phenolics, which are believed to be safer by providing additional health benefits compared to synthetic antioxidants. The antioxidant potential of different types of plant extracts including rosemary extract, *Pandanus amaryllifolius* leaf extract and Inca muña leaf extracts have been reported (Mereno et al., 2006; Nor et al., 2008; Chirinos *et al.*, 2011) and suggested the possibility of incorporating these natural antioxidants for applications in food industry. Canola oil, seeds and meal contain various minor constituents such as tocopherols, carotenoids, phytic acid and sinapic acid derivatives (SADs) such as free sinapic acid (SA), sinapoyl glucose (SG), and sinapine (SP). Rapeseed press cake and proteins contain significant amounts of SA, in the free and esterified form (Thiyam et al., 2009) and such phenolics have potential antioxidant activities in food systems.

Deodistillates are excellent starting materials for the production/ recovery of variety bioactive components such as tocopherols, phenolics, phytosterols or squalene (Chu *et al.*, 2004; Moreira & Baharin, 2004). During deodorization of vegetable oils these compounds are lost in significant amounts in deodistillates. A recent study demonstrated the potential of dimers and trimers of canolol (CAN) or 4-vinyl-2,6-dimethoxyphenol in a rapeseed oil deodistillate (DDL) (Harbaum-Piayda et al., 2010). This minor component can be incorporated into many food and non-food products. It has been shown that canolol isolated from crude rapeseed oil exhibited antioxidative, anti-inflammatory and strong scavenging capacity against the endogenous mutagen, peroxynitrite ($ONOO^2$) (Kuwahara *et al.*, 2004). However, the efficacy of CAN and DDL for improving the frying stability of oils remained to be confirmed. Previously, olive oil deodorizer distillate was used to obtain unsaponifiable matter and its antioxidative effect was tested on the sunflower oil stability during frying and

on the quality of potato chips (Abdalla, 1999). However, there are no other reports on the use of deodorizer distillates in frying.

The main objectives of this work were (1) to extract and quantify phenolics canola oil deodistillates, and (2) to evaluate the antioxidant efficacy of DDL phenolic extracts and CAN in protecting refined high-oleic canola oil against oxidation during frying of French fries. This is the first report showing the efficacy of CAN and DDL to stabilize canola oil during deep-fat frying. The study is also novel in its industrial application perspective, as the results obtained are expected to create new market opportunities for underutilized oil refining byproducts especially DDL, as "natural plant extracts" for various food applications.

5.3 Materials and Methods

5.3.1 Materials

All chemicals were of analytical grade. Ammonium thiocyanate, *p*-anisidine, barium chloride dihydrate, butylhydroxy-toluene (BHT), n-hexane, iron (III) chloride dihydrate, iron (II) sulfate heptahydrate, petroleum ether, methanol, and cyclohexane were purchased from Sigma Aldrich, Canada. Refined rapeseed oil was purchased from a local supermarket, Winnipeg, Manitoba, Canada. Canola deodistillate was supplied by Bunge Canada, as a part of the collaborative research. Canola oil was purchased from a local market in Winnipeg. Standard sinapic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA).

5.3.2 Extraction of canola oil deodistillate (DDL) phenolics

The DDL (0.5 g) was first solubilized with hexane (1:20 w/v), followed by extraction with 3 ml of 50% methanol thrice (Harbaum-Piayda *et al.*, 2010). For production of the extract, the same procedure of extraction was maintained and 400 g of DDL was solubilized in 1600 ml of hexane for 15 minutes, followed by extraction with 400 ml of 50% methanol, five times. Methanolic extracts from each step were analyzed for phenolic content. The pooled methanolic extracts were evaporated to dryness/ minimum volume in a rotary

evaporator (at 50 ^{o}C until methanol evaporates, and at 70 ^{o}C for 30 minutes for the evaporation of water). A minimum quantity (10 mg) of dried DDL extract was dissolved in 5 ml 50% methanol for further quantification.

5.3.3 Production and extraction of canolol from canola seed/meal

Canola seeds/ meals were subjected to oven treatment at 180 ^{o}C in oven for 15 min (pre-treated) prior to Accelerated Solvent Extraction (ASE) (ASE 300, Dionex). This oven temperature was based on earlier studies conducted in our lab for maximizing the canolol production (unpublished data). The substrates were extracted with hexane in ASE under the following conditions: 50 g of canola meal/ seed and 50 g of Ottawa sand were mixed thoroughly using a spatula. Two filter papers were placed at the bottom of each sample cells followed by completely filling it with canola seeds/ meal. Cell caps were hand tightened securely for both sides and were placed in ASE cell holder and extraction was carried out using hexane. The hexane extracts were concentrated using a rotary evaporator at 45 ± 5 ^{o}C to dryness and were then re-dissolved in a minimum amount of refined canola oil. This was further used for frying experiments. The quantification of canolol in the extract was performed in HPLC-DAD by following detection at 270 nm and the concentration was expressed as sinapic acid equivalents.

5.3.4 Synthesis of canolol

For HPLC-DAD identification, canolol was synthesized from syringaldehyde and malonic acid following a previously reported method (Sinha *et al.*, 2007) with slight modifications. The highly viscous reaction mixture was appropriately diluted and filtered using a syringe filters (0.45 μm) before HPLC analysis as explained in section 2.5. An authentic standard of canolol kindly donated by Dr. Amy Logan of CSIRO Animal Food and Health Sciences, Werribee, Australia was also used for identification purpose.

5.3.5 HPLC-DAD analysis of DDL extracts and Canolol

DDL extracts were analyzed by reversed-phase HPLC-DAD (Ultimate 3000; Dionex, Sunnyvale, CA, USA) equipped with on-line degasser, binary pump, auto sampler, column heater and diode array detector (Khattab *et al.*, 2010). A gradient elution was performed using water/methanol (90:10) with 1.2% *O*-phosphoric acid as solvent A, and methanol (100%)with 0.1% *O*-phosphoric acid as solvent B, using the column- Synergi 4l Fusion-RP 80 A °; 150 9 4.0 mm 4 micron (Phenomenex, Canada) at 0, 7, 20, 25, 28, 31 and 40 min with 10, 20, 45, 70, 100, 100 and 10% B. Solvent A and B as well as DDL extracts were filtered through a 0.45µ filter. The column was maintained at 25 ℃ with a flow rate of 0.8 ml/min. Chromatograms were acquired at 219, 270, and 330 nm and data were analyzed using the Chromeleon software (Version 6.8). Peaks were identified by comparing their relative retention times with those of the authentic standards of sinapic acid (SA). Each extract was injected two times in two different vials and results of duplicate analyses from each extract were used for quantification. The major peak in the DDL extract was quantified at 219 nm as standard SA equivalents, while canolol was identified at 270 nm based on UV spectrum characteristics and retention time of synthetic canolol and quantified as standard SA equivalents.

5.3.6 Frying experiment

Frying experiments were conducted in deep fryers of 3L working capacity. The four treatment of high oleic canola oil used were: (1) Canola oil without any added antioxidant or extracts (Control, CNT), (2) Canola oil with 200 ppm BHT, (3) Canola oil with 200 ppm DDL extracts, and (4) Canola oil with 200 ppm canolol (CAN). BHT was added to the oil by dissolving it in minimum quantity of methanol and then mixed with oil under N_2. Rotary evaporated DDL extract/ canolol extracts were first dissolved in minimum amount of oil, and then mixed uniformly with the canola oil under inert conditions.

On the first day of frying, the canola oil (3L) was heat conditioned at 185 ± 5 ^{o}C and then held at this temperature for 30 min before start frying the French fries. Frozen potato pieces of uniform size were fried in 30 g batches at a constant frying temperature of 185 ± 5^{o}C for 5 minutes. The fryers were left uncovered during the frying period. Twelve batches of French fries (30 g each) were fried each day, 30 min apart for a total of 6 h of frying. The same oil was used for frying for 5 days for a total of 30 h of frying. On the second and consecutive frying days, the oil was filtered and weighed before frying to determine the amount of fresh oil needed to replenish the oil in the fryer. Everyday, the oil was conditioned prior to frying of the first batch of French fries. Approximately 40 ml of oil samples were taken for further analysis every morning before it was measured and replenished with fresh oil. The 0 h oil was collected after the conditioning of the oil in the first day. A total of six samples of oil were collected (0 h, day 1, day 2, day 3, day 4, and day 5) from each treatment, flushed with nitrogen gas and stored at -20 ^{o}C until further analysis.

5.3.7 Analytical methods

The *p*-anisidine values (AnV), conjugated dienes (CD) and conjugated trienes (CT) were determined according to the recommended methods of AOCS (1998). The AnV method is based on the reactiveness of the aldehyde carbonyl bond on the *p*-anisidine amine group, leading to the formation of a Schiff base that absorbs at 350 nm (Laguerre *et al.*, 2007). For CD and CT values, canola oil samples were diluted with iso-octane and then absorbance was measured at 234 and 268 nm for CD and CT values, respectively using Beckman Coulter DU 800 UV Spectrophotometer (Beckman Coulter Inc, Fullerton, California, USA).

5.3.8 Colour index of frying oil

Lovibond PFx 995Tintometer, a PFX*i* series of spectrophotometric colorimeter, was used to measure oil color. The frozen oil samples were brought to room temperature, mixed with diatomaceous earth (0.16% w/w), filtered using Whatman wet strengthened qualitative

circles and then transferred to the cuvettes (1 cm optical path). The absolute measurements were displayed in Lovibond RYBN color scale. Since it was found that the R and Y values were significantly correlated with the time of deep-fat frying, only these values were considered for the analysis, eliminating the B (blue) and N (neutral) values. The higher the R and Y values, the darker and lower quality of the oil is.

5.3.9 Statistical Analysis

Single batch of frying was performed with canola oil with or without added extracts/ antioxidants to fry French fries and oil samples were collected from each batch in triplicate. Means and standard deviations were determined using Microsoft Excel (Version 5.0, Microsoft, Corporation, Redmond, WA). One way analysis of variance (ANOVA) was carried out using Minitab 17.0 software (Minitab Inc., USA). Statistical significance was determined using Tukey t-tests. Statistical significance was accepted at $p < 0.05$.

5.4 Results and discussions

A review on previous studies on deep-frying showed that the sampling of oil was carried out only after cooling the oil to ambient temperature by keeping it overnight after each day of frying and we followed the same sampling procedure (Ramadan *et al.*, 2006; Aladedunye & Przybylski, 2009; Aydeniz & Yilmaz, 2012), even though some researchers reported the sampling at the same day of frying (Abdalla *et al.*, 1999). The oxidative stability of frying oils were assessed mainly based on (1) peroxide value (PV) (2) conjugated dienes and trienes (CD and CT) (3) *p*-anisidine value (AV) and (4) TOTOX values. The overall results indicated better performances of oil with DDL or CAN extracts than the control oil or BHT-added oil. The major objective was to compare the efficacy of DDL and CAN extracts against BHT in deep-frying. Therefore, the maximum concentration of BHT allowed for such applications (200 ppm) was selected for the frying experiment. All the extracts were added at 200 ppm level on weight basis, after quantification using HPLC-DAD.

5.4.1 Assessment of oil stability based on primary oxidation products

Initial increase in the PV during frying period indicates increased formation of peroxides due to oxidation. However, peroxides are unstable under deep-frying conditions (especially at higher frying temperatures) and as the oil deterioration continues, the hydroperoxides decomposes forming carbonyl and aldehydic compounds causing the PV to decrease (Shahidi *et al.*, 2002). This makes PV an unreliable parameter to assess the extent of oil deterioration. DDL and CAN significantly ($P<0.05$) reduced the oil oxidation process during frying (Figure 5.1). All the four samples (CNT, BHT, DDL and CAN) showed a rapid increase in the hydroperoxides within first day of frying, which decreased thereafter in the case of CNT, DDL and CAN oils. However, oil with BHT showed a slight increase in PV even after the first day of frying. The PV expressed as cumene hydroperoxides reached its maximum value on the first day in the case of DDL (361.38 ±0.6μM), CAN (182.41 ±2.17μM) and CNT (830.97 ±61.46μM) treatments. However, oils enriched with DDL and CAN extracts showed a considerable reduction in the hydroperoxides after the first day of frying, indicating the potential of these extracts to reduce or inhibit further primary oxidation product. This observation may also attribute to the formation of specific oxidation products formed during frying, which might reduce the concentration of primary oxidation products.

A considerable difference in PV was observed between control oil and oil enriched with CAN. The results showed that the DDL controlled PV appreciably, as PV was lower than BHT and CNT at every interval, confirming the antioxidant efficacy in stabilizing the oils by delaying hydroperoxides formation. Based on the results of PV, the following order of inhibition of hydroperoxides was observed with oils with different extracts/antioxidants-CAN>DDL>BHT>CNT. At the 5^{th} day of frying the PV reduced to 110.30 ±5.65, 207.91 ±4.93, 278.59 ±1.39 and 120.00 ±5.47μM respectively for oils enriched with CAN, DDL, BHT and without any added antioxidants/extracts.

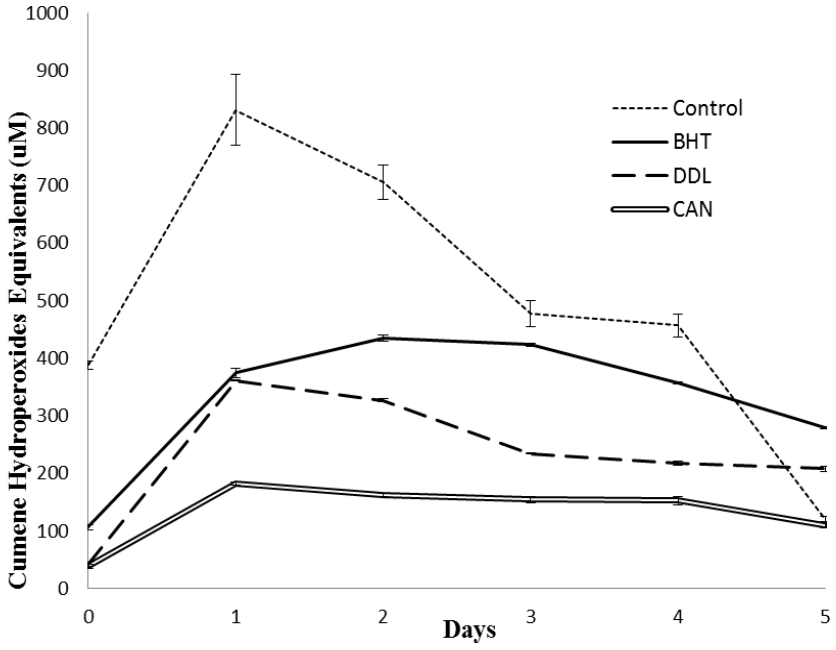

Figure 5.1 Changes in peroxide value (PV) during frying of canola oils with or without BHT, DDL or CAN extracts (200 ppm). (Mean and standard deviations for n=3)

The PV decreased after some hours of heating, indicating formation of secondary oxidation products, such as ketones, aldehydes, hydrocarbons and epoxides, which could be measured using the anisidine test. To assess the extent of fats and oils deterioration during frying, PV alone is not a reliable parameter, even if there exists a very good trend with respect to changes in hydroperoxides. However, the controlled formation of PV by antioxidants during deep-fat frying initially could be used as a preliminary assessment of antioxidant capacity.

5.4.2 Assessment of oil stability based on secondary oxidation products

The efficacy of DDL was also confirmed by the reduced formation of secondary oxidation products as measured as *p*-anisidine value (AnV). Hydroperoxides, the products of primary oxidation, react to form secondary products of which aldehydic components are measured by the anisidine test (Augustin & Berry, 1983). The AnV increased progressively over 5 days of frying (Figure 5.2). In this study, there was a marked increase in AnV on the

first day. After first day of frying, the AnV (mmol/kg oil) for control increased from 21.97±1.49 to 116.44±0.87, while for BHT, DDL and CAN were from 32.33± 0 to 54.61±0.10, from 24.64±0.35 to 40.20±0.44and from 15.29±0.22 to 43.24±0.1, respectively. It was also shown that during frying, for all treatments, the time of frying significantly influenced the AnV. At day 5, the AnV of control reached 221.72±0.07, while, for BHT, DDL and CAN treatments the values were, 149.37±0.99, 98.83±0.51, and 79.90±0.30 mmol/kg oil.

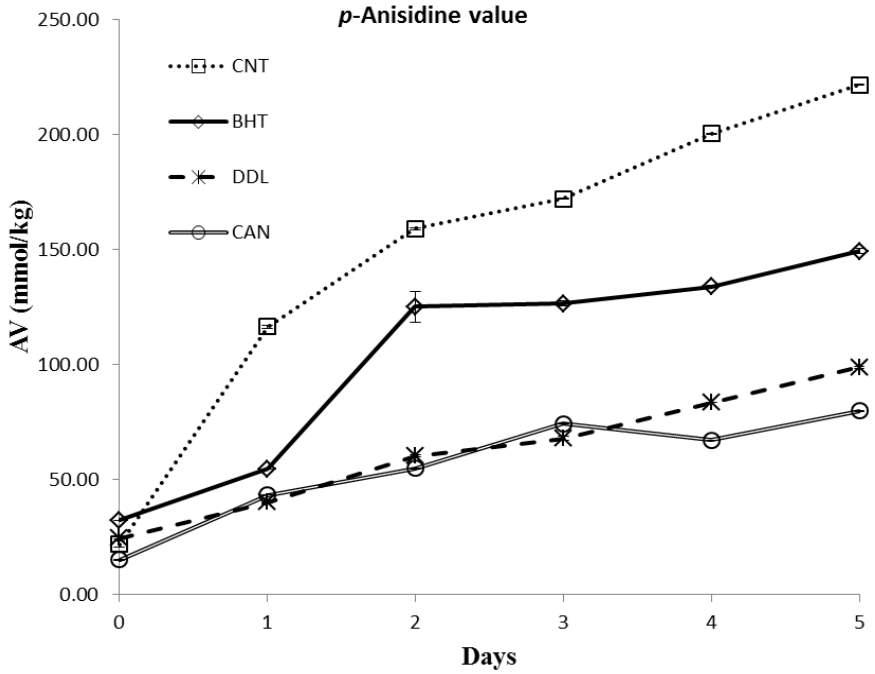

Figure 5.2 Changes in *p*-anisidine Value (AnV) during frying of canola oils with or without BHT, DDL or CAN extracts (200 ppm). (Mean and standard deviations for n=3)

Generally there were increases of AnV for all samples with definite pattern correlating with PV values. Based on the results of AV, the following order of inhibition of *p*-AnV observed with oils with different extracts/antioxidants- CAN>DDL>BHT>CNT. Even though BHT is reported to be more effective in retarding the formation of secondary oxidation products compared to primary oxidation products, the 200 ppm level used in the frying experiment was insufficient to obtain this effect. Moreover, it is well known that the concentration of BHT in frying oil is reduced under frying conditions. The rate at which the

AnV increased during the frying of canola oil was significantly less in the case of oil enriched with CAN extracts followed by DDL extracts. Both the CAN and DDL extracts can be recommended as a potent source of antioxidants for the stabilization of food systems, especially unsaturated vegetable oils.

5.4.3 Changes in conjugated diene and triene values of oil during frying

The concentration of both CD and CT are important determinants of oil stability, where in CD values are mainly related to the degree of primary oxidation and CT values are more correlated to the secondary oxidation.

Even though, all samples showed increase in CD and CT values during frying, the increase was less significant in the case of CAN compared to DDL, BHT or control treatments (Table 5.1). Generally, the CD values increase with frying duration as more hydroperoxides that possess CD structures are formed with more hydroperoxides degraded into secondary oxidation products with CT structures (Table 1). The results indicated that the CD values (expressed as percentage conjugated dienoic acid) after five days of frying reached maximum in the case of control oil, followed by BHT treatments (1.49 ±0.004 % and 1.40 ±0.004 % respectively). The CD values of oil enriched with CAN extract at fifth day of frying was the lowest (1.00 ±0.001) followed by DDL treatments (1.14 ±0.004%). Similarly, the levels of CT throughout the frying period were lowest in CAN treatments followed by DDL, with highest levels found in BHT treatment and control oils. Moreover in every treatment, the level of CT was lower than the corresponding CD values. The low levels of both CD and CT in CAN treatments indicate the good oxidative stability of the oil due to the presence of canola.

Table 5.1 Changes in conjugated diene and conjugated triene during frying of canola oils with or without BHT, DDL or CAN extracts (200 ppm) expressed as percentage conjugated dienoic acid

Days	**Conjugated diene (234 nm)** [a]				**Conjugated triene (268 nm)** [a]			
	Control	BHT	DDL	Canolol	**Control**	BHT	DDL	Canolol
0	0.41 ± 0.00^{Fb}	0.44 ± 0.00^{Ea}	0.39 ± 0.01^{Fc}	0.32 ± 0.00^{Fd}	0.09 ± 0.00^{Fb}	0.10 ± 0.01^{Fa}	0.09 ± 0.01^{Fb}	0.07 ± 0.00^{Ec}
1	0.85 ± 0.00^{Ea}	0.59 ± 0.02^{Db}	0.53 ± 0.01^{Ec}	0.47 ± 0.00^{Ed}	0.29 ± 0.00^{Ea}	0.17 ± 0.01^{Eb}	0.14 ± 0.00^{Ec}	0.12 ± 0.00^{Dd}
2	0.99 ± 0.00^{Da}	0.82 ± 0.00^{Cb}	0.51 ± 0.00^{Dd}	0.66 ± 0.01^{Dc}	0.33 ± 0.00^{Da}	0.25 ± 0.00^{Db}	0.15 ± 0.00^{Dc}	0.17 ± 0.01^{Cc}
3	1.18 ± 0.00^{Ca}	1.10 ± 0.01^{Bb}	0.84 ± 0.00^{Cd}	0.75 ± 0.00^{Cc}	0.35 ± 0.00^{Ca}	0.33 ± 0.00^{Cb}	0.29 ± 0.00^{Cc}	0.19 ± 0.00^{Bd}
4	1.36 ± 0.00^{Ba}	1.15 ± 0.01^{Bb}	0.98 ± 0.00^{Bd}	0.90 ± 0.00^{Bc}	0.37 ± 0.00^{Ba}	0.32 ± 0.01^{Bc}	0.34 ± 0.00^{Bb}	0.22 ± 0.00^{Ad}
5	1.50 ± 0.00^{Aa}	1.40 ± 0.00^{Ab}	1.15 ± 0.00^{Ad}	1.00 ± 0.00^{Ac}	0.39 ± 0.00^{Aa}	0.38 ± 0.00^{Ab}	0.36 ± 0.00^{Ac}	0.22 ± 0.00^{Ad}

[a] mean and standard deviations for n=3; the different lower-case (for row) or upper-case superscripts (for column) indicated significant difference between different treatments or day of frying at the level $p = 0.05$.

5.4.4 Total oxidation value of frying oils

The TOTOX values of the oils tested followed almost the same trend of PV, wherein the within first day of frying, a rapid increase in TOTOX value was observed (Figure 5.3). The TOTOX values of oils treated with DDL and CAN were lower compared to the BHT and control samples and a lower TOTOX-value corresponds to good quality of oil (Matthaeus *et al.*, 2010). Moreover, after first day of frying the TOTOX values of DDL/CAN treated oils were not changed significantly, indicating their potential to stabilize or inhibit the lipid oxidation of canola oil during frying. The highest TOTOX value of 1778.4 ±122.31, 762.97 ±1.39, and 408.07 ±4.34 were observed after first day of frying for control oil and oils treated with DDL and CAN respectively. However, the oil enriched with BHT showed the highest value of TOTOX (993.88 ±10.13) after the second day of frying.

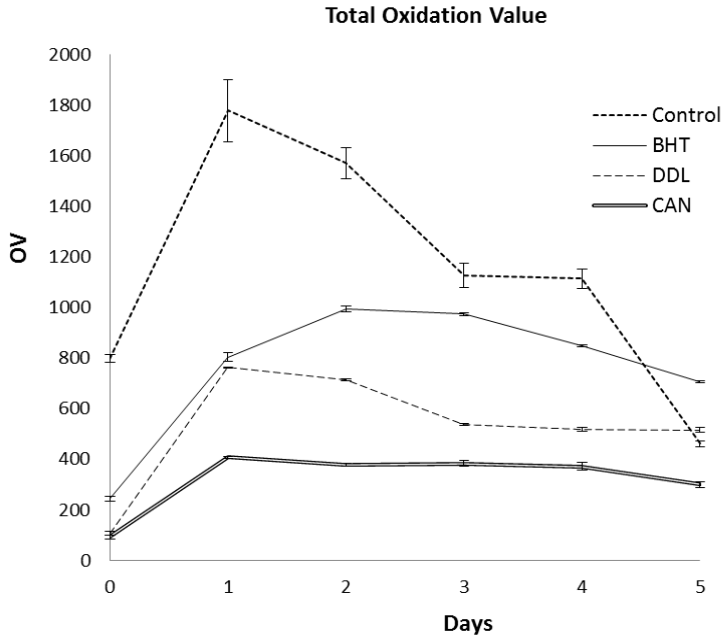

Figure 5.3 Changes in total oxidation values (TOTOX value, OV) during frying of canola oils with or without BHT, DDL or CAN extracts (200 ppm) calculated as OV= 2PV + 1AnV. (Mean and standard deviations for n=3)

As mentioned above, the BHT was not as effective as CAN or DDL extracts in the present study. The low antioxidant activity of BHT in stabilizing the canola oil was mainly

due to the lower efficacy in delaying hydroperoxides formation as evidenced by high total increase of PV. Additionally, BHT exhibits better antioxidative properties in oil-in-water emulsion rather than in bulk oils due to its lipophilic nature. This contributed to the less efficacy of BHT in the frying experiments compared to oil treated with DDL or CAN, as indicated by the TOTOX values.

5.4.5 Color index of frying oil

The color indices of frying oil are highly correlated to the content of total polar compounds in the oils (Xu, 1999; Xu, 2000). In the present study, the redness (R) and yellowness (Y) values of frying oil from day 0 to 5 were analyzed. The R-values of all the samples increased significantly from 0 day to 5^{th} day as indicated by the color data (Table 5.2). Even though the initial and final R value (at 0 day and 5 day respectively) of the control oil and BHT oil are relatively low in comparison to the oils enriched with CAN and DDL, the percentage increase in the R value is more in the case of control oil and BHT oil (approx. 10 times) than the oils enriched with DDL (9 times) and CAN (3 times). This indicated that, addition of extracts, especially canolol, suppressed the redness development in oil during frying. Similarly, among all the samples, the oil enriched with DDL showed a significantly higher Y value after 5^{th} day of frying (approx. 51), which was approximately 24 times increase from the Y values of 0 day sample. The oils enriched with BHT, and CAN showed a significantly lower change in the Y values after 5^{th} day of frying (9.8, and 4.7 times respectively). The results suggested that addition of CAN did not increase the color values in comparison to the respective 0 day samples, however it should be noted that the control oil showed better Y values as the addition of extracts itself imparted color to the oil.

Table 5.2 Changes in color values (R and Y)[a] of the canola oils with or without BHT, DDL or CAN extracts (200 ppm) during frying.

Color value	Time (day)	Control oil	Oil with BHT	Oil with DDL	Oil with CAN
Red	0	0.1±0.21	0.15±0.07	0.5±0.00	0.5±0.14
	1	0.1±0.00	0.1±0.00	0.9±0.00	0.5±0.00
	2	0.4±0.00	0.2±0.00	1.8±0.00	0.6±0.00
	3	0.7±0.00	0.4±0.00	2.5±0.14	1.0±0.00
	4	1.05±0.07	0.6±0.00	3.4±0.28	1.2±0.00
	5	1.3±0.00	1±0.00	4.6±0.14	1.6±0.14
Yellow	0	0.85±0.21	0.7±0.14	2.1±0.00	1.75±0.21
	1	1.7±0.00	1.1±0.00	3.8±0.00	1.9±0.00
	2	3.4±0.00	2.1±0.00	8.2±0.00	2.7±0.00
	3	5.1±0.00	3.3±0.00	18±0.00	4.0±0.00
	4	8.45±0.21	4.4±0.00	28.5±0.71	5.2±0.00
	5	12±0.00	6.9±0.28	51.0±0.00	8.35±0.35

[a] Mean and standard deviations for n=3

Unlike the linear correlation between TAA and AnV, the color (Y value) was highly correlated (R^2=0.822-0.985) with AnV in an exponential model (Figure 5.4). This was in agreement with a canola oil frying study conducted by Aladedunye & Przybylski (2009) who observed a low first order correlation between color and AnV. A color change accompanying an increase in AnV indicated that the color darkening in frying oil resulted from increased oxidation. The increase in color intensity is attributed to accumulation of nonvolatile decomposition products from triacylglycerols and free fatty acid oxidation (Abdulkarim *et al.*, 2007). However, the results suggested that the correlation between color and AnV was time independent. At the beginning of frying (day 0 to 2), the color darkening with AnV was in a lower rate in all oils. After second day of frying, the color intensity rapidly increased, perhaps due to the reduction of the antioxidant potential and increase of colored oxidation products. Overall, color can be used as a good alternative of assessment of oil oxidation.

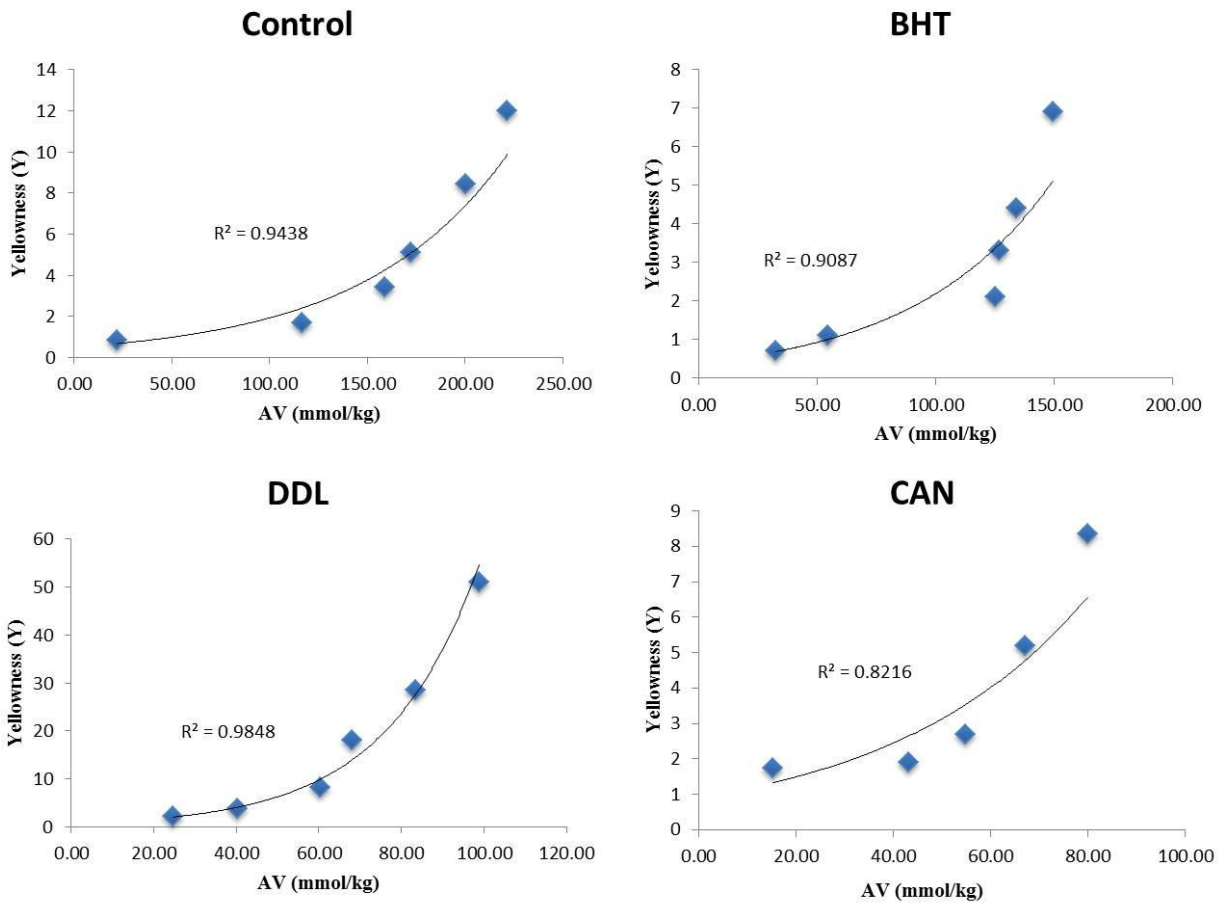

Figure 5.4 Correlation between the color (yellowness) and *p*-anisidine value (AnV) during frying of canola oils with or without BHT, DDL or CAN extracts (200 ppm) (Mean and standard deviations for n=3)

5.5 Conclusion

The formation of PV, AnV, TOTOX, CD, CT, and color suggested that the degradation, mainly thermal, was significantly lower in the oils enriched with crude DDL and CAN extracts compare to the control oil.These results lead to a conclusion that the rate of the oxidative degradation of the different oil enriched with DDL and CAN were far more effective in limiting oxidative degradation compared to the control at 200 ppm level. A comparison of the crude DDL and CAN extracts showed that the CAN extract enriched oil was much effective in improving the oxidative stability, by lowering levels of PV, AnV, CD, CT and TOTOX values. The DDL extract used in the present study was a crude extract so that

better results might be obtained with further purification. The color values of the frying oils were highly correlated to the secondary oxidation products. The crude extracts (CAN and DDL) did not show significant quality characteristics based on color attributes. The results suggested that both crude DDL and CAN extracts can be promoted as novel additives that provide oxidative stability to frying oil. The study also indicated the value added potential of canola meal and canola oil DDL. However, the exact nature of the phenolic compound in the DDL extract was not addressed in this study and warrants further studies. The novelty of the study has two aspects: (i) canolol, the decarboxylation product of sinapic acid, was tested and proved as an efficient antioxidant for deep frying, in comparison to a synthetic antioxidant and (ii) the crude phenolic extracts of DDL also improved oil stability, and hence had value added potential for industrial uses.

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CHAPTER 6

MANUSCRIPT 4

IMPACT OF CANOLOL ENRICHED EXTRACT FROM HEAT-TREATED CANOLA MEAL TO ENHANCE OIL QUALITY PARAMETERS IN DEEP-FRYING: A COMPARISON WITH ROSEMARY EXTRACT AND TBHQ-FORTIFIED OIL SYSTEMS

6.1 Abstract

Canolol enriched extracts obtained from the extraction of fluidized bed treated canola meal with supercritical carbon dioxide have been added to high-oleic canola oil in different concentrations (200, 500 and 750 mg/kg). After 30 h of deep-fat frying, oils fortified with canolol enriched extracts showed a two to three times better frying performance in comparison to the commonly used antioxidants (TBHQ, 200 mg/kg; rosemary extract, 40 and 200 mg/kg) and a control without antioxidants with regards to the formation of di-and polymer triacylglycerols, total polar compounds, secondary degradation products (*p*-anisidine value) and the iodine value. The canolol enriched extracts were also able to slow down the degradation of α- and γ-tocopherol during frying resulting in significant amounts of tocopherols after 30 h of frying in comparison to the other oils. The influence of the canolol enriched extracts indicated strongly concentration-dependent performance. With increasing concentration of the extract, the thermal stability of the fortified oil was improved. The only disadvantage of the addition of the extracts was an increase of the initial acid value, but within the frying time, only oil fortified with 750 mg canolol enriched extract/kg reached the limit given in different countries.

Keywords: canolol, di- and polymer triacylglycerols, frying, tocopherols, total polar compounds

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6.2 Introduction

Deep-fat frying is a simple and fast process for the preparation of food with a high acceptance by the consumer because of the palatability and the typical smell and taste of the products. From the chemical side the reactions occurring during deep-fat frying are very complex and not understood comprehensively today. During the process, oil is heated to temperatures between 160 and 180 ℃ in the presence of water from the food being fried and oxygen from the atmosphere over a longer period of time resulting in many reactions and subsequent products that impair the quality of the oil (Velasco *et al.*, 2009; Zhang *et al.*, 2012). Since the oil becomes part of the food being fried, the oil quality is of great importance for the quality of the food.

Main factors for the stability of oils during the frying process are the fatty acid composition and the presence of antioxidants (Przybylski & Eskin, 2006). Today oils with a high content of favorable mono-unsaturated oleic acid are preferred to combine health aspects with a desirable oxidative stability (Matthaus, 2006; Petersen *et al.*, 2013). On the other side synthetic and natural antioxidant active compounds are used to improve the oxidative and thermal stability of frying oils and to enlarge the time of application before discharging the oil. Effective synthetic antioxidants under moderate conditions are butylated hydroxyl toluene (BHT) or tert-butylhydroquinone (TBHQ), but unfortunately their use during frying is limited due to disadvantages during application or concerns to consumers as their safety has been questioned due to their health hazards in animals and humans (Sharayei *et al.*, 2011; Deshpande *et al.*, 1996).

Consequently, there is considerable interest in the use of natural substances or extracts that have antioxidant properties. Such naturally derived plant extracts are rich source of phenolic compounds, which are believed to be safer by providing additional health benefits compared to synthetic antioxidants. The antioxidant potential of different types of plant

extracts including rosemary *(Rosmarinus officinalis*) extract, *Pandanus amaryllifolius* leaf extract and Inca muña leaf extracts have been reported (Moreno *et al.*, 2006; Nor *et al.*, 2008; Chirinos *et al.*, 2011) showing the possibility of incorporating natural antioxidants into food products.

In comparison to other oilseeds, canola is rich in phenolic acids in free, esterified or insoluble form (Naczk *et al.*, 1986). The major phenolic compounds of canola are sinapic acid and its derivatives, mainly sinapine, the choline-ester of sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid) (Krygier *et al.*, 1982). During processing these phenolic compounds mainly remain in the press cake. However, Koski *et al.* (2003) and Wakamatsu *et al.* (2005) showed that oil-soluble 2,6-dimethoxy-4-vinylphenol (vinylsyringol or canolol) can be found in crude canola oil as degradation product of the oil insoluble sinapic acid by decarboxylation after heating of canola. Various authors found that temperatures of about 160 °C are necessary for the fast formation of canolol while higher temperatures result in a reduction of the canolol content after heating (Spielmeyer *et al.*, 2009; Pudel *et al.*, 2014).

In literature a high antioxidant activity is described for canolol in different systems (Kuwahara *et al.*, 2004; Sorensen *et al.*, 2013; Nowak *et al.*, 1992; Aachary *et al.*, 2014). Sorensen et al. (2013) found an order of BHT > canolol > sinapine > canola extract > sinapic acid in an oil-in-water emulsion and Matthäus (2012) described a remarkable antioxidant activity of canolol isolated from roasted canola in the DPPH and ß-carotene-linoleic acid assay. Recently, Aachary *et al.* (2014) showed that the formation of peroxides, conjugated dienes and trienes as well as secondary oxidation products during deep-fat frying was significantly lower in oils fortified with canolol or a deodistillate phenolic extract obtained from canola oil in comparison to a canola oil without added antioxidant or canola oil with BHT.

For the isolation of canolol containing extracts from canola two different approaches

are described. Harbaum-Piayda *et al.* (2010) isolated a polyphenol rich fraction from the deodistillate of the refining process which additionally to canolol and its dimer also contained tocopherols and other substances. Pudel *et al.* (2014) produced a canolol enriched extract from fluidized bed treated canola meal at 165 ℃ after extraction with super-critical carbon-dioxide. This process produced an extract with 2.5 percent canolol.

The aim of the present paper is to compare the effectiveness of a canolol enriched extract (CAN) obtained by extraction with supercritical carbon-dioxide from a fluidized bed treated rapeseed meal with a commercially available natural antioxidant (Rosemary extract (RM)) and a standard synthetic antioxidant (TBHQ). CAN was evaluated at three concentration levels (200, 500 and 750 mg/kg), RM at two levels (40 and 200 mg/kg) and TBHQ at one level (200 mg/kg).

6.3 Materials and Methods

6.3.1 Materials

All chemicals were of analytical grade. Food grade tert-butylhydroquinone (TBHQ), sinapic acid and tocopherol standards were purchased from Sigma Aldrich, Canada. A commercial rosemary extract product namely INOLENS 4 (solution of natural rosemary extract in vegetable oil containing 4% carnosic acid) was purchased from Vitiva d.d., Slovenia. Refined high oleic canola oil was provided by Viterra Canola processing, Winnipeg, Manitoba, Canada.

6.3.2 Processing of canolol enriched extract (CAN)

Commercial canola meal was purchased from Altmärkisches Kraftfutterwerk Rittleben GmbH, Germany. The meal was grinded by a roller mill and partially screened by sieving. The fraction of 0.4 – 0.8 mm particle size was subsequently heated by hot air within a fluidized bed up to a temperature of 165 ℃. Immediately after reaching that temperature the meal was cooled down (Pudel *et al.*, 2014).

The heat treated meal was extracted by Flavex Naturextrakte GmbH, Germany, using CO_2 extraction. Before extraction the heat treated meal was moistened from 1.7% to about 20%. The CO_2 extraction was carried out at 50 ℃, 200 bar pressure and a solvent ratio of 20 kg CO_2/kg meal. The canolol content of the resulted extract was 3%.

6.3.3 Frying experiment

Frying experiments were conducted in 3L-capacity stainless steel deep fryers (Hamilton Beach Company, Picton, Canada). The seven treatments of high-oleic canola oil used were: (1) canola oil without any added antioxidant or extracts (Control, CNT), (2) Canola oil with 200 mg/kg TBHQ, (3) canola oil with canolol enriched extract (CAN) at three concentrations: 200, 500 and 750 mg/kg, and (4) canola oil with rosemary (RM) extract at two concentrations: 40 and 200 mg/kg. All treatments were conducted in duplicates at Richardson Center for Functional Food and Nutraceuticals (RCFFN), University of Manitoba, Winnipeg, Canada. TBHQ was added to the oil by dissolving it in minimum quantity of methanol and then mixed with oil under N_2. CAN and RM rich oils were mixed uniformly with the canola oil under inert conditions.

Frying procedure was conducted following Archary's *et al.* (2014) protocol with slight modifications. High-oleic canola oil (3L) was conditioned by heating to 185 $\pm$ 5 ^{o}C and then held at this temperature for 30 min before start frying every day. A batch of 30 g of uniform size frozen French fries was fried at a constant frying temperature of 185 $\pm$ 5 ^{o}C for 5 minutes. The fryers were left uncovered during the frying period. Twelve batches of French fries (30 g each) were fried each day, 30 min apart for a total of 6 h of frying. The same oil was used for frying for 5 days for a total frying time of 30 h. A total of six samples of oil were collected (0, 6, 12, 18, 24, 30 h) from each treatment, flushed with nitrogen gas and stored at -20 ^{o}C until further analysis. Fryers were shut off and left to cool overnight at the end of each frying days. Oil was replenished to 3 L every second and consecutive days.

6.3.4 Anisidine value (AnV), acid value (AV), iodine value (IV)

Anisidine value (AnV), acid value (AV), iodine value (IV) were determined by FT-NIR transmission measurement (Guillermo *et al.*, 1999). The measurement was done according to the method DGF C-VI 21a (DGF, 2013). In brief, oil samples were filled in 8 mm disposable vials. All spectra were recorded in triplicate at 50 ℃ after a thermal preconditioning for 10 minutes in a separate thermoblock to avoid turbid solutions. Spectra were obtained in transmission mode from 12.500 cm^{-1} to 4.000 cm^{-1}. Each spectrum was time-averaged based on 32 scans at a resolution of 8 cm^{-1} using Bruker MPA FT-NIR spectrometer (Bruker Optik GmbH, Ettlingen, Germany), equipped with OPUS software version 7.2.

6.3.5 Di and polymerized triacylglycerols (DPTG)

The content of oligomer triacylglycerols was determined with gel-permeation chromatography by high pressure liquid chromatography (HPLC) according to the method DGF C-III 3c (DGF, 2013).

6.3.6 Polar compounds

The determination of the polar compounds was carried out according to the method DGF C-III 3b (13) (DGF, 2013).

6.3.7 Colour index of frying oil

Lovibond PFx 995Tintometer, a PFX*i*series of spectrophotometric colorimeter was used to measure oil color. The frozen oil samples were brought to room temperature, mixed with diatomaceous earth (0.16% w/w), filtered using Whatman wet strengthened qualitative circles and then transferred to the cuvettes (1 cm optical path). The absolute measurements were displayed in Lovibond RYBN color scale. Since, it was found that the R and Y values were significantly correlated with the time of deep-fat frying, only these values were considered for the analysis, eliminating the B (blue) and N (neutral) values. The higher the R

and Y values, the darker and lower quality of the oil is.

6.3.8 Determination of canolol and tocopherol residue content in canola oil

For determination of canolol content, 2 grams of canola oil was extracted with 70% methanol for twice. The phenolic profiling of canolol extract was established following a reversed-phase HPLC-DAD analysis (Khattab *et al.*, 2010) using an HPLC system (Ultimate 3000; Dionex, Sunnyvale, CA, USA), which consisted of a diode array detector and other accessories. Solvent A, 90% methanol (aqueous) acidified with *o*-phosphoric acid (1.2%) and solvent B, 100% methanol acidified with o-phosphoric acid (0.1%) were used as mobile phases in a gradient elution, where in the concentration of mobile phase B (%, indicated in brackets) changed in the following sequences at specified time periods (minutes) 0 (10), 7 (20), 20 (45), 25 (70), 28 (100), 31 (100) and 40 (10). Synergi 4μ Fusion-RP 80 Å; 150 × 4.0 mm- 4 micron (Phenomenex, Canada) column was used for peak separation. Both the mobile phases and phenolic extracts were passed through syringe filters (0.45 μm). Other conditions of analysis were strictly maintained which included: flow rate (1 ml/min), column compartment temperature (25 ℃) and wavelengths of analysis (270 nm). Peaks were identified by comparing their relative retention times and spectrum with those of the authentic standards of sinapic acid.

Analysis of tocopherol was conducted according to AOCS official method (Ce 8-89) (AOCS, 1992). Simply, 2 grams of canola oil was mixed with n-hexane to a final volume of 25 ml. The tocopherols were detected with the same HPLC system described above. Spherisorb ODS2 column (80Å, 5 μm, 4 mm X 250 mm) was used for peak separation. Isopropanol in hexane (0.5:99.5, v/v) was used as mobile phase in an isocratic elution at flow rate of 1.0 ml/min for 30 min. The injection volume was maintained at 20 μl. Chromatograms were acquired at 292 nm where in identification of tocopherols was achieved by comparing the relative retention times and spectrum with the standards of α-, β and γ-tocopherol.

6.3.9 Statistical Analyses

All analyses were carried out at least in triplicate and results were presented as experimental mean ± SD. One way analysis of variance (ANOVA) was done using SAS 9.4 software (SAS Institute Inc., USA). Linear regressions were produced by Microsoft Excel 2007 (Microsoft Corporation, Redmond, WA). Statistical significance was determined using least significant difference *t*-test. Statistical significance was accepted at $p < 0.05$.

6.4 Results and Discussions

During deep-fat frying oil undergoes complex reactions resulting in fast degradation of the oil. Some of these degradation products are desirable since they are responsible for the typical taste and smell of fried products, but others impair oil quality with subsequent frying time (Boskou, 2003). Antioxidants are used to improve the thermal stability of the oil during processing since they can scavenge free radicals, inactivate pro-oxidant metals, quench singlet oxygen, or inactivate sensitizers depending on their structure feature. A suitable mean to prove the effectiveness of new antioxidant active compounds during food processing in comparison to common used antioxidants is the performance of a frying experiment and analysis of relevant parameters characterizing the quality of the used frying oil.

Such parameters are defined in the recommendations for the assessment of used frying oils of the German Society of Fat Science (Anon, 2000). The most important parameters for the assessment of used frying oils are the content of polar compounds as well as polymer and oligomer triacylglycerols. In many countries limits for these parameters have been established to monitor the suitability of used frying fat and oils for human consumption since the high uptake of these compounds decreases the nutritional value of the oil and also the product being fried (Kanner, 2007). On the other side, the toxicological influence of these compounds is under suspicion depending on the species in question. Other parameters to follow the degradation of the used frying oils and to assess the oil quality are the anisidine

value, changes of color or decrease of unsaturated fatty acids.

6.4.1 Di- and polymerized triacylglycerols

During frying a continuous formation of high molecular weight degradation products mainly di- and polymer triacylglycerols (DPTG) can be observed. The amount of DPTG in used frying oils is used as a reliable parameter for the assessment of the oil quality (Gertz, 2001) and the limit in different countries has been set between 10 and 12 % indicating that the used frying oil is no more suitable for human consumption and should be discarded.

As shown in Figure 6.1, DPTG increased significantly in all samples during the frying experiment independently on the added antioxidant, but significant differences ($p < 0.05$) were found between the slopes of the curves, representing differences in the rate of formation. All antioxidants showed an effect on the formation of DPTG in the frying oil in comparison to the control and were able to reduce the formation of DPTG under frying conditions. CNT without added antioxidant reached nearly 10 % DPTG after 30 h of frying indicating that the oil was almost unsuitable for human consumption. On the other hand all fortified oils remained below 8.5 % showing protection against polymerization reactions by the different antioxidants during continuous heating. The anti-polymerization effects of RM (200 mg/kg) and TBHQ (200 mg/kg) were comparable and resulted in less than 8 % DPTG after 30 h frying. In contrast to other synthetic antioxidants such as BHA or BHT which are relatively ineffective to improve thermo-stability of oils during frying (Tsakins *et al.*, 2002) due to decomposition and evaporation (steam volatility), TBHQ is more effective in extending the life of frying oils in comparison to other commonly used synthetic antioxidants because the degradation products of TBHQ are also strong antioxidants. Phenolic antioxidants such as TBHQ have been reported to be particularly effective against polymerization. In different frying studies the effectiveness of TBHQ in comparison to other synthetic and natural antioxidants has been shown (Man & Liu, 1999).

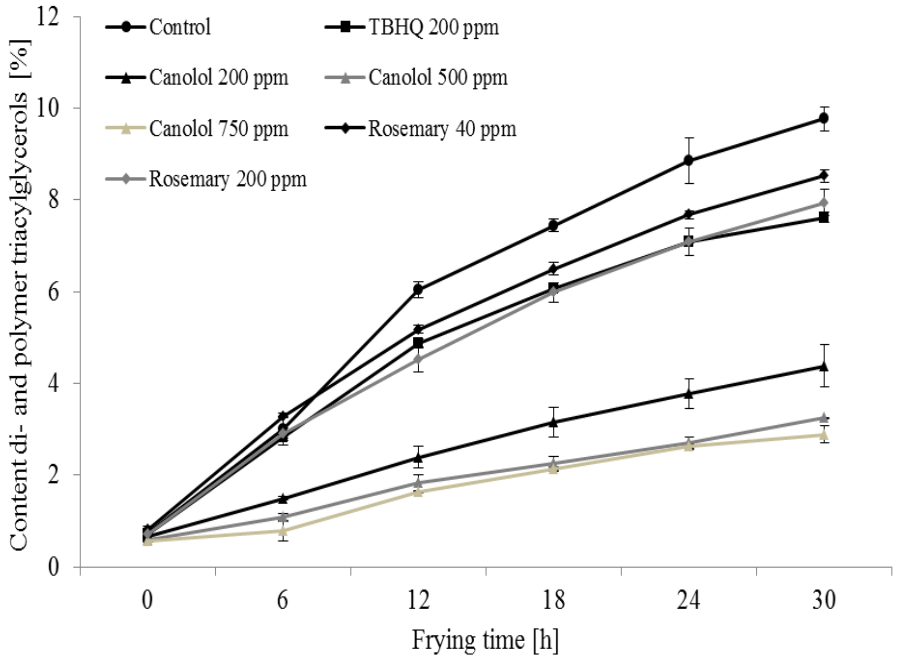

Figure 6.1 Formation of di- and polymer triacylglycerols during deep-fat frying with high-oleic canola oil fortified with different antioxidants.

The strongest antipolymerisation effect was found for CAN. During 30 hours of frying the content of DPTG did not reach 4.5 % which was only about half the polymerization rate of oils fortified with RM or TBHQ.

The results also showed a clear concentration-dependent effect of CAN on the polymerization of the oil during frying. The strongest effect was found for oil fortified with 750 mg/kg CAN resulting in an increase of DPTG to only 2.9 % after 30 hours of frying. That was less than one third of the polymerization rate of the control and a little more than one third in comparison to TBHQ or RM.

6.4.2 Polar compounds

Another important parameter to evaluate the quality of used frying oils is the content of total polar compounds. Since these substances are not volatile and represent the major reaction products at frying temperatures measuring the content of polar compounds gives a

reliable parameter for assessing frying oil stability. Since the polar fraction of used frying oils is suspected to contain most of the harmful substances several countries have established a limit for the content of total polar compounds of used frying oils between 24 and 27% (Paul *et al.*, 1997).

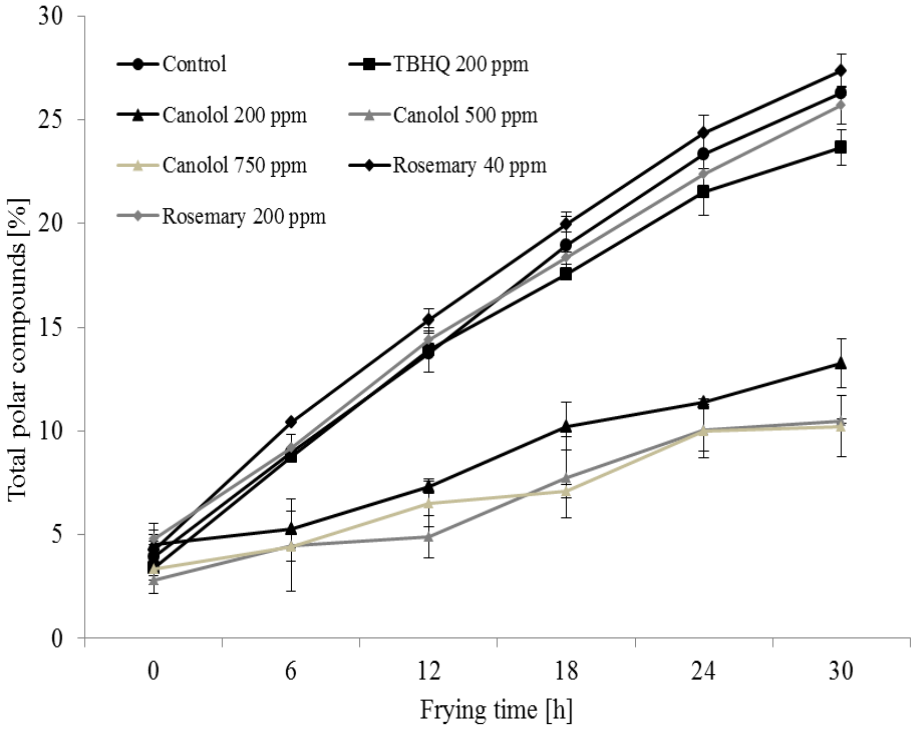

Figure 6.2 Formation of total polar compounds during deep-fat frying with high-oleic canola oil fortified with different antioxidants.

After 30 h of frying the control as well as the oils fortified with RM exceeded the limit of 25% total polar compounds (Figure 6.2). The differences between the oils were not significant ($p < 0.05$), but the addition of 40 mg rosemary extract/kg oil seems to have a slight pro-oxidative effect resulting in a more rapid formation of polar compounds during frying than for CNT. The content of total polar compounds in the oil fortified with 200 mg TBHQ/kg remains just below the limit after 30 h of frying, but the amounts were very close to the polar compounds of RM and CNT.

A very strong effect on the formation of polar compounds during frying was found for

CAN. While oil fortified with 200 mg CAN/kg reached a value of 13.2 % total polar compounds, the formation of polar compounds was significantly reduced for 500 and 750 mg CAN/kg with 10.5 and 10.2 %, respectively. The increase of the polar compounds was only about half of that found for the other antioxidants or the control.

6.4.3 Acid value

In different countries the content of free fatty acids is used as criteria for the assessment of the quality of used frying oils. According to these regulations the frying media should be discarded if the acid value exceeds 2 or the content of free fatty acids exceeds 1 g/100 g (Fox, 2001). On the other hand, the recommendations of the 7th Symposium on Deep-fat frying do not consider the content of free fatty acids as regulatory index to monitor and compare the degree of degradation of frying oils (Stier & Gertz, 2013).

During the frying experiment a continuous increase of the content of the acid value was monitored. No significant differences were found between CNT without antioxidant and the oils fortified with RM and TBHQ, respectively ($p < 0.05$). The acid value increased from 0.1 g/100 g to 0.6 g/100 g (Figure 6.3).

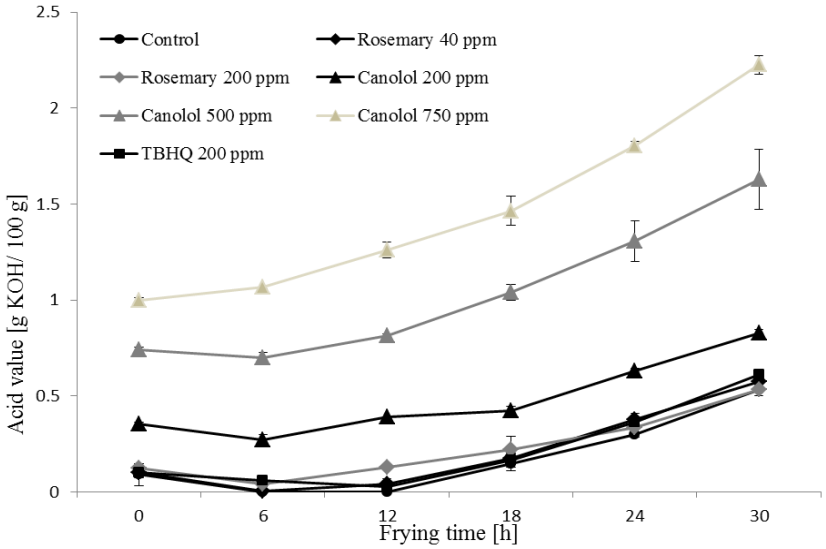

Figure 6.3 Development of the acid value during deep-fat frying with high-oleic canola oil fortified with different antioxidants.

In contrast, the situation for oils fortified with CAN had different effects. The addition of CAN resulted in a higher acid value in the initial oils, depending on the concentration of the added extract. While the addition of 200 mg CAN/kg resulted in an initial acid value of 0.36 g/100 g, after addition of 500 and 750 mg CAN/kg the initial acid value was 0.74 g/100 g and 1.0 g/100 g, respectively. This could be partially explained by the influence of the free fatty acids perhaps formed during the short heating of the meal in the fluidized bed treatment at 165 °C in the presence of moisture from the meal and extracted with supercritical carbon dioxide.

During frying the acid value of the oil fortified with 200 mg CAN/kg increased comparable to the other oils and after 30 h of frying the acid value was 0.83 g/100 g. In contrast to this, the increase of the acid value was markedly steeper for oils fortified with 500 and 750 mg CAN/kg, respectively, resulting in acid values of 1.63 g/100 g and 2.2 g/100 g after 30 h of frying. Depending on the concentration of CAN added to the frying oil the development of the acid value was accelerated. While the slope of the curves calculated from 12 to 30 h of frying for the CNT, TBHQ, RM and CAN 200mg/kg was about 0.03 the addition of 500 and 750 mg CAN/kg, respectively, increased the slope of the curve to 0.04 and 0.05, respectively. That shows that a higher initial acid value of the frying oil at the beginning of the experiment resulted in a faster increase of the acidity during frying.

6.4.4 Anisidine value

During deep-fat frying a huge number of volatile and non-volatile components are formed as a result of the thermal degradation of triacylglycerols. The most prominent secondary oxidation products are carbonyl compounds, which contribute to the flavor of the oil and account for almost 50% of the volatiles produced during frying (Przybylski & Eskin, 1995). The content of non-volatile aldehydes can be monitored by the anisidine value, resulting from the reaction of p-anisidine with 2,4-dienals and 2-alkenals. Some authors also

described a highly significant correlation between the anisidine value and the flavor scores of oils (List *et al.*, 1974; Tompkins & Perkins, 1999).

Figure 6.4 shows the continuous increase of the anisidine value with the frying time for CNT and all fortified oils. After 30 h of frying, CNT reached an anisidine value of about 100, similar to that of oils fortified with RM. The anisidine value of oil fortified with 200 mg TBHQ/kg was a little lower; this oil reached an anisidine value of about 90, which was significant ($p < 0.05$) lower than the previously discussed oils. The anisidine values for oils fortified with extract obtained from fluidized bed treated canola meal were also lower. Depending on the added concentration the anisidine values were found in the range between 25 and 46, with highly negative linear correlation between the amount of added antioxidant and the anisidine value ($R = -0.9630$).

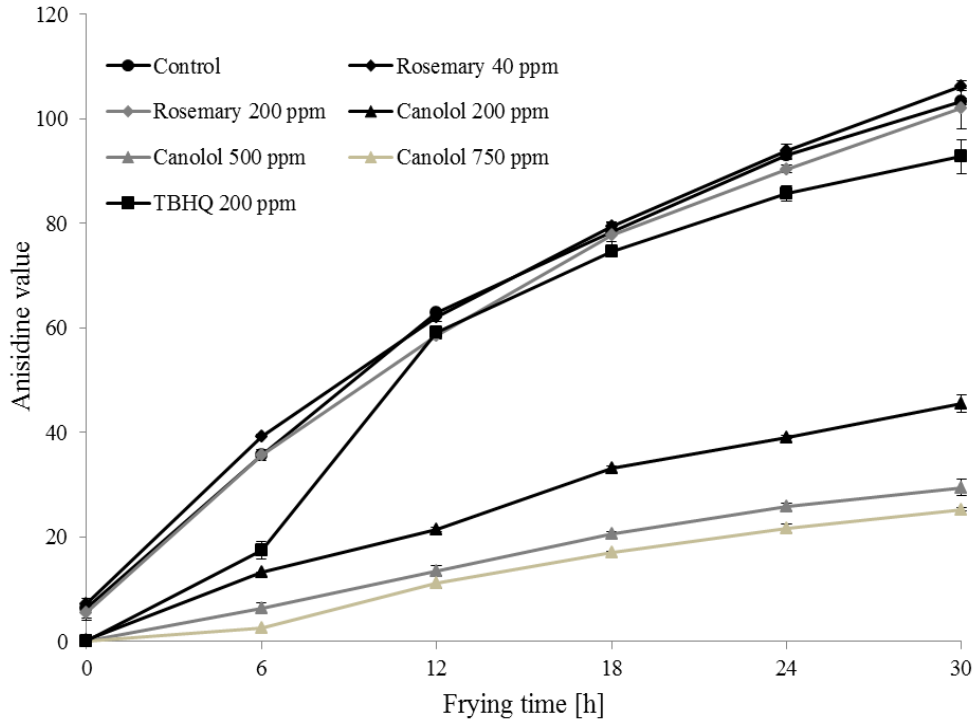

Figure 6.4 Development of non-volatile compounds during deep-fat frying with high-oleic canola oil fortified with different antioxidants measured as anisidine value.

6.4.5 Iodine value

The iodine value gives some information about the amount of unsaturation of fatty acids and the higher the iodine value the more C-C double bonds are present in the molecule. As a result of the degradation of the fatty acid molecules during frying the number of double bonds decreases resulting in a decrease of the iodine value.

The initial iodine value of the high-oleic canola oil was approximately 97. With continuous frying the iodine value of all fortified frying oils decreased linearly with frying time (Figure 6.5). After 30 h of frying the lowest iodine values were found for oil fortified with RM with 87.8 and 88.7, respectively for 40 and 200 mg/kg, however the difference was not significant between the CNT (89.0) and RM 200 mg/kg ($p < 0.05$). The use of TBHQ resulted in a lower decline of the iodine value during frying (90.1) and the lowest degradation of the double bonds was found for oils fortified with CAN.

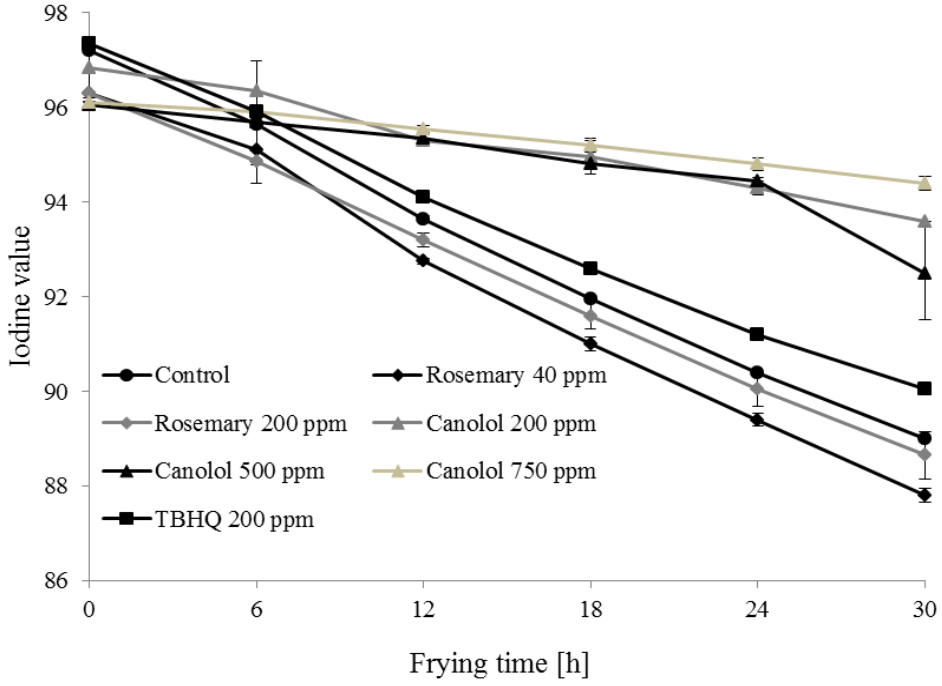

Figure 6.5 Decrease of the grade of unsaturation during deep-fat frying with high-oleic canola oil fortified with different antioxidants.

6.4.6 Color changes of frying oils

Color darkening is a complicated process involving interactions with fatty acids, dimers, polymers and other decomposition compounds present in the oil. The rate of oil darkening during deep-fat frying is also affected by some minor compounds such as pigments. Additionally, various compounds such as carbohydrates, lipids, proteins, phosphoric and sulphuric compounds and trace metals introduced during frying of different kinds of food will also influence the color of frying oil. The color indices of frying oil are highly correlated to the content of total polar compounds in the oils (Xu, 2000).

In the present study, the redness (R) and yellowness (Y) values of frying oil from 0 to 30 h were analyzed (Figure 6.6). The initial R and Y values of oils rich in CAN and RM extracts were higher than that of CNT and TBHQ rich oils. This is due to the presence of red and yellow color in the CAN and RM extract samples. Both R and Y values rapidly increased as the frying time increased. The degradation in CAN treated oils was much slower than that in the non-treated oils. Although the Y values of CAN treated oils are higher than CNT oil after 12 h of frying, both the values were lower in the CAN treated oil with all concentrations after 18 h due to its lower color degradation rate. This result indicated that CAN had a significant effect on prevention of color degradation during frying. On the other side RM extracts seems to have no protective effect on color degradation during frying at both 40 and 200 mg/kg concentrations. The most rapid color degradation was found in oil fortified with TBHQ rich oil which showed the highest value for redness (R=1.55, Y=12) after the 30 h of frying. This negative effect of TBHQ on oil color during frying has been reported earlier. One explanation is that some phenolic antioxidants may form colored compounds in the presence of heat and metals (Asap & Augustin, 1986). The instrumental analyzed color results were consistent with the oil color observed with eyes (Figure 6.7). Overall, the color degradation of the oils during frying with different treatments was increased with the following order:

CAN<CNT<RM<TBHQ.

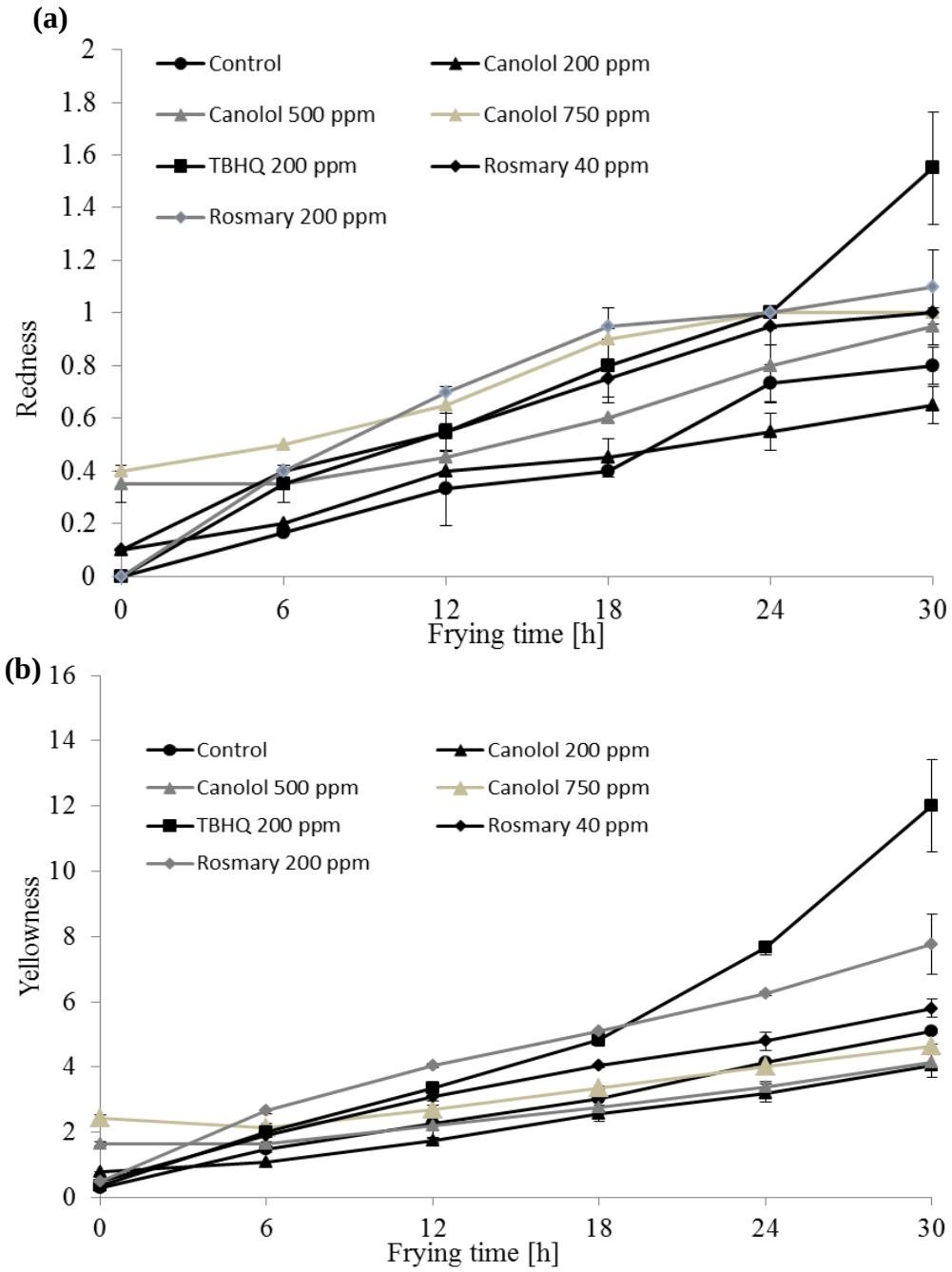

Figure 6.6 Influence of different antioxidants during deep-fat frying on color values ((a) redness and (b) yellowness) of the frying medium.

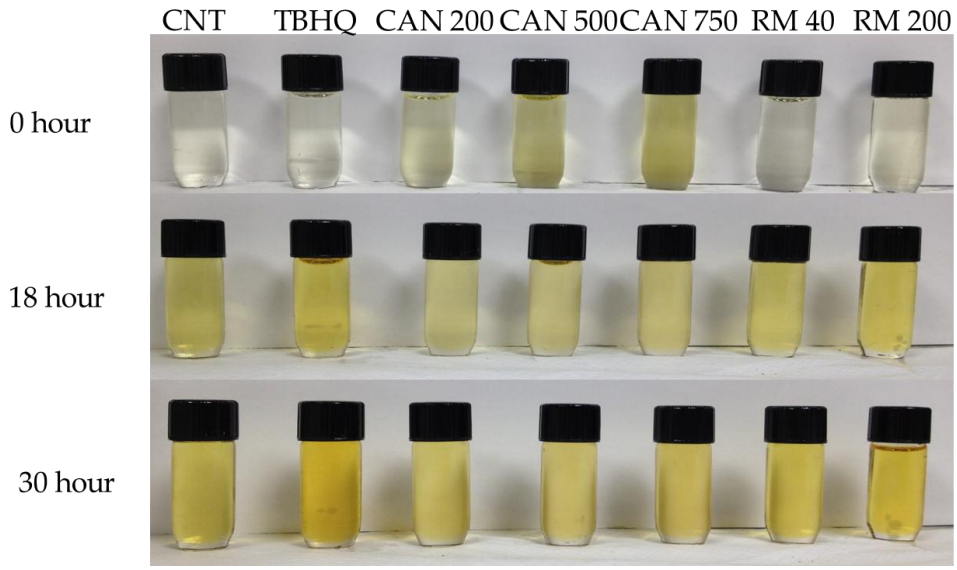

Figure 6.7 Images of canola oil samples at different frying period with different antioxidant treatment (CNT: Control; CAN: Canolol treatment; RM: Rosemary treatment)

6.4.7 Degradation of CAN and tocopherol during frying

Antioxidants added to frying oils during extensive heating over a longer period of time are not only interesting to improve its thermal stability, but they could also improve the storage stability of food after frying if the antioxidant compounds survive the frying period and is taken up by the food. The amount of antioxidant coming into the food depends on the type of antioxidant and on the level of antioxidant in the frying medium at the time of each frying operation (Augustin & Berry, 1983).

As expected canolol was not detected in the CNT, TBHQ and RM treated oils. Figure 6 summarizes the degradation of CAN through the frying process. The highest degradation of CAN was observed in the first day of frying which resulted in a degradation of CAN by more than half. In oil fortified with 200 mg/kg CAN after 18 h of frying only traceable amounts of canolol remained and after 30 h no CAN was detected. A similar degradation trend was observed for oils fortified with 500 and 750 mg CAN /kg, respectively. The residual CAN concentrations after 30 h of frying were 22 and 38 mg/kg for oils fortified with 500 and 750

mg/kg, respectively.

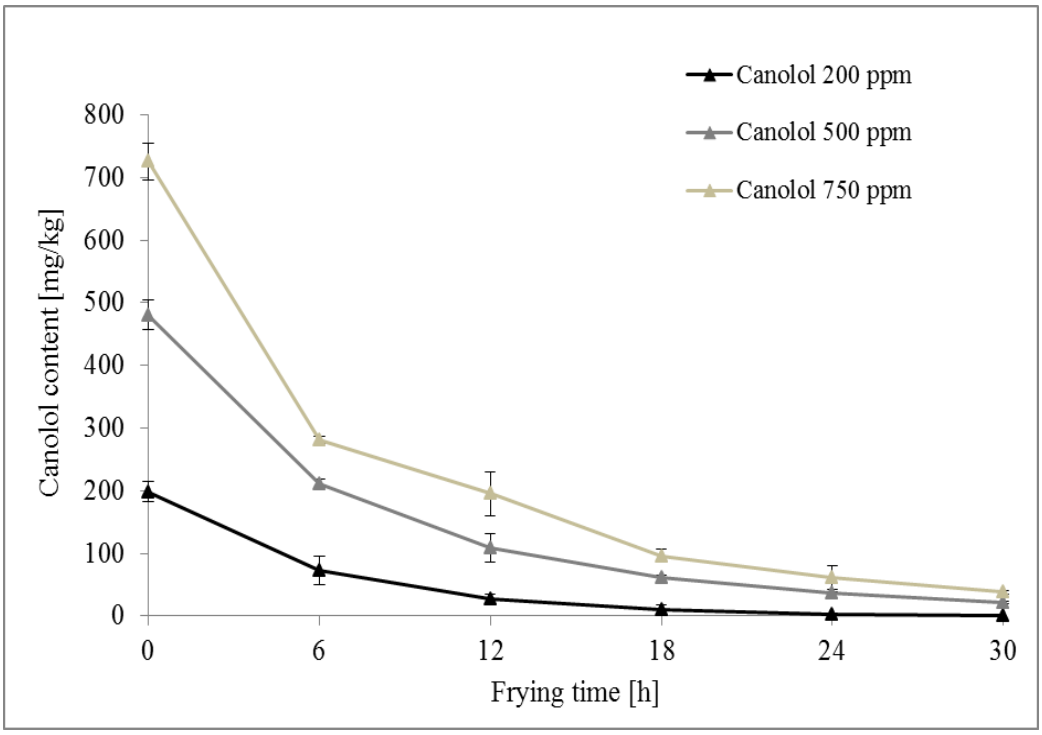

Figure 7: Influence of the concentration of CAN on degradation of CAN during deep-fat frying with high-oleic canola oil.

In order to assess the degradation rate of CAN during the frying period, correlations between frying time and log transformation of CAN concentration in oils were calculated. The correlations between the two parameter of all three concentration levels were very significant (R >0.98). Interestingly, with increasing initial concentration of CAN, the degradation rate of canolol was decreased. While the degradation rate of CAN in canola oils fortified with 500 and 750 mg/kg were very close together (slope: -0.25 and -0.26, respectively), the degradation rate for oil fortified with 200 mg CAN/kg was much higher (slope: -0.47). This indicates that CAN may have a role in stabilizing itself during frying. This is the first report on canolol degradation kinetics as we know which can provide more information for the food application of CAN during frying temperature.

The main vitamin-E-active compounds of high-oleic canola oil are α.tocopherol and γ-tocopherol, with average concentrations of about 182.9 and 295.0 mg/kg, respectively.

During frying the tocopherols are degraded and after 18 h no tocopherols were found in CNT and oils fortified with TBHQ and RM 40 mg/kg. After 24 h also in oil fortified with RM 200 mg/kg the concentration of tocopherols was almost zero. Only in oils fortified with CAN, significant amounts of α- and γ-tocopherols were found after 30 h of frying, with a strong dependency on the concentration of added CAN. The degradation rates of α- and γ-tocopherol were lower for oil fortified with 750 mg CAN/kg than for 500 mg/kg or 200 mg/kg with slopes of the curves as -4.4, -4.2 and -3.9 for α-tocopherol, respectively, and -8.3, -7.4 and 7.3 for γ-tocopherol, respectively. The faster degradation rate of γ-tocopherol in comparison to α-tocopherol is in agreement with the finding of Aggelousis & Lalas (1997) who described the relative decomposition rates after 5 days of frying as δ > γ > α-tocopherol. Also (Carlson & Tabacchi, 1986) showed that the decomposition rates of tocopherols in fried soybean oils were γ- > α-tocopherol.

The addition of natural antioxidants like CAN can improve the stability of tocopherols and increases the half-life of these compounds during deep-fat frying. While for CNT, RM (40 and 200 mg/kg) and TBHQ the initial concentration of α- and γ-tocopherol was degraded to 50 % (LD_{50}-value) within 5.4, 6.1, 8.5 and 4.7 hours (α-tocopherol) and 5.8, 6.5, 8.3 and 5.0 hours (γ-tocopherol), the half life of α-and γ-tocopherol was longer for oils fortified with CAN (17.9, 23.6 and 28.7 hours (α-tocopherol) and 15.8, 18.1 and 19.6 hours (γ-tocopherol)). This result is in agreement with findings of Reblova and Okrouhla (2010) who showed that different phenolic compounds were able to extend the half life of α-tocopherol significantly.

The results also show that the addition of TBHQ has only a negative effect on the degradation of tocopherols. In comparison to CNT without additional antioxidant the degradation of both tocopherols was faster in oil fortified with TBHQ 200 mg/kg.

Table 6.1 Depletion of tocopherols during frying with high oleic canola oil fortified with antioxidants

	Control	TBHQ 200 ppm	Canolol 200 ppm	Canolol 500 ppm	Canolol 750 ppm	Rosemary 40 ppm	Rosemary 200 ppm
Frying time [h]				**α-tocopherol [mg/kg]**			
0	$166.5 \pm 3.1^{B}_{a}$	$171.7 \pm 4.5^{AB}_{a}$	$171.1 \pm 7.3^{AB}_{a}$	$186.8 \pm 9.4^{AB}_{a}$	$199.2 \pm 9.2^{A}_{a}$	$190.7 \pm 8.4^{AB}_{a}$	$194.6 \pm 9.7^{AB}_{a}$
6	$60.4 \pm 19.8^{CD}_{b}$	$28.3 \pm 4.2^{D}_{b}$	$161.1 \pm 4.3^{A}_{a}$	$176.8 \pm 0.5^{A}_{a}$	$186.8 \pm 2.0^{A}_{a}$	$99.0 \pm 9.1^{BC}_{b}$	$141.2 \pm 25.7^{AB}_{b}$
12	$0.0 \pm 0.0^{D}_{c}$	$0.0 \pm 0.0^{D}_{c}$	$113.4 \pm 2.0^{B}_{b}$	$138.3 \pm 11.6^{B}_{b}$	$177.6 \pm 8.1^{A}_{ab}$	$0.0 \pm 0.0^{D}_{c}$	$28.0 \pm 10.0^{C}_{c}$
18	$0.0 \pm 0.0^{D}_{c}$	$0.0 \pm 0.0^{D}_{c}$	$83.4 \pm 1.7^{C}_{c}$	$117.8 \pm 14.9^{B}_{bc}$	$151.4 \pm 14.2^{A}_{b}$	$0.0 \pm 0.0^{D}_{c}$	$0.0 \pm 0.0^{D}_{c}$
24	$0.0 \pm 0.0^{C}_{c}$	$0.0 \pm 0.0^{C}_{c}$	$78.1 \pm 1.7^{B}_{c}$	$88.6 \pm 6.2^{B}_{cd}$	$115.5 \pm 6.9^{A}_{c}$	$0.0 \pm 0.0^{C}_{c}$	$0.0 \pm 0.0^{C}_{c}$
30	$0.0 \pm 0.0^{C}_{c}$	$0.0 \pm 0.0^{C}_{c}$	0.0^{C}_{d}	$68.1 \pm 5.5^{B}_{d}$	$84.9 \pm 0.1^{A}_{c}$	$0.0 \pm 0.0^{C}_{c}$	$0.0 \pm 0.0^{C}_{c}$
LD_{50}-value	5.4 ± 0.4^{E}	4.7 ± 0.1^{E}	17.9 ± 0.5^{C}	23.6 ± 0.8^{B}	28.7 ± 1.2^{A}	6.1 ± 0.1^{DE}	8.5 ± 1.0^{D}
Frying time [h]				**γ-tocopherol [mg/kg]**			
0	$280.0 \pm 3.7^{A}_{a}$	$291.1 \pm 4.8^{A}_{a}$	$271.0 \pm 22.5^{A}_{a}$	$289.5 \pm 12.7^{A}_{a}$	$299.9 \pm 15.7^{A}_{a}$	$313.5 \pm 11.7^{A}_{a}$	$320.0 \pm 29.3^{A}_{a}$
6	$89.9 \pm 15.0^{CD}_{b}$	$75.9 \pm 5.2^{D}_{b}$	$230.9 \pm 21.0^{A}_{ab}$	$244.9 \pm 17.8^{A}_{a}$	$242.8 \pm 6.2^{A}_{b}$	$148.2 \pm 28.3^{B}_{b}$	$157.5 \pm 28.0^{BC}_{b}$
12	$15.3 \pm 13.3^{B}_{c}$	$0.0 \pm 7.7^{B}_{c}$	$160.2 \pm 39.9^{A}_{bc}$	$173.9 \pm 3.5^{A}_{b}$	$207.0 \pm 21.1^{A}_{b}$	$20.9 \pm 29.5^{B}_{c}$	$49.8 \pm 17.4^{B}_{c}$
18	$0.0 \pm 0.0^{C}_{c}$	$0.0 \pm 0.0^{C}_{c}$	$94.5 \pm 7.5^{B}_{cd}$	$123.2 \pm 23.9^{AB}_{bc}$	$152.6 \pm 12.2^{A}_{c}$	$0.0 \pm 0.0^{C}_{c}$	$33.4 \pm 3.9^{C}_{cd}$
24	$0.0 \pm 0.0^{C}_{c}$	$0.0 \pm 0.0^{C}_{c}$	$63.0 \pm 3.0^{B}_{d}$	$95.6 \pm 18.5^{A}_{c}$	$114.8 \pm 2.1^{A}_{cd}$	$0.0 \pm 0.0^{C}_{c}$	$0.0 \pm 0.0^{C}_{d}$
30	$0.0 \pm 0.0^{C}_{c}$	$0.0 \pm 0.0^{C}_{c}$	$34.6 \pm 2.6^{B}_{d}$	$79.6 \pm 3.1^{A}_{c}$	$81.8 \pm 1.5^{A}_{d}$	$0.0 \pm 0.0^{C}_{c}$	$0.0 \pm 0.0^{C}_{d}$
LD_{50}-value	5.8 ± 0.5^{B}	5.0 ± 0.1^{B}	15.8 ± 2.4^{A}	18.1 ± 0.1^{A}	19.6 ± 1.1^{A}	6.5 ± 0.4^{B}	8.3 ± 0.5^{B}

LD_{50}-value = Time in hours, necessary to reduce the initial concentration of tocopherol for 50%.

All data are expressed as mean ± standard deviation (experiment replications n=2).

The different capital letters in superscript indicates significant difference between the means of different antioxidant treatment ($p<0.05$). The different small letters in subscript indicate significant difference between the means of frying time ($p<0.05$).

6.5 Conclusion

This chapter shows for the first time the very strong effect of a canolol enriched extract obtained from fluidized bed treated canola meal after extraction with supercritical carbon dioxide on the thermal stability of high-oleic canola oil during deep-fat frying. The most important parameters used for the assessment of the quality of the used oils, di- and polymer triacylglycerols and total polar compounds, were lower after 30 h of frying in comparison to commonly used antioxidants, TBHQ and rosemary extract.

This shows that reactions typical for the degradation of oil during frying are slowed down by the addition of CAN. Additionally CAN is able to prevent oil color degradation during frying, even though the oil was more yellow at the first day of CAN addition . RM extracts and TBHQ have adverse effect on the color of oil during frying. The addition of CAN also improves the life-time of α- and γ-tocopherol in the oil, which may have a positive effect on the product being fried.

The study also shows a strong influence of the concentration of the added CAN on the improvement of the thermal stability of the frying medium. The higher the concentration of CAN, the better the quality of the oil after 30 hours frying. From the results it can be concluded that the shelf-life of the frying medium can be improved by the addition of CAN for at least two times taking into account that the formation of polar compounds and di- and polymer triacylglycerols is about half of that formed in the control or in oils fortified with RM or TBHQ.

The addition of CAN increases the acid value of frying oil in comparison to the other used oils. Depending on the concentration of CAN this results in a faster increase of the acid value and finally the limit set by different countries for the acid value of used frying oils can be exceeded. Despite the higher initial acid value the thermal stability of oils fortified with CAN seems not to be affected with regard to other parameters applicable for the assessment

of used frying oils.

In summary, it can be concluded that CAN enriched extracts obtained from fluidized bed treated canola meal by supercritical extraction with carbon dioxide are very strong antioxidants that can be used to improve the thermal stability of frying oils remarkably. Still open is the question whether canolol alone is responsible for the strong antioxidative effect of the extracts or whether also other compounds contribute. Shrestha & De Meulenaer (2014) showed that phospholipids and Maillard type browning reaction products could also be responsible for a better oxidative stability of mustard and rapeseed oil obtained from roasted seed material. Further investigations have to be done on the composition of the CAN enriched extracts and on the contribution of the individual compounds on the antioxidant activity of the extract.

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CHAPTER 7

7. GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

7.1 General conclusions

Chemical refining resulted in significant loss of sinapic acid derivatives (SADs) and tocopherols in expelled canola oil. This thesis demonstrated the potential of the refining byproducts of expeller canola oil as sources of SADs and tocopherols. Sinapic acid (42.9 μg/g), sinapine (199 μg/g), and canolol (344 μg/g) were found in the refining byproducts, namely, soapstock, spent bleaching clay and wash-water for the first time. Tocopherols (3.75 mg/g) and other non-identified phenolic compounds (2.7 mg SAE/g) were found in deodistillates. A primary assessment of the antioxidant capacity of these byproducts by DPPH scavenging activity assay confirms that the deodistillates exhibited the highest antioxidant activity due to high phenolic content, followed by the wash-water, spent bleaching clay and soapstock.

The antioxidant capacities of the phenolics from deodistillates (DDL) and canola meal extracts have been further confirmed by three *in vitro* non-biological relevant assays: Folin-Ciocalteu, DPPH and FRAP assays. Canola meals extracted using ultrasound-assisted extraction and accelerated solvent extraction (ASE) produced an extract rich in sinapine (SP) and another extract rich in canolol (CAN). The results revealed that the phenolic extracts of DDL and CAN presented lower antioxidant activities than that of SP, and sinapic acid standard. In contrast, CAN and DDL extracts exhibited significant stronger activity to reduce the hydrogen peroxide induced oxidative stress in both CHO and Caco-2 cells. The results indicated that CAN and its derivatives might reveal higher biological relevant antioxidant and cytoprotective activities than sinapic acid and sinapine.

Similarly, crude CAN and DDL extracts also showed significant antioxidant activities in the canola oil deep-frying model. CAN and DDL extracts exhibited higher protection against

frying oil deterioration than BHT, with a considerable reduction in the peroxide value (PV), anisidine value and conjugated diene and trienes after deep-frying, indicating their efficacy in reducing both primary and secondary oxidation (CAN > DDL > BHT > Control). Further study examining the concentration effect of CAN on improving high-oleic canola oil stability indicated that addition of CAN in the concentration from 200-750 mg/kg can significantly improve the quality of the used oils based on several important parameters including di- and polymer triacylglycerols (DPTG), total polar compounds (TPC), secondary degradation products (anisidine value) and the iodine value. Additionally, they also helped to slow down the degradation of color and tocopherols in the canola oil during the 5-day frying period.

To our knowledge, this work is the first to demonstrate the retention and recovery of the sinapic acid derivatives and tocopherols using byproducts generated in a commercial expeller-pressed facility. The results from cell culture and deep-fat frying studies further confirmed the value added potential of canola phenolics as promising food additives and nutraceuticals. The results open up opportunities for the utilization of both canola meal and refining byproducts as new side streams for commercial utilization in the context of canola and rapeseed oil processing.

7.2 Future Perspectives

In order to enhance the utilization of canola byproducts as sources of biophenols and their food and health related applications, further studies still need to be carried out.

(1) The antioxidative and bioactive properties of deodistillates phenolics have been demonstrated in this thesis, further molecular structure characterization of the deodistillates phenolics would help to understand the mechanism insights.

(2) More investigations for canola oil refining byproducts especially techno-functional perspectives to improve the recovery efficiency and purity of phenolics by involving novel extraction techniques such as membrane filtration, molecular distillation and

solid phase extraction etc. are needed.

(3) Few studies have demonstrated the bioavailability and mechanisms canola byproducts derived phenols. Thus, such research can be extended to *in vitro* cellular mechanism studies and *in vivo* animal and clinical studies in order to enhance their application as nutraceuticals.

(4) Further investigations about the effect of canolol on sensory properties of frying oils and fried products are of interest.

(5) Commercialization of canolol as food additives will be of interest for the food industry.