SUBCRITICAL WATER EXTRACTION OF ANTIOXIDANT COMPOUNDS FROM CANOLA MEAL

A Thesis

Submitted to the College of Graduate Studies and Research

In Partial Fulfillment of the Requirements

For the Degree of

Master of Science

In the

Department of Food and Bioproduct Sciences

of the

University of Saskatchewan

Saskatoon, Saskatchewan

Canada

By

Majid Hassas Roudsari

PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a Postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work or, in their absence, by the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use made of any material in my thesis.

Requests for permission to copy or to make other use of material in this thesis, in whole or in parts, should be addressed to:

Head
Department of Food and Bioproduct Sciences
University of Saskatchewan
51 Campus Drive
Saskatoon, SK S7N 5A8
Canada

ABSTRACT

Antioxidant compounds were extracted from canola meal by subcritical water extraction (SWE), hot water (80°C) extraction and ethanolic (95%) extraction. The highest extract yields were obtained with SWE at 160°C, and the lowest with ethanolic extraction (SWE 160°C > SWE sequential > SWE 135°C > SWE 110°C = hot water extraction > ethanolic extraction). Ethanolic extracts exhibited the highest total phenolics contents and Trolox equivalent antioxidant capacity (TEAC) values on a per gram of extract basis, and hot water extracts, the lowest (ethanolic extraction > SWE 110°C > SWE 160°C > hot water extraction). Extraction pressure (3.44-6.89 MPa) had no effect on the yields, total phenolics contents or TEAC values of extracts from SWE. The use of buffered water (pH 2-8) for SWE increased extract yield but had adverse effects on the total phenolics contents and TEAC values of extracts. No increase in efficacy of SWE at 110 or 160°C was observed at extraction times longer than 25-30 min.

The total phenolics contents and antioxidant capacities of extracts were assessed by the total phenolics assay, the 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) scavenging method, TEAC method, the β-carotene-linoleic acid (linoleate) model system, the reducing power assay and the stripped oil model system. Ethanolic extracts exhibited the highest total phenolic contents and antioxidant capacities on a per gram of extract basis. Subcritical water extraction at 160°C exhibited the highest total phenolic contents and antioxidant capacities on a per gram of meal basis. Results from the total phenolics assay and the antioxidant capacity assays were significantly correlated, with the exception of those from the stripped oil model system.

ACKNOWLEDGEMENTS

I would like to sincerely thank my supervisors, Drs. Bob Tyler and Peter Chang for their generous support, academic advice and expertise and criticism in the preparation of this manuscript. I would like also to thank the other members of my advisory committee, Dr. Phyllis Shand, former chair of my committee, Dr. George Khachatourians, and my former academic supervisor, Dr. Ron Pegg, for their helpful and valuable suggestions during my research work. My appreciation is also expressed to Dr. Lope Tabil for serving as External Examiner.

I also express my thankfulness to my loving parents, Drs. Mohammad-Reza Hassas Roudsari and Shokooh Hamzehpour-Dastforoosh, for their endless support, lofty love and motivation. Special thanks go to my brothers, their families and my sister for their invaluable encouragements.

Special thanks to Drs. Murray Drew, Gerald Langner, Janitha Wanasundara, Andrew Olkowski, Takuji Tanaka, Nicholas Low, Michael Nickerson, Martin Reaney and Dennis Bayrock for their valuable suggestions, comments, support and time. Sincere thanks and appreciation are also extended to Mrs. Debbie Anderson, Tara McIntosh and Heather Silcox for their technical support. Thanks to Mrs. Patty Posnikoff and Sharon Greenough for their dedicated help on administrative work.

I would also like to thank all of my friends for their encouragement and Agriculture and Agri-Food Canada (Saskatoon Research Centre) for the use of their facilities.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

%ANT	antioxidant activity percentage
AAPH	2,2' azobis (2-amidopropane) dihydrochloride
ABTS	2,2' azobis (3-ethylbenzthiazoline-6-sulphonic acid)
ANOVA	analysis of variance
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
CD	conjugated diene
CL	column
$DPPH^{\bullet}$	2,2-diphenyl-1-picrylhydrazyl radical
$E^{1\%}_{1cm}$	extinction value
EDTA	ethylenediamine tetraacetic acid
ET	electron transfer
FC	Folin & Ciocalteu
HAT	hydrogen atom transfer
HPP	high pressure pump
MGC	monoglyceride citrate
PF	protection factor
PG	propyl gallate
PUFA	polyunsaturated fatty acids
RSA	radical scavenging activity
SFE	supercritical fluid extraction
SWE	subcritical water extraction

TBHQ	*tert*-butylhydroquinone
TEAC	Trolox equivalent antioxidant capacity
TPC	temperature controller
UV	ultraviolet

1.0 INTRODUCTION

1.1 Rationale

Several degradation reactions, both on heating and on long term storage, deteriorate fats, oils and lipid-based foods. Oxidation reactions and the decomposition of oxidation products are the main deterioration processes which result in decreased nutritional value and sensory quality. The prevention or retardation of these oxidation processes is essential for the food producer and for all persons involved in the value chain. Various methods may be used to inhibit oxidation, including prevention of oxygen access, use of lower temperature, inactivation of enzymes catalyzing oxidation, reduction of oxygen pressure, and the use of suitable packaging (Pokorný and Korczak 2001).

Another method of protection against oxidation is the use of specific additives, which inhibit or retard the reaction. These oxidation inhibitors are generally known as "antioxidants". Antioxidants represent a class of substances that vary widely in chemical structure and have varied mechanisms of action. Antioxidant activity depends on many factors, including lipid composition, antioxidant concentration, temperature, oxygen pressure, and the presence of other antioxidants and food constituents (Pratt and Hudson 1990).

Antioxidants were first used prior to World War II as food preservatives (Pokorný and Korczak 2001). These early antioxidants were natural substances but soon were replaced by synthetic ones. Synthetic antioxidants were not only cheaper, but also

exhibited more consistent purity and more uniform antioxidant properties than did early natural antioxidants. However, consumers challenged the increased use of various synthetic food additives. Consequently, a growing demand for the use of natural compounds to preserve food was shown by consumers as natural materials were considered to be more acceptable as dietary components. Industrial producers have tried to act in accordance with consumers' wishes, and have moved towards the increased use of natural antioxidants. Natural antioxidants may be found in any plant part. Fruits, vegetables, spices, nuts, seeds, leaves, roots and barks have been considered as potential sources of natural antioxidants (Pratt and Hudson 1990). Antioxidants in flaxseed, sunflower, soybean, cottonseed and canola typify those found in oilseeds. Canola contains endogenous antioxidant compounds, whose isolation and application to food systems have been targeted by numerous research investigations over the past two decades (Naczk and Shahidi 2006).

Antioxidant compounds are usually present in rather low amounts in natural materials. Therefore, large additions of antioxidant-containing material would be required to obtain a significant improvement in stability against oxidation, which may be accompanied by a negative effect on the flavour or functional properties of the product. The easiest way to prepare more concentrated materials is to remove water by a suitable drying procedure. The next most optimal procedure is extraction. The choice of solvent is of crucial importance. Conventional methods to extract natural antioxidants from plants are generally based on the employment of organic solvents, which may generate residue issues and have detrimental effects such as left residues, on the environment and food constituents. Recently, the use of subcritical water (*i.e.*, hot water under pressure sufficient to maintain the water in the liquid state) for the extraction of bioactive

compounds from plant material has shown great potential. This technique may be a promising method for the selective and efficient extraction of antioxidant constituents from canola meal.

1.2 Hypothesis

Subcritical water extraction (SWE) is a water-based extraction technology that may be as effective in extracting natural antioxidants from canola meal as ethanol extraction and more effective than conventional hot water extraction.

1.3 Aims and objectives

(1) To extract natural antioxidants from canola meal using subcritical water extraction (SWE), ethanolic (95%) extraction and conventional hot water (80^{o}C) extraction;

(2) To investigate the effects of temperature, pressure, time and pH on the extraction of antioxidant compounds from canola meal using SWE; and

(3) To evaluate the antioxidant capacities of the extracts obtained from canola meal by SWE, ethanolic and hot water extraction using the total phenolics assay, the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) scavenging method, the Trolox equivalent antioxidant capacity (TEAC) method, the β-carotene-linoleic acid (linoleate) model system, the reducing power assay and a stripped corn oil model system.

2.0 LITERATURE REVIEW

2.1 Antioxidants (oxidation inhibitors)

Several degradation reactions, which may occur on heating or during long term storage, deteriorate fats and oils and the lipid constituents of foods. Oxidation reactions and the decomposition of oxidation products are the main processes which result in decreased nutritional value and sensory quality (Gordon 2001a). Research has implicated oxidative and free-radical-mediated reactions in degenerative processes related to ageing and diseases such as cancer, coronary heart disease and neurodegenerative disorders such as Alzheimer's disease (Lachance and others 2001). The prevention or retardation of these oxidation processes is essential for the food producer and almost everyone involved in the entire food chain from “farm to fork”. Various methodologies may be employed to inhibit oxidization, including prevention of oxygen access, use of lower temperature, inactivation of enzymes catalyzing oxidation, reduction of oxygen pressure and the use of suitable packaging (Yanishlieva 2001). Another common method of protection against oxidation is to use specific additives which inhibit or retard oxidation. These oxidation inhibitors are generally known as antioxidants (Huang 2005).

2.1.1 Antioxidant mechanisms

Antioxidants represent a class of compounds that vary widely in chemical structure and have varied mechanisms of action. The most important mechanism is their

reaction with lipid free radicals, forming inactive products (Pokorný and Korczak 2001). The mechanisms of antioxidant activity are shown in Table 2.1.

Antioxidants can be classified into two groups based on the way they inhibit or retard oxidation. The first group is primary (chain-breaking) antioxidants, which react directly with lipid radicals and convert them into stable products. The second group is secondary (preventive) antioxidants, which can lower the rate of oxidation by different mechanisms. Direct scavenging of free radicals is not involved (Decker and others 2005). Most primary antioxidants act by donating a hydrogen atom, and are consumed during the induction period (Gordon 2001a). Secondary antioxidants may act by binding metal ions able to catalyze oxidative processes, by scavenging oxygen, by absorbing UV radiation, by inhibiting enzymes or by decomposing hydroperoxides (Schwarz and others 2001). It has been reported that some natural phenolic compounds function as both primary and secondary antioxidants (Gordon 2001a). Antioxidant capacity assessment monitors either a decrease in the radical or the antioxidant, or the formation of products (Decker and others 2005). This is discussed further in section 2.3.

2.1.2 Natural versus synthetic antioxidants

Increased intake of dietary antioxidants may help to maintain an adequate antioxidant status, defined as the balance between antioxidants and oxidants in living organisms (Halliwell and others 1995). Antioxidants are widely used in the manufacture, packaging and storage of lipid-containing foods. Much interest has developed during the last few decades in naturally occurring antioxidants because of the adverse attention received by synthetic antioxidants, and also the worldwide trend to avoid or minimize

Table 2.1 – Mechanisms of antioxidant activity[a]

Antioxidant class	*Mechanism of antioxidant activity*	*Examples of antioxidants*
Proper antioxidants	Inactivating lipid free radicals	Phenolic compounds
Hydroperoxide stabilizers	Preventing decomposition of hydroperoxides into free radicals	Phenolic compounds
Synergists	Promoting activity of proper antioxidants	Citric acid, ascorbic acid
Metal chelators	Binding heavy metals into inactive compounds	Phosphoric acid, Maillard reaction compounds, citric acid
Singlet oxygen quenchers	Transforming singlet oxygen into triplet oxygen	Carotenes
Substances reducing hydroperoxides	Reducing hydroperoxides in a non-radical way	Proteins, amino acids

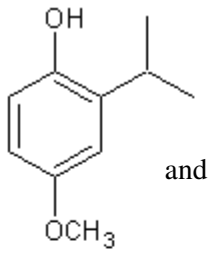
[a] Hall 2001.

the use of artificial food additives (Pokorný and Korczak 2001).

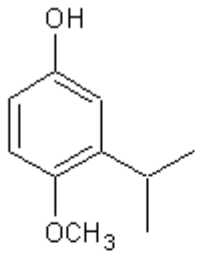
Phenolic compounds, in synthetic or natural forms, are typical antioxidants (Barlow 1990). The most commonly used synthetic antioxidants are BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene), PG (propyl gallate) and TBHQ (*tert*-butylhydroquinone) (Barlow 1990). The chemical structures of these compounds are shown in Figure 2.1. They are added to an extensive variety of foods in order to prevent or retard oxidation. Shi and others (2001) stated that natural antioxidants are safer, more potent and more efficient than synthetic antioxidants. For example, α-tocopherol (the most active form of vitamin E) is more effective than synthetic racemic α-tocopherol, primarily because α-tocopherol transfer protein selectively recognizes natural α-tocopherol (Shi and others 2001). In addition, the possible activity of synthetic antioxidants as promoters of carcinogenesis has become a concern (Barlow 1990). Therefore, replacing synthetic antioxidants with natural alternatives, or simply replacing all synthetic food additives with natural choices, has attracted great interest over the past two decades (Wanasundara and others 1998).

2.1.3 Plants as sources of antioxidants

Natural antioxidants may be found in any plant part. Fruits, vegetables, spices, nuts, seeds, leaves, roots and barks have been considered as potential sources of natural antioxidants (Pratt and others 1990). Antioxidants in flaxseed, sunflower, soybean, cottonseed and canola typify those found in oilseeds. The majority of natural anioxidants are phenolic compounds, and the most important groups of natural antioxidants are the tocopherols, flavonoids and phenolic acids that are common to all plant sources (Naczk and Shahidi 2006).



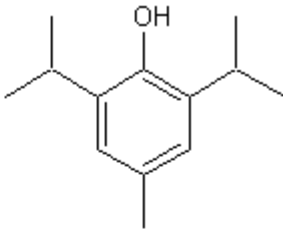
and



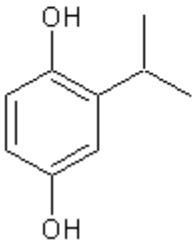
4-methoxy-2-*tert*-buthyl phenol (2-BHA)

4-methoxy-3-*tert*-buthyl phenol (3-BHA)

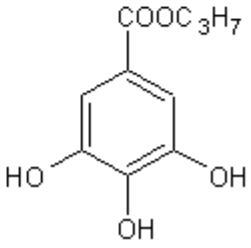
butylated hydroxyanisole (BHA)



2,6-di-*tert*-butyl-*p*-hydroxytoluene (BHT)



tert-butyl hydroquinone (TBHQ)



propyl gallate (PG)

Figure 2.1 – Chemical structures of food-grade synthetic phenolic antioxidants (modified from Yanishlieva 2001).

Tocopherols are considered the best known and most widely used antioxidants (Blokhina and others 2003). They are classified into two groups, namely tocopherols and tocotrienols. There are four isomers (α-, β-, γ- and δ-) in each group, for a total of eight tocopherol isomers (Figure 2.2) (Blokhina and others 2003; Yanishlieva 2001). Tocopherols exist in nearly all parts of plants (Yanishlieva 2001).

Flavonoids represent a large group of phenolics that occur naturally in plants and are found in fruits, vegetables, grains, barks, roots, stems, flowers, tea and wine (Blokhina and others 2003). They are characterized by the carbon skeleton C_6–C_3–C_6. The basic structure of these compounds consists of two aromatic rings linked by a three-carbon aliphatic chain (Yanishlieva 2001). Several classes of flavonoids are delineated on the basis of their molecular structure, but the four main groups that occur in plant tissues are flavones, flavanones, catechins and anthocyanins (Figure 2.3) (Nijveldt and others 2001).

Phenolic acids and their derivatives occur widely in the plant kingdom, *e.g.*, legumes, cereals, fruits and plant products such as tea, cider, oil, wine, beverages and medicinal plants (Odaci and others 2007). Phenolic acids (Figure 2.4) can found in free and conjugated forms in cereals (Naczk and Shahidi 2006). They are present in highest concentration in the aleurone layer of grains, but are also found in the embryo and seed coat (Naczk and Shahidi 2006). The level of phenolics in plant sources also depends on such factors as cultivation techniques, cultivar, growing conditions, ripening process, processing and storage conditions, as well as stress conditions such as UV radiation, infection by pathogens and parasites, wounding, air pollution and exposure to extreme temperatures (Naczk and Shahidi 2006).

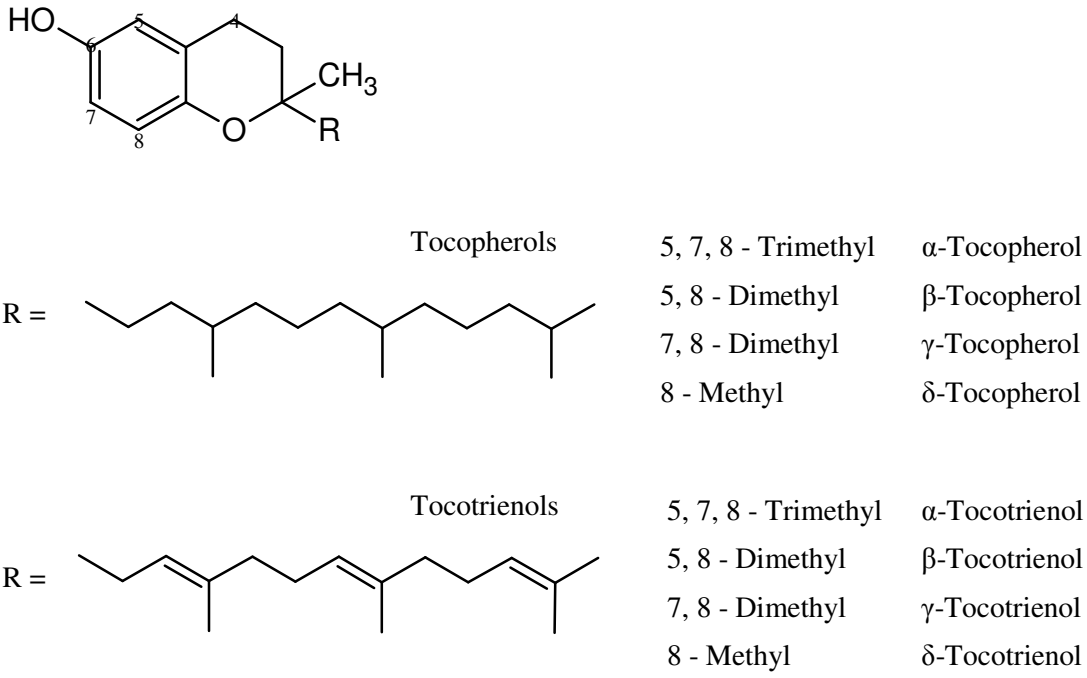


Figure 2.2 – Chemical structures of tocopherols and tocotrienols and their isomers (modified from Yanishlieva 2001).

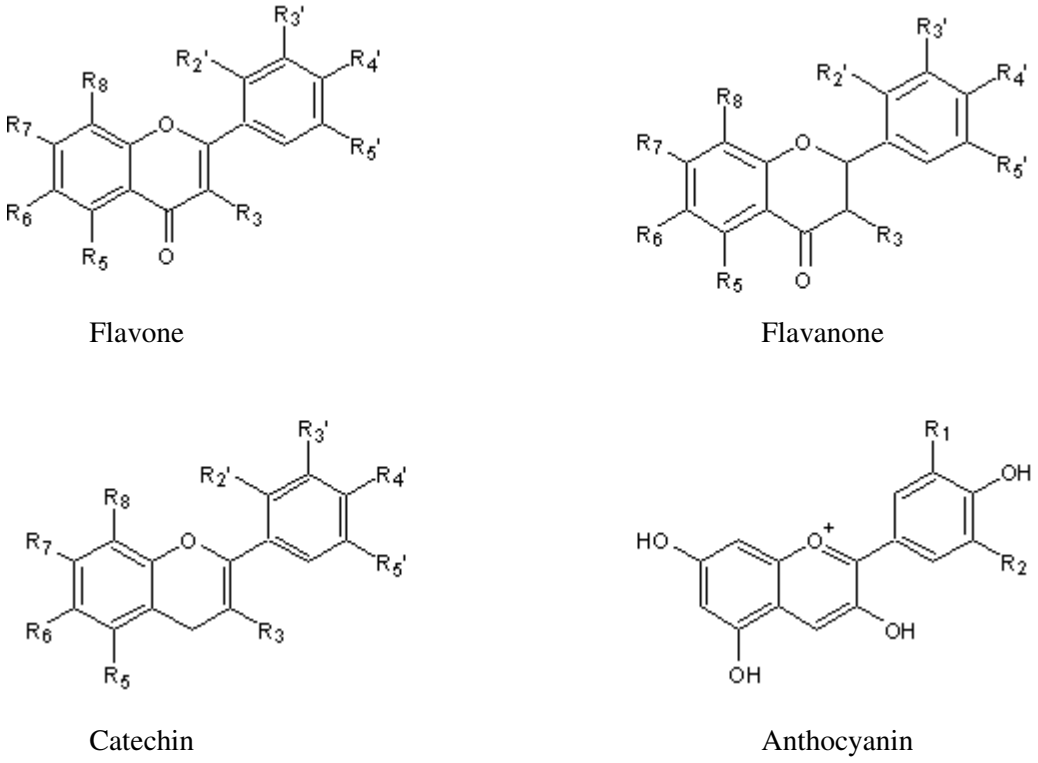
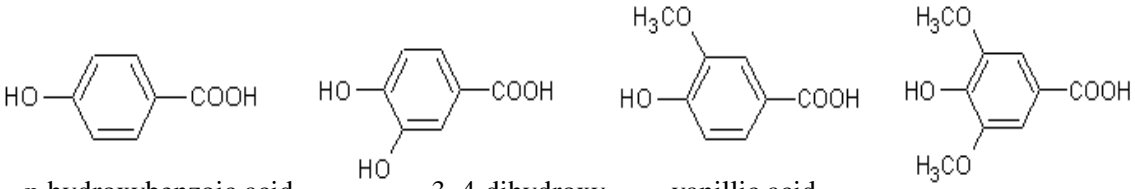


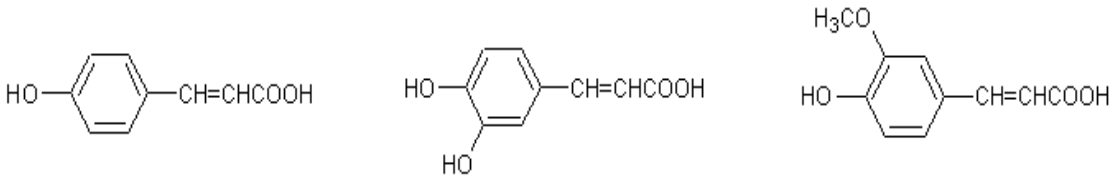
Figure 2.3 – The molecular structures of the four main flavonoid groups (modified from Nijveldt and others 2001).

Benzoic acid derivatives

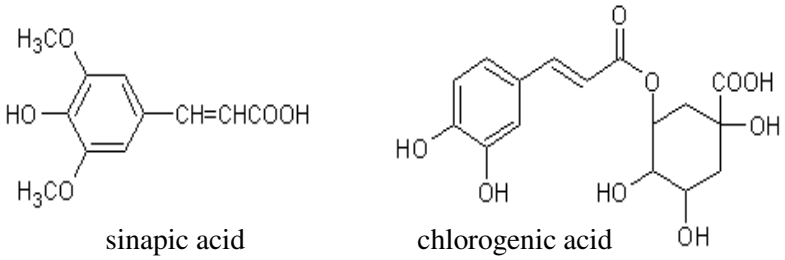


p-hydroxybenzoic acid | 3, 4-dihydroxy-benzoic acid | vanillic acid | syringic acid

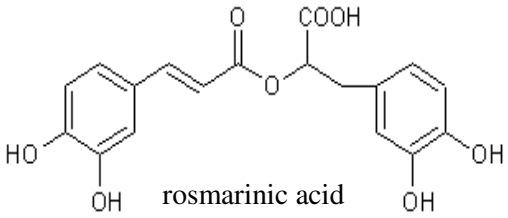
Cinnamic acid derivatives



p-coumaric acid | caffeic acid | ferulic acid



sinapic acid | chlorogenic acid



rosmarinic acid

Figure 2.4 – Phenolic acids as examples of common natural antioxidants (modified from Yanishlieva 2001).

2.1.4 Antioxidants in canola

Canola is a derivative of rapeseed with low glucosinolate and erucic acid contents (Hall 2001). Antioxidant compounds identified in canola include phenolic acids (both benzoic and cinnamic acid derivatives) (Kozlowska and others 1988), flavonoids (Hall 2001) and condensed tannins (Shahidi and Naczk 1989). A diversity of phenolic compounds is present in canola and rapeseed flours (dehulled, defatted seed), meals (defatted, whole seed) or extracts, indicating that these products could protect a food against rancidity by any of several mechanisms (Table 2.1). The high antioxidant activity of a canola fraction containing several groups of phenolics demonstrated protection via multiple mechanisms (Amarowicz and others 2003).

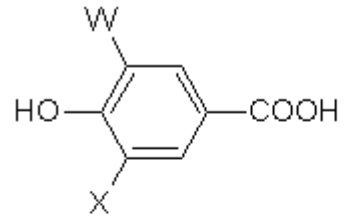
Canola meal has been reported to contain 15.4-18.4 g/kg (dry basis, defatted meal) of phenolic acids (Shahidi and Naczk, 1992). The phenolic compounds of oilseeds include hydroxylated derivatives of benzoic acid and *trans*-cinnamic acid, coumarins, flavonoids and lignans (Pink and others 1994). Phenolic acids in canola meal are found in free, esterified or insoluble-bound forms (Naczk and others 1998). These authors reported that canola meal may contain more than 2 g of free phenolic acids per kg of meal, more than 15 g of esterified phenolic acids per kg of meal and approximately 1 g of insoluble-bound phenolic acids per kg of meal (dry basis in all cases).

A high concentration of sinapic acid in canola meal was reported by Naczk and others (1992). These authors also reported that sinapic acid, the predominant phenolic acid found in canola, exists in free form, in esterified form, and in soluble-bound form. Wanasundara and others (1995) reported that sinapic acid and its analogues contributed significantly to antioxidant activity in canola meal. Several compounds with high antioxidant activity were identified as phenolic compounds having one, two or three

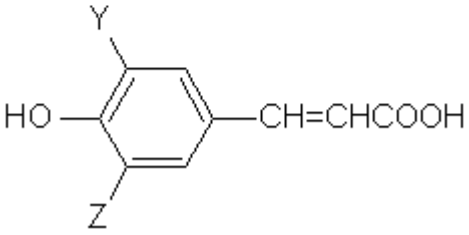
hydroxy groups (Figure 2.5), which were identified (by thin layer chromatography) as sinapic acid, *p*-hydroxybenzoic acid, flavonoids and 1-*O*-β-D-glucopyranosyl sinapate (Wanasundara and others 1995).

Wanasundara and Shahidi (1994) reported that the antioxidant activity of a crude ethanolic extract of canola meal (500 and 1000 ppm) against the oxidation of canola oil was equivalent to that of TBHQ (200 ppm), and stronger than that of BHA (200 ppm), BHT (200 ppm) or BHA/BHT/monoglyceride citrate (MGC) (250 ppm) on a mass basis. Wanasundara and others (1994) isolated the most active component of the extract and identified it as 1-*O*-β-D-glucopyranosyl-3, 5-dimethoxy-4-hydroxycinnamate (1-*O*-β-D-glucopyranosyl sinapate; Figure 2.6). Shahidi and others (1995) observed that the addition of 0.5-5% canola flour provided 73-97% inhibition of fat oxidation in meat. The amount of phenolic acids in canola flour ranged from approximately 6.2 to 12.8 g per kg of meal (Naczk and others 1998).

Canola also contains tocopherols (Hall 2001) and condensed tannins (Shahidi and Naczk 1989). Tocopherol contents in canola meal ranged from 580 to 850 ppm and Gamma-tocopherol represented 66% of the tocopherols, and α- and δ-tocopherols accounted for 32 and 2%, respectively (Warner and Mounts 1990). Condensed tannins in canola meal ranged from 0.2 to 22% of hulls. However, the level of condensed tannins extractable by solvent systems commonly used for isolation of polyphenols was not more than 0.1% (Naczk and others 1998). Naczk and others (1998) mentioned that discrepancies in the reported data on tannin contents may be due to the different solvent systems employed for extraction or the quantification methods used for tannin analysis. The same authors indicated that canola meals contained 0.68-0.77% of condensed tannins.



Acid	W	X
Protocatechuic	**H**	**OH**
Vanillic	**OCH_3**	**H**
Syringic	**OCH_3**	**OCH_3**
Gallic	**OH**	**OH**
***p*-hydrobenzoic**	**H**	**H**



Acid	Y	Z
***p*-Coumaric**	**H**	**H**
Caffeic	**H**	**OH**
Ferulic	**H**	**OCH_3**
Sinapic	**OCH_3**	**OCH_3**

Figure 2.5 – Structures of phenolic acids found in canola (modified from Naczk and others 1998).

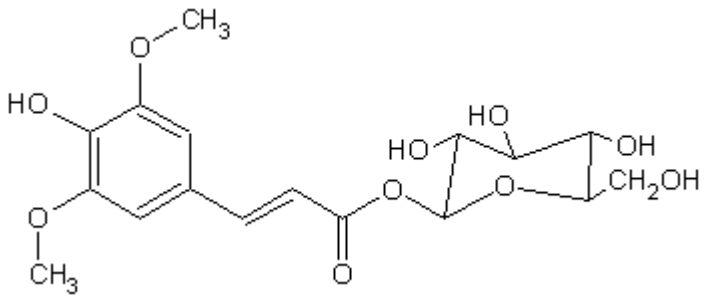


Figure 2.6 – 1-O-β-D-Glucopyranose sinapate isolated from canola meal and characterized as an antioxidant (adapted from Wanasundara and others 1995).

2.1.4.1 Characteristics of phenolics as antioxidants

The overall effectiveness of a natural antioxidant (*e.g.*, polyphenolics and phenolic acid derivatives) depends on the involvement of the phenolic hydrogen in radical reactions, the stability of the natural antioxidant radical formed during radical reactions, and chemical substitutions present on the structure (Hall 2001). The substitutions on the structure are probably the most significant with respect to the ability of a natural antioxidant to participate in the control of radical reactions and to form resonance-stabilized, natural antioxidant radicals (Barlow 1990).

Miller and Quackenbush (1957a,b) found that alkyl substitutions could enhance antioxidant activity. The antioxidant activity of phenol was enhanced by the electron-donating capability of methyl, ethyl and tertiary butyl substitutions at positions *ortho* and *para* to the hydroxyl groups. Phenolic acids (Figure 2.4) participate in hydrogen donating and radical scavenging reactions. The antioxidant activity of phenolic acids is due to the phenolic hydrogens (Naczk and Shahidi 2004). Hydroxyl substitutions at *ortho* and *para* positions also will enhance antioxidant activity (Hall 2001). Intramolecular hydrogen bonds are formed by *ortho* substituted phenols (*e.g.*, 1, 2-dihydroxybenzene) during radical reactions (Figure 2.7), which increases the stability of the phenoxy radical (Baum and Perun 1962). It has been reported that caffeic acid was a better antioxidant than ferulic acid or *p*-coumaric acid (Hall 2001), mainly because of intramolecular hydrogen bonding due to the presence of the second hydroxyl group in the caffeic acid. Ferulic acid contains *ortho* methoxy substitution in its structure (Figure 2.4) that may provide a stabilizing effect on the phenoxyl radical, which enhance the antioxidnat activity of ferulic acid over *p*-coumaric acid (Hall 2001). The superior antioxidant activity of trihydroxybenzoic acid (*i.e.*, gallic acid) over 3, 4-

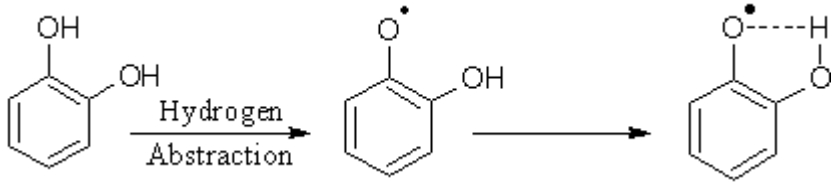


Figure 2.7 – Intramolecular hydrogen bonding of *ortho*-substituted phenols (modified from Baum and Perun 1962).

dihydroxybenzoic acid (*i.e.*, protocatechuic acid) was due to the presence of three hydroxyl groups in trihydroxybenzoic acid. The hydroxy group allows the formation of hydrogen bonds, which results in stronger antioxidant activity than those of compounds containing a methoxy (OCH_3) substitution *ortho* to the hydroxy group (Baum and Perun 1962).

The acid proton appears to have little impact on antioxidant activity. Both caffeic acid and chlorogenic acid, the resultant compound after replacement of the acid proton of caffeic acid with quinic acid via an ester bond (Figure 2.4), were equally effective in controlling lipid oxidation (Hall 2001). The allylic group, as found in cinnamic acid derivatives (Figure 2.4), provides enhanced antioxidant activity when compared to benzoic acid derivatives. Pratt and Hudson (1990) reported that caffeic acid (3, 4-dihydroxycinnamic acid) was a better antioxidant than protocatechuic acid (3, 4-dihydroxybenzoic acid) in a lard system. The allylic group may improve the resonance stability of the phenoxyl radical (Cuvelier and others 1992).

2.2 Extraction of antioxidants

Antioxidant compounds are usually present in rather low amounts in natural materials. Therefore, antioxidants materials, in non-concentrated form, need to be amployed in relatively large amounts to obtain a significant improvement in stability against oxidation. However, there could be a negative effect on the flavour or functional properties of a product by using such a large amount of an antioxidant preparation. The easiest way to prepare materials that are more concentrated is to remove water by a suitable drying procedure. The next most optimal procedure is extraction, where the choice of extraction solvent is of critical importance.

2.2.1 Extraction with organic solvents

Organic solvents are commonly used for the extraction of antioxidants from plant material. Two priliminary factors determine the choice of solvent for extraction, namely the nature of the antioxidants and that of the plant material (Pokorný and Korczak 2001). Table 2.2 provides some examples which demonstrate that even in such closely related materials as rosemary and sage leaves, the optimum solvent may be different (Pokorný and Korczak 2001).

Organic solvents, including, hexane, acetone, ethyl acetate and methanol, have been compared for the extraction of antioxidants (Pokorný and Korczak 2001). These authers reported that solvents of intermediate polarity seemed to be preferable to either non-polar or highly polar solvents. For the extraction of antioxidants from lentil seed, mixtures of acetone, methanol or ethanol with water (8:2, v/v) were tested, and aqueous acetone was found to be the best (Amarowicz and others 2003).

Duh and Yen (1997) used methanol for extraction of phenolic antioxidants from peanut hull. The same solvent was used for extraction of antioxidant compounds from spices (Banias and others 1992). Pokorný and Korczak (2001) reported that less polar solvents (*e.g.*, acetone or ethyl acetate) were more suitable for spices. They also reported that using chloroform or ethyl acetate for extraction of tea leaf catechins was more effective than using methanol. Oregano leaves were extracted with ethanol, and the obtained extract was extracted with petroleum ether, diethyl ether, ethyl acetate or butanol. The diethyl ether extract was found to be very efficient in lard in terms of antioxidant activity (Vekiari and others 1993).

There exist many procedures to remove from extracts the impurities which may cause strong odour, bitter taste or undesirable colour. It was proposed that washing of

Table 2.2 – Relative activities of 0.05 % rosemary and sage extracts when extracted with different organic solvents [a, b]

Extracted material	*Extraction solvent*	*PF in sunflower oil*	*PF in rapeseed oil*
Rosemary	hexane	2.4	3.9
	ethyl acetate	3.2	3.1
	acetone	2.6	3.3
	methanol	2.2	2.3
Sage	hexane	1.5	2.3
	ethyl acetate	2.4	2.2
	acetone	1.8	2.6
	methanol	1.3	1.7

[a] PF = protection factor; determined using Schaal oven test at 40°C
[b] Pokorný and Korczak 2001.

crude extracts prepared by organic solvent extraction with cold or hot water removed bitter substances (López-Sebastián and others 1998). The water soluble fraction from the same material exhibited weak antioxidant activity in comparison with the starting material and the water-insoluble fraction. Therefore, antioxidant constituents extracted by organic solvent extraction were not extractable with water.

The effect of extraction method on the yield of catechins was determined when tea leaves were extracted with methanol or acetone. Refluxing with methanol resulted in higher yields of catechins than did extraction in a shaking incubator with acetone or methanol (Pokorný and Korczak 2001). Green tea extracts exhibited a pro-oxidant effect in marine oils under Schaal oven test conditions at 65°C, which was attributed to the catalytic effect of the chlorophyll constituents. A green tea extract exhibited excellent antioxidant activity when dechlorophyllized by column chromatography (Wanasundara and Shahidi 1998). The efficacy of a green tea extract (at >200 ppm) with respect to antioxidant activity was higher than that of α-tocopherol (500 ppm), BHA (200 ppm) or BHT (200 ppm), but less than that of TBHQ (200 ppm).

In order to remove sugars and other undesirable water-soluble, inactive substances, extracts obtained by organic solvent extraction may be concentrated by a subsequent extraction with water (López-Sebastián and others 1998). However, some efficient antioxidants may also be removed in this step (Pokorný and Korczak 2001), hence an increase in the activity of re-extracted material may not occur. The removal of interfering components occasionally compensates for this disadvantage. For example, sugars as initiators of Maillard reactions could impart foreign flavours to the product and could cause the deterioration of colour (Kitts and Hu 2005).

Aqueous alkaline solution has been used to extract antioxidants from rosemary and sage leaves (Pokorný and Korczak 2001). Alkaline solution also can be used to wash the active acidic fraction from a crude extract prepared by organic solvent extraction (Stashenko and others 1999). Antioxidant constituents from rosemary and sage can also be extracted during the process of aromatizing vinegar with these spices (Pokorný and Korczak 2001). It was reported by these authors that aromatized vinegar extended the shelf-life of mayonnaise by inhibiting lipid oxidation.

Aeschbach and Rossi (1996) extracted hydrosoluble (polar) antioxidants, using propylene glycol as a polar carrier, by a purely mechanical procedure from herbs, spices, tea, coffee, fruit and vegetable peel, and cereals. They recommended these extracts for direct application in food systems.

Strong antioxidative activity of ethanolic and methanolic extracts of rapeseed phenolics has been reported by Naczk and others (1998) and Nowak and others (1992). Moreover, Shahidi and others (2000) observed that the addition of 0.5-5% of canola flour to meat resulted in a 73-97% inhibition of lipid oxidation. Therefore, extraction of phenolic compounds from canola meal and their possible use as natural antioxidants to delay lipid oxidation may present a new opportunity for the canola industry.

The utility of the extraction should be estimated for every case of industrial application since the costs are rather high, and sometimes increased by a subsequent re-extraction to remove impurities. In some cases, the extracts may not be considered as natural food materials, but extracts from spices would probably be acceptable as they have already been used as food ingredients for other purposes (Shan and others 2005).

2.2.2 Subcritical water extraction (SWE)

Most conventional methods for extraction of natural antioxidants from plants are based on organic solvents, which may have undesirable effects on the environment and on food components. Several methods have been employed to extract antioxidants from aromatic plants. These include solid-liquid extraction, aqueous alkaline extraction, extraction with vegetable oils, extraction with aqueous alkanol solutions and supercritical fluid extraction (SFE) (Kubátová and others 2001). Water may be used as an extraction solvent, and has gained an increasing amount of attention due to its unique solvation properties, which can be altered by changing the temperature (Rovio and others 1999). Subcritical water extraction is considered a recent alternative for the isolation of antioxidant constituents. Subcritical water extraction, also known as hot water extraction, pressurized (hot) water extraction, pressurized low polarity water extraction, high-temperature water extraction, superheated water extraction or hot liquid water extraction, is a promising "green" technique based on the use of water as the sole extraction solvent (Smith 2002). Temperatures between 100 and 374^{o}C (the critical point of water is at 374^{o}C and 22 MPa) are generally applied and the pressure is sufficient to keep water in the liquid state (Ramos and others 2002). In some reports, room temperature (25°C) has been employed for SWE (Ibáñez and others 2003). At temperatures above 100°C, the dielectric constant of water, ε, (*i.e.*, its polarity) can be lowered easily and significantly by increasing the temperature. Pure water at ambient temperature and pressure has an ε of 79, whereas increasing the temperature to 250^{o}C at a pressure of 5 MPa (necessary to maintain the liquid state) yields a significant reduction in ε to ~27. This value is similar to that of ethanol at 25^{o}C and 0.1 MPa and,

consequently, low enough to dissolve many compounds of intermediate or low polarity (Ramos and others 2002).

Subcritical water extraction has been applied in the determination of organic pollutants in soils, sludges and sediments, and also is used for the extraction of volatiles from plant material (Rovio and others 1999). Ibáñez and others (2003) extracted the most active antioxidant compounds from rosemary, such as carnosol, rosmanol, carnosic acid, methyl carnosate and flavonoids such as cirsimaritin and genkwanin, by SWE. The data indicated high selectivity for this method, and the antioxidant activity of the fractions so obtained by extraction at different water temperatures was very high. Kim and Mazza (2006) reported that SWE of phenolic compounds, including *p*-hydroxybenzaldehyde, vanillic acid, vanillin, acetovanillone and ferulic acid, from flax shive was maximized at the combined conditions of high temperature and high NaOH concentration. Lignans were also extracted from whole flaxseed by SWE (Cacace and Mazza 2006). Maximum amounts of lignans and other flaxseed bioactives, including proteins, were extracted at 160°C. However, these authors reported that on a dry weight basis, the most concentrated extracts in terms of lignans and other phenolic compounds were extracted at 140°C. Ho and others (2007) extracted lignans, carbohydrates and proteins from flaxseed meal. The maximum yield of lignans and proteins was obtained at pH 9 at temperatures of 170°C and 160°C, respectively. Maximum recovery of carbohydrates was at pH 4 and 150°C. In another study, Rodriguez-Meizoso and others (2006) demonstrated that the combined use of SWE and high-performance liquid chromatography-diode array detection (HPLC-DAD) was a suitable protocol to obtain and characterize nutraceuticals from natural sources, *i.e.*, oregano. They also reported that changing the water temperature could be used as a means of fine tuning the

extraction selectivity of pressurized water for the extraction of antioxidant compounds from oregano using SWE. Subcritical water has been applied as an HPLC analytical solvent to extract and quantify caffeine, chlorophenols and anilines (Li and others 2000).

García-Marino and others (2006) stated that SWE would be an appropraite extraction technique for obtaining a greater quantity of polyphenolic compounds (catechins and proanthocyanidins) from winery by-products, and compared SWE with extraction with MeOH/H_2O (75:25, v/v). Pongnaravane and others (2006) compared the effectiveness of SWE of anthraquinones from *Morinda citrifolia* with that of other extraction methods, such as ethanol extraction in a stirred vessel, Soxhlet extraction and ultrasound-assisted extraction. The results of their study showed that SWE extracts presented comparable antioxidant activities to those of Soxhlet extracts, and that SWE extracts were more effective than ethanol extracts and ultrasound-assisted extracts in terms of antioxidant activity. Subcritical water extraction has been used to extract ginsenosides from American ginseng (Choi and others 2003); catechins and epicatechin from tea leaves and grape seeds (Piñeiro and others 2004); anthraquinones (antibacterial, antiviral and anticancer compounds) from roots of *Morinda citrifolia* (Shotipruk and others 2004); and, flavones, anilines and phenols from orange peels (Lamm and Yang 2003).

A major disadvantage of SWE is the high operating pressure, which requires expensive equipment (Smith 2002). Thus, the cost of the process is relatively high, making it unsuitable for the extraction of major food components, such as lipids. In the case of antioxidants, price would not play a crucial role, since they are an expensive group of food compounds and costs are compensated by other advantages, such as the high purity of extracts and the efficiency of the process (Ramos and others 2002). The

use of water also allows for a substantial saving in maintenance costs, although maintaining high pressure can be expensive (Ramos and others 2002). The possibility of fine tuning the selectivity of antioxidant extraction through a small change in water temperature is another advantage of SWE (Smith 2002; Ramos and others 2002).

2.3 Antioxidant capacity assessment techniques

Numerous tests have been developed for measuring the antioxidant capacity of food and biological samples. However, there is no universal method that can measure the antioxidant capacity of all samples accurately and quantitatively (Prior and others 2005). Individual antioxidants may act by single or multiple mechanisms in a particular system (Ishige and others 2002). Therefore, it is beneficial to understand the reaction mechanisms of antioxidants in different assays.

2.3.1 Reaction mechanisms

Based on the chemical reactions involved, most antioxidant capacity assays can be classified as hydrogen atom transfer (HAT) reaction based assays or electron transfer (ET) reaction based assays (Huang and others 2005). These reactions may occur in parallel and usually occur together in all samples (Prior and others 2005). The factors that determine the mechanism of antioxidant activity in radical deactivation in a given system are antioxidant structure and properties, antioxidant solubility and partition coefficient, pH and the nature of the system (Huang and others 2005). Hydrogen atom transfer based and single electron transfer based assays measure the radical (or oxidant) scavenging capacity of a sample instead of the preventive antioxidant capacity (Prior and others 2005). The mechanism and the efficacy of antioxidants may be also

determined by bond dissociation energy and ionization potential (Wright and others 2001).

Hydrogen atom transfer based methods detect the capacity of an antioxidant to quench free radicals by donating hydrogen (AH = any H donor) (Prior and others 2005). Therefore, a synthetic free radical generator, an oxidizable molecular probe and an antioxidant are the main components of any HAT-based method (Wright and others 2001).

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

Hydrogen atom transfer based reactions are not dependent on the nature of the solvent or pH (Wright and others 2001). They are usually relatively rapid, typically completed in seconds to minutes. Reducing agents, including metals, interfere with HAT-based reactions and may lead to erroneously high apparent capacities (Prior and others 2005).

Single electron transfer based methods measure the capability of a potential antioxidant to transfer one electron to reduce any compound, including metals, carbonyls and radicals (Wright and others 2001).

$$X^{\bullet} + AH \rightarrow X^{-} + AH^{\bullet +}$$

$$AH^{\bullet +} \xleftrightarrow{H_2O} A^{\bullet} + H_3O^{+}$$

$$X^{-} + H_3O^{+} \rightarrow X + H_2O$$

$$M\,(III) + AH \rightarrow AH^{+} + M\,(II)$$

Single electron transfer based reactions are pH dependent (Wright and others 2001). Relative capacity in ET-based methods is based primarily on two factors, namely deprotonation (Lemańska and others 2001) and ionization potential (Sartor and others

1999) of the reactive functional group. In general, an increase in pH leads to increased electron-donating capacity with deprotonation, and consequently reduces ionization potential values (Lemańska and others 2001). Antioxidant capacity calculations in ET-based reactions are based on the percent decrease in product rather than kinetics, since ET-based reactions are usually slow and can require long times to reach completion (Wright and others 2001). The presence of trace components and contaminants (particularly metals) in samples could account for the high variability and poor reproducibility and consistency of results (Sartor and others 1999).

In this study, the following assays were used to assess the antioxidant capacities of samples: determination of total phenolics content (ET), the Trolox equivalent antioxidant capacity (TEAC) assay (ET), the β-carotene-linoleic acid (linoleate) assay (HAT), the radical-scavenging activity (RSA) assay (ET), the reducing power assay (ET) and the bulk stripped corn oil model system (other).

2.3.2 The total phenols assay using the Folin-Ciocalteu (FC) reagent

The total phenols assay originally was designed and used for the analysis of protein by reaction between the reagent and tyrosine (which contains a phenol group) residues in proteins. Molybdotungstate reagent oxidizes phenols and yields a coloured product with an absorption maximum at 745-750 nm (Folin and Ciocalteu 1927). Singleton and others (1999) adopted this assay for the analysis of total phenols in wine. Since then, the total phenols assay has been used in many studies and is now commonly known as the total phenols (or phenolics) assay. The total phenols assay actually measures the reducing capacity of a sample (Singleton and others 1999). Numerous

publications reported excellent linear correlations between "total phenolic profiles" and "the antioxidant capacity" (Huang and others 2005).

Despite the undefined chemical nature of FC reagent, it is believed to contain heteropolyphosphotungstates-molybdates. Under basic conditions, FC reagent reacts with phenolic compounds and, consequently, a phenolate anion is formed, possibly $(\text{phenol-MoW}_{11}\text{O}_{40})^{4-}$, by dissociation of a phenolic proton. This sequence of reversible one- or two-electron reduction reactions leads to blue-coloured compounds being formed between phenolate and FC reagent. In essence, it is believed that the complex and electron-transfer reaction between Mo(VI) and reductants reduces the molybdenum (Huang and others 2005; Prior and others 2005):

$$\text{Mo (VI) (yellow)} + e \rightarrow \text{Mo (V) (blue)}$$

A number of papers have reported different reference standards for the total phenolics assay, including catechin equivalents (Katsube and others 2003; Vinson and others 2001), tannic acid equivalents (Nakamura and others 2003), chlorogenic acid equivalents (Wang and others 2003), caffeic acid equivalents (Maranz and others 2003), protocatechuic acid equivalents (Cai and others 2003), vanillic acid equivalents (Jayasinghe and others 2003) and ferrulic acid equivalents (Velioglu and others 1998).

The total phenolics assay has become a routine assay in studying phenolic antioxidants and it is simple, sensitive and precise (Huang and others 2005). However, a number of substances, particularly sugars, aromatic amines, sulfur dioxide, ascorbic acid and other enediols and reductones, organic acids, Fe(II) and Cu(I), interfere with the total phenolics method, so correction for interfering substances should be considered. In addition, reaction between the FC reagent and some nonphenolic organics, including adenine, adenosine, alanine, aniline, aminobenzoic acid, ascorbic acid, benzaldehyde,

creatinine, cysteine, cytidine, cytosine, dimethyaniline, diphenylamine, ethylenediamine tetraacetic acid (EDTA), fructose, guanine, guanosine, glycine, histamine, histidine, indole, methylamine, nitriloacetic acid, oleic acid, phenylthiourea, proteins, pyridoxine, sucrose, sulfanilic acid, thiourea, thymine, thymidine, trimethylamine, tryptophan, uracil, uric acid and xanthine, may interfere with the assay. Also, reaction between FC reagent and some inorganic substances, such as hydrazine, hydroxyammonium chloride, iron ammonium sulfate, iron sulfate, manganese sulfate, potassium nitrite, sodium cyanide, sodium metabisulfite, sodium phosphate, sodium sulfite and tin chloride, may also lead to overestimated phenolics concentrations (Prior and others 2005).

2.3.3 The Trolox equivalent antioxidant capacity (TEAC) assay

Miller and Rice-Evans (1993) first described the TEAC assay, which is based on the ability of antioxidants to scavenge the long-life radical cation 2, 2'-azobis-(3-ethylbenzthiazoline-6-sulphonic acid) $ABTS^{\bullet+}$ (Figure 2.8). The TEAC assay was improved by Re and others (1999). In this assay, peroxyl radicals or other oxidants oxidize ABTS to its radical cation, $ABTS^{\bullet+}$ (intense blue colour). The antioxidant capacities of test compounds are determined by measuring decreases in the blue colour as a result of reaction between the $ABTS^{\bullet+}$ radical and the antioxidant compounds in the sample. It has been shown that $ABTS^{\bullet+}$ has an absorption maximum at 415 nm (Cano and others 2000). In the most recent version of the TEAC method, the absorbance decrease of $ABTS^{\bullet+}$ in the presence of the test sample or Trolox (Figure 2.9) at a fixed time point is measured, and the antioxidant capacity is expressed as Trolox equivalents (Prior and others 2005).

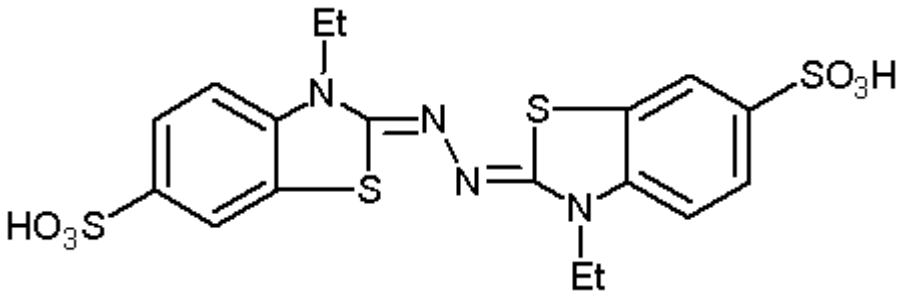


Figure 2.8 – Structure of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) (modified from Prior and others 2005).

2.3.4 The β-carotene-linoleic acid (linoleate) assay

Oxidation results in the bleaching of carotenoids (Huang and others 2005). Oxidation is induced by light or heat (Burda and Oleszek 2001) or by peroxyl radicals [*e.g.*, 2, 2'-azobis-(2-amidopropane)-dihydrochloride (AAPH) or oxidizing lipids] (Ursini and others 1998). Classical antioxidants that can donate hydrogen atoms to quench radicals and prevent decolorization of carotenoids:

$$\beta\text{-Carotene–H (orange)} + ROO^{\bullet} \rightarrow \beta\text{-Carotene}^{\bullet}\text{ (bleached)} + ROOH$$

$$\beta\text{-Carotene–H (orange)} + ROO^{\bullet} + AH \rightarrow \beta\text{-Carotene–H (orange)} + ROOH + A^{\bullet}$$

Decolorization of β-Carotene (Figure 2.10) can be monitored spectrophotometrically at 470 nm (Burda and Oleszek 2001); this decolorization can be employed as an essay of antioxidant activity. Heat-induced oxidation of an aqueous emulsion system of β-carotene and linoleic acid was reported as an antioxidant test reaction (Miller 1971). An advantage of the β-carotene bleaching method is that it requires no specialized instrumentation. Phenolic antioxidants can neutralize any free radicals formed within the system (*e.g.*, the linoleate free radical) and, consequently, may delay decolorization of β-carotene (Amarowicz and others 2004).

Beta-carotene bleaching can occur by multiple pathways, so interpretation of results may be complicated (Prior and others 2005). Also, there are no standard formats for expressing results, hence studies may utilize different methods for calculating inhibition kinetics (Burda and Oleszek 2001; Amarowicz and others 2004; Prior andothers 2005).

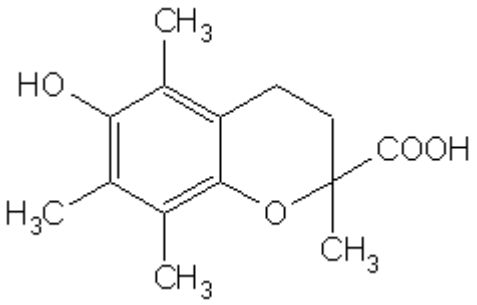


Figure 2.9 – Structure of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (modified from Wang and others 2004).

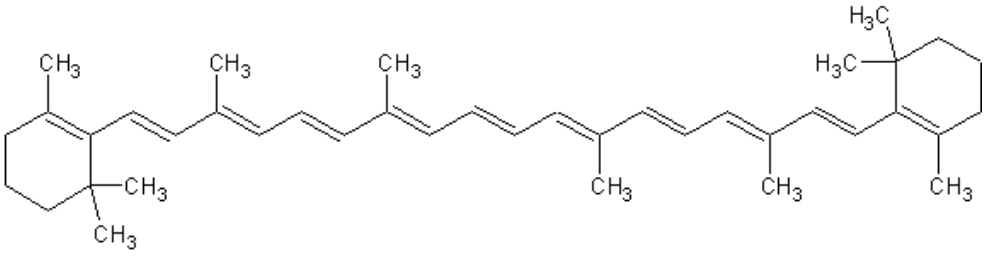


Figure 2.10 – Structure of β-carotene ($C_{40}H_{56}$) (modified from Anonymous 2006).

2.3.5 The radical-scavenging activity (RSA) assay

The 2, 2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$, Figure 2.11) is a stable organic nitrogen radical, is commercially available and has a deep purple colour. The RSA assay measures the reducing capacity of antioxidants toward $DPPH^{\bullet}$. Upon reduction, the colour of $DPPH^{\bullet}$ solution fades and this colour change is conveniently monitored spectrophotometrically at 515 nm. Therefore, test compounds with high antioxidant activity result in a rapid decline in the absorbance of the $DPPH^{\bullet}$ (Amarowicz and others 2004). Brand-Williams and others (1995) first described this widely used assay.

The DPPH test is simple and quick. It has widespread use in antioxidant capacity screening, probably due to the simplicity of the equipment required. The 2,2-diphenyl-1-picrylhydrazyl radical remains unaffected by certain side reactions (*e.g.*, metal-ion chelation and enzyme inhibition), which is dissimilar to laboratory-generated free radicals such as the hydroxyl radical and the superoxide anion (Amarowicz and others 2004).

The interpretation of results is complicated when the test compounds have spectra that overlap $DPPH^{\bullet}$ at 515 nm, *e.g.*, carotenoids (Nomura and others 1997). Either radical reaction (HAT) or reduction (ET) can decolorize $DPPH^{\bullet}$. Steric accessibility of an antioxidant compound determines the type of reaction (HAT or ET) (Prior and others 2005). Hence, small molecules that have better access to the radical site show higher antioxidant capacity with this test. Since $DPPH^{\bullet}$ is a stable nitrogen radical, unlike the highly reactive and transient peroxyl radicals involved in lipid peroxidation, many antioxidants that react quickly with peroxyl radicals may react slowly with, or may even be inert to, $DPPH^{\bullet}$, due to steric inaccessibility (Prior and

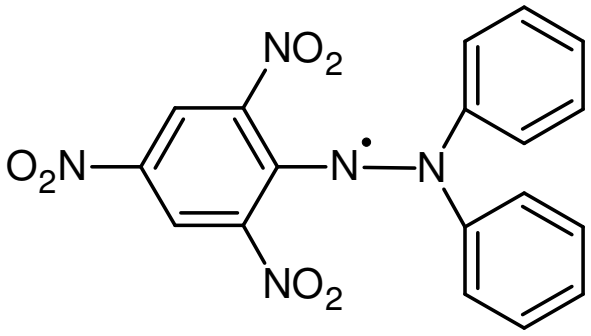


Figure 2.11 – Structure of 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH•) (modified from Prior and others 2005).

others 2005). It was reported that the reaction of $DPPH^{\bullet}$ with eugenol was reversible (Bondet and others 1997), which leads to falsely low readings for antioxidant capacity of samples containing eugenol and other phenols with similar structures, *e.g.*, *o*-methoxyphenol.

2.3.6 The reducing power assay

In the reducing power assay, the reductants (antioxidants) in the test compounds reduce the Fe^{3+}/ferricyanide complex [$FeCl_3/K_3Fe(CN)_6$] to the ferrous (Fe^{2+}) form (Chung and others 2002). Therefore, depending on the reducing power of the test compounds, the yellow colour of the test solution changes to various shades of green or blue (Amarowicz and others 2004) or the presence of Fe^{2+} results in formation of Perl's Prussian blue, which can be measured spectrophotometrically at 700 nm (Yen and Chen 1995).

The reducing power assay is considered to be a sensitive method for the "semi-quantitative" determination of dilute concentrations of polyphenolics, which participate in the redox reaction (Amarowicz and others 2004).

2.3.7 The bulk stripped oil model system

The term conjugated diene (CD) (Figure 2.12) implies two double bonds separated by a single bond, which is an unusual structure for polyunsaturated fatty acids (PUFA). The presence of CDs in a PUFA can be interpreted, therefore, as an indication of autoxidation of fatty acid moieties (Corongiu and Banni 1994). Conjugation of the pentadiene structure is a result of the formation of hydroperoxides from PUFA (Gordon 2001b). This structure causes absorption of UV radiation at 233–234 nm. Hence, the

(A) =

(B) =

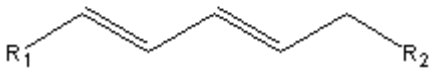


Figure 2.12 – (A) A polyunsaturated fatty acid (PUFA). (B) A PUFA with a conjugated diene (CD) (modified from Pegg 2005).

bulk stripped oil model system represents a simple and rapid method of monitoring the oxidative deterioration of marine oils or vegetable oils (Decker and others 2005). Oils need to be stripped to prevent any possible interaction of minor old constituents before adding test compounds having antioxidant capability. Therefore, oil samples containing antioxidants exhibit a lower number of CDs, compared to oil samples without antioxidant after a specific period.

2.4 Summary

Antioxidants have gained interest by researchers since it was reported that an increased intake of dietary antioxidants may help maintain an adequate antioxidant status, defined as the balance between antioxidants and oxidants in living organisms (Halliwell and others 1995). Antioxidants are found in all biological systems including plants (Barlow 1990). Phenolic compounds represent the majority of natural antioxidants and the most important groups of natural antioxidants are the tocopherols, flavonoids and phenolic acids that are common to all plant sources (Naczk and Shahidi 2006). Oilseeds, namely flaxseed, sunflower, soybean, cottonseed and canola are known as sources of natural antioxidants (Naczk and Shahidi 2006).

In canola, phenolic acids (both benzoic and cinnamic acid derivatives) (Kozlowska and others 1988), flavonoids (Hall 2001) and condensed tannins (Shahidi and Naczk 1989), were identified as antioxidant compounds. A diversity of phenolic compounds is present in canola or rapeseed flours (dehulled, defatted seed), meals (defatted, whole seed) or extracts, indicating that these products could protect food against rancidity (Amarowicz and others 2003). Wanasundara and Shahidi (1994) reported that the antioxidant activity of a crude ethanolic extract of canola meal (500 and

1000 ppm) could protect against the oxidation of canola oil. The effect was equivalent to that of TBHQ (200 ppm), and stronger than that of BHA (200 ppm), BHT (200 ppm) or BHA/BHT/monoglyceride citrate (MGC) (250 ppm) on a mass basis. Therefore, canola meal showed great potential as a natural source of antioxidant compounds.

Conventional extraction methods based on organic solvents were applied for the extraction of natural antioxidants from canola (Amarowicz and others 2003; Naczk and others 1998; Nowak and others 1992; Wanasundara and Shahidi 1994). These methods may have undesirable effects on the environment and on food components. Water has gained increasing attention as an extraction solvent, due to its unique dissolving properties, which can be altered by changing the temperature (Rovio and others 1999). Subcritical water extraction is an example of an extraction method using water as a solvent that is considered as a recent alternative for the isolation of antioxidant constituents.

Ibáñez and others (2003) extracted the most active antioxidant compounds from rosemary by SWE. García-Marino and others (2006) stated that SWE would be the most adequate extraction technique for obtaining a greater quantity of polyphenolic compounds from winery by-products. Kim and Mazza (2006) extracted phenolic compounds from flax shive by SWE. Lignans were also extracted from whole flaxseed by SWE (Cacace and Mazza 2006). Subcritical water extraction has been used to extract anthocyanins from red grape skin (Ju and Howard 2005); ginsenosides from American ginseng (Choi and others 2003); catechins and epicatechin from tea leaves and grape seeds (Piñeiro and others 2004); anthraquinones (antibacterial, antiviral and anticancer compounds) from the roots of *Morinda citrifolia* (Shotipruk and others 2004); and, flavones, anilines and phenols from orange peels (Lamm and Yang 2003).

In the SWE system, temperatures between 100 and 374^{o}C (the critical point of water is at 374^{o}C and 22 MPa) are generally applied and the pressure should be high enough to keep water in the liquid state (Ramos and others 2002). At temperatures above 100°C, the dielectric constant of water, ε, (*i.e.*, its polarity) can be lowered easily and significantly by increasing the temperature to that of ethanol and methanol and, consequently, low enough to dissolve many compounds of intermediate or low polarity (Ramos and others 2002). A major disadvantage of SWE is the high operating pressure, which requires expensive equipment (Smith 2002). However, in the case of antioxidants, price would not play a crucial role, since they are an expensive group of food compounds and costs are compensated by other advantages, such as the high purity of extracts and the efficiency of the process (Ramos and others 2002). Therefore, SWE may be a great candidate for the extraction of antioxidant compounds from canola meal.

There are numerous assays developed for measuring the antioxidant capacity of food and biological samples. However, there is no universal method that can measure antioxidant capacity of all samples accurately and quantitatively (Prior and others 2005). Based on the chemical reactions involved, most antioxidant capacity assays can be classified as HAT reaction-based assays or ET reaction-based assays (Huang and others 2005). These reactions may occur in parallel and usually occur together in all samples (Prior and others 2005). In this study, the following assays were used to assess the antioxidant capacities of samples: determination of total phenolics content (ET), the Trolox equivalent antioxidant capacity (TEAC) assay (ET), the β-carotene-linoleic acid (linoleate) assay (HAT), the radical-scavenging activity (RSA) assay (ET), the reducing power assay (ET) and the bulk stripped corn oil model system (other). These assays are suitable for measuring antioxidant capacities and a comparison of their attributes may

elucidate a singular assay that is appropriate for the determination of antioxidant capacity in extracts from canola meal.

The objective of this study was to investigate the use of SWE for the extraction of antioxidant compounds from canola meal.

3.0 MATERIALS AND METHODS

3.1 Canola samples

Three batches of commercial canola meal were provided by ADM, Lloydminster, SK. Batches were received in May 2005 (Batch 1), January 2006 (Batch 2) and April 2006 (Batch 3).

3.2 Extraction of antioxidants from canola meal

Three methods of extraction were applied in this study namely, subcritical water extraction, ethanolic extrcation and hot water extrcation:

3.2.1 Subcritical water extraction (SWE)

Subcritical water extraction of canola meal was conducted at temperatures of 110, 135 and 160°C, at pressures of 500, 750 and 1000 psi (3.44, 5.14 and 6.89 MPa) and at a flow rate of 1.0 mL/min for 30 min in a homebuilt apparatus (constructed by Agriculture and Agri-Food Canada, Saskatoon, SK) (Figure 3.1). The following procedure was employed.

A 1.0-g sample of canola meal was filled into an extraction column (Type 304 Stainless Steel, 151.2 mm × 6.9 mm ID). Two pieces of filter paper (GF/B and Whatman No. 5) and glass-wool were inserted at the top of the column, and a piece of filter paper (GF/B) and glass-wool at the bottom, to prevent the frits (2 µm on the bottom of the column and 10 µm on the top) from plugging. Chromatography column end fittings

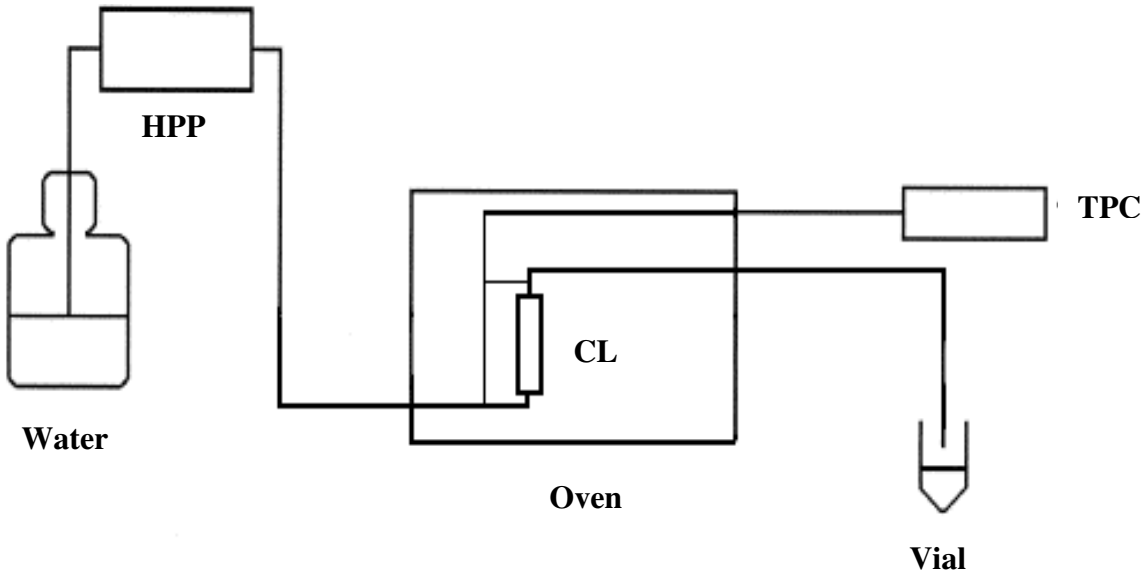


Figure 3.1 – Schematic diagram of the subcritical water extractor. HPP, high-pressure pump; CL, column; TPC, temperature controller.

(Chromatographic Specialties Inc, Brockville, ON) were used to connect the extraction column to the system. Connections and tubing were stainless steel. Tubing (1/16 inch o.d.) and fittings were adequate for high pressure (34 MPa). Water was purged with nitrogen to remove dissolved oxygen prior to extraction. The column was attached to the system after warming up the oven (programmable forced-air convection oven, max temp 325°C) to an initial temperature of 50°C. When the oven reached its operating temperature, extraction commenced at the desired pressure [controlled with a back pressure regulator to 3.44, 5.14 or 6.89 MPa (500, 750 or 1000 psi; Upchurch Scientific, Oak Harbor, WA) at a flow rate of 1.0 mL/min using an HPLC pump (Waters 515 HPLC Pump, Waters Co., Milford, MA). The extract was collected in a 50-mL graduated cylinder over a 30-min period. Operating parameters (time, temperature, pressure and the volume collected) were monitored and recorded periodically. After the extraction was completed, the extract was transferred to a freeze drying flask and then lyophilized (Flexi-Dry™, FTS SYSTEMS, Stonebridge, NY). The dried sample was weighed and then transferred into an air-tight vial which was stored at -20^{o}C.

3.2.1.1 Single temperature extraction

The above procedure was applied at single temperatures of 110, 135 or 160°C for 30 min each at pressures of 3.44, 5.14 or 6.89 MPa (500, 750 or 1000 psi). A 1.0-g sample of canola meal was extracted at each temperature. The extractions were performed in triplicate.

3.2.1.2 Sequential extraction

A 1.0-g sample of canola meal was extracted at 110°C at 3.44, 5.14 or 6.89 MPa (500, 750 or 1000 psi) for 15 min. Then, the extraction was stopped and the system was heated to 135°C. After a 15-min extraction of the same sample at 135°C, the extraction was stopped again and the system was heated to 160°C prior to an additional 15-min extraction at that temperature. Extractions were performed in triplicate.

3.2.2 Ethanolic extraction (95%)

Canola meal was extracted with 95% ethanol for 30 min using a reflux system based on procedures described by Amarowicz and Shahidi (2003) and Wanasundara and others (1994), with slight modifications. Thirty-six grams of canola meal was placed in a 100-mL conical flask, to which 600 mL of 95% ethanol was added. The contents of the flask were heated for 30 min in a water bath equipped with a temperature controller (Precision, Danville, VA) set at 80^{o}C. The slurry then was filtered under slight vacuum through Whatman No. 3 filter paper, and the solid residue was extracted twice more under the same conditions. The ethanol extracts were pooled and the solvent was evaporated using a rotary evaporator (BÜCHI Rotavapor 114 equipped with a BÜCHI Water bath B-480, Flawil, Switzerland and OAKTON Aspirator pump Model WP-15, Metex Corporation Limited, Toronto, ON) at <40°C. The dried sample was weighed and then transferred into an air-tight vial and stored at -20°C. Ethanol extractions were performed in triplicate.

3.2.3 Hot water extraction (80°C)

The extraction was carried out on canola meal using HPLC grade, deoxygenated water using the procedure described by Bergeron and others (2005). Canola meal (36 g) was placed in a 1000-mL conical flask containing 600 mL of water and was heated for 30 min at 80°C in a shaking water bath equipped with a temperature controller (Precision, Danville, VA). The solid residue was extracted twice more under the same conditions and the slurry was filtered under slight vacuum through Whatman No. 3 filter paper. The extracts so obtained were lyophilized. The dried sample was weighed and then transferred into an air-tight vial and stored at -20°C. Hot water extractions were performed in triplicate.

3.2.4 Determination of crude protein contents of extracts

Crude protein contents of extracts was measured by the Department of Plant Sciences, University of Saskatchewan, Saskatoon, SK, using the combustion method (AACC method 46-30) and an FP-528 LECO instrument (LECO Corporation, St. Joseph, MI). Crude protein was calculated using a nitrogen to protein conversion factor of 6.25.

3.3 Effects of temperature, pressure, time and pH on SWE of canola meal

Canola meal received from ADM, Lloydminster, SK on January 2006 (batch 2) was chosen arbitrarily for these studies.

3.3.1 Effect of extraction temperature

To determine the effect of temperature on SWE of antioxidant constituents from canola meal, 1.0 g of meal was extracted at 160, 135 or 110^{o}C for 30 min, or sequentially at 110, 135 and 160^{o}C for 15 min at each temperature, at 6.89 MPa (1000 psi). Extractions were carried out in triplicate. The total phenolics contents and antioxidant capacities (TEAC assay) of extracts were determined using procedures described in sections 3.4.1 and 3.4.2, respectively.

3.3.2 Effect of extraction pressure

To determine the effect of pressure on SWE of antioxidant constituents from canola meal, 1.0 g of meal was extracted at 110^{o}C (the temperature was chosen arbitrarily from 110^{o}C, 135 ^{o}C and 160^{o}C) for 30 min at 5.17 MPa (750 psi) or 3.44 MPa (500 psi). Extractions were performed in triplicate. Results were compared with those obtained previously at the same temperature and extraction time but at 6.89 MPa (1000psi). The total phenolics contents and antioxidant capacities (TEAC assay) of extracts were determined using procedures described in sections 3.4.1 and 3.4.2, respectively.

3.3.3 Effect of extraction time

To determine the effect of extraction time on SWE of antioxidant constituents from canola meal at 110^{o}C or 160^{o}C, 1.0 g of meal was extracted to 6.89 MPa (1000 psi) for 10-40 min in 5-min intervals. Extractions were performed in triplicate. The total phenolics contents and antioxidant capacities (TEAC assay) of extracts were determined using procedures described in sections 3.4.1 and 3.4.2, respectively.

3.3.4 Effect of pH and ionic strength

To determine the effect of pH on the efficacy of SWE of antioxidant constituents from canola meal, 1.0 g of meal was extracted with phosphoric acid/monosodiumphosphate/disodium phosphate solution (0.2 M) adjusted to pH 2 or 4, or phosphate buffer (0.2 M) at pH 6 or 8 (Anonymous 2000) at 110^{o}C for 15 min at 6.89 MPa (1000 psi). Extractions were performed in triplicate. The total phenolics contents and antioxidant capacities (TEAC assay) of extracts were determined using procedures described in sections 3.4.1 and 3.4.2, respectively.

To obtain phosphoric acid/monosodiumphosphate/disodium phosphate solution (0.2 M) adjusted to pH 2 or 4, or phosphate buffer at pH 6 or 8, the following procedures were employed:

Phosphoric acid/monosodium phosphate/disodium phosphate solution (0.2 M, pH 2) was made by dissolving 27.60 g monosodium phosphate and 0.01 g disodium phosphate in 500 ml of deionized water. A few drops of phosphoric acid (85%) was added to the solution to obtain pH 2 and then the volume was brought to 1000 ml with deionized water.

Phosphoric acid/monosodium phosphate/disodium phosphate solution (0.2 M, pH 4) was made by dissolving 27.56 g monosodium phosphate and 0.07 g disodium phosphate in 500 ml of deionized water. A few drops of phosphoric acid (85%) was added to the solution to obtain pH 4 and then the volume was brought to 1000 ml with deionized water.

Phosphate buffer (0.2 M, pH 6) was made by dissolving 24.29 g monosodium phosphate and 6.44 g disodium phosphate in 500 ml of deionized water. Phosphoric acid

solution (85%) was added to obtain pH 6 and then the volume of solution was brought to 1000 ml with deionized water.

Phosphate buffer (0.2 M, pH 8) was made by dissolving 1.88 g monosodium phosphate and 49.94 g disodium phosphate in 500 ml deionized water.VSodium hydroxide (0.1 N) was added to obtain pH 8 and then the volume was brought to 1000 ml with deionized water.

3.4 Determination of total phenolics contents and antioxidant properties of extracts of canola meal

Methods and equations that were used to determine the total phenolics contents and antioxidant properties of extracts obtained by hot water, ethanolic (95%) extraction and SWE are described hereafter.

3.4.1 Total phenolics contents

The total phenolics content of extracts and meals were estimated by a colorimetric assay (Amarowicz and others 2004) based on the reduction of a heteropolyphosphotungstate-molybdate complex by phenolics to blue-coloured products (Stratil and others 2006). In brief, freeze-dried canola extracts and ground meals were dispensed in ethanol. Extracts (or standard) in ethanol solution (0.25 mL) were added to test tubes containing 4 mL of deionized water. Then, 0.25 mL of Folin & Ciocalteu's phenol reagent (Sigma Aldrich Canada Ltd., Oakville, ON) was added to each tube, and after vortexing, 0.5 mL of saturated sodium carbonate was added to each tube. The contents were vortexed for 15 s and then left to stand at room temperature for 30 min. Sinapic acid was used as the standard, as it is the predominant free phenolic acid in

canola (Naczk and others 1998). Absorbance was recorded using a spectrophotometer (Ultraspec 2000, Pharmacia Biotech, Buckinghamshire, UK) at 755 nm. Sample calculations are provided in Appendix A.

3.4.2 Trolox equivalent antioxidant capacity (TEAC) assay

The Trolox equivalent antioxidant capacity assay evaluates the capacity of a crude extract to scavenge 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) $ABTS^{\bullet+}$ radicals (Liyana-Pathirana and Shahidi 2006; Arts and others 2004; Re and others 1999). In short, a 7 mM solution of ABTS in water was prepared and $ABTS^{\bullet+}$ was formed after the addition of potassium persulfate to the solution at a final concentration of 2.45 mM. After 12–16 h incubation in darkness at room temperature, the stock solution was diluted with ethanol until an absorbance of 0.7 ± 0.02 at 734 nm was reached. After addition of 4.0 mL of diluted $ABTS^{\bullet+}$ solution to 40 µL of sample (or Trolox standard, 0.5-4 mM), the reaction mixture was incubated for 6 min in plastic cuvettes at 37°C. The decrease in absorbance at 734 nm (Ultraspec 2000 spectrophotometer) was determined at exactly 6 min after initial mixing for all samples. The absorbance of $ABTS^{\bullet+}$ without sample, *i.e.*, the control, also was measured. Sample calculations is provided are Appendix B. The TEAC value was calculated using the following formulae:

$$\% \text{ Inhibition} = [(A_C - A_S)/A_C] \times 10 \qquad \text{(eq. 3.1)}$$

$$\text{TEAC value} = \% \text{ Inhibition}/m$$

where A_C is the absorbance of the control at t=6 min, A_S is the absorbance of the sample (or Trolox standard) at t=6 min, and m is the slope of the standard curve.

3.4.3 β-Carotene-linoleic acid (linoleate) assay

The antioxidant capacities of the extracts were estimated using a β-carotene-linoleic acid (linoleate) model system (Amarowicz and others 2004). In brief, 2 mg of β-carotene dissolved in chloroform (10 mL) was added to a small, round-bottom flask. Then, 40 mg of linoleic acid and 400 mg of Tween 40 were added. The contents of the flask were mixed by hand shaking and the chloroform was evaporated using a rotary evaporator (BÜCHI Rotavapor 114 equipped with a BÜCHI water bath B-480, Flawil, Switzerland and OAKTON Aspirator pump Model WP-15, Metex Corporation Limited, Toronto, ON) at <40°C. Then, 100 mL of aerated deionized water were transferred to the flask with vigorous stirring. Five millilitres of the prepared emulsion was transferred to a series of tubes containing 0.5 mL of ethanolic solution of extracts from SWE at 110°C or 160°C (2 mg/mL), ethanolic extracts (2 mg/mL), hot water extracts (2 mg/mL) or BHA (0.0002-0.5 mg/mL) or 0.5 mL of 95% ethanol as a control. After initial mixing, the test systems were incubated in a water bath (Precision, Danville, VA) at 50°C for 2 h. The absorbance of each sample was measured every 15 min using a spectrophotometer (Ultraspec 2000 spectrophotometer) at 470 nm. Sample calculations are provided in Appendix C.

The antioxidant activities of SWE, ethanolic and hot water extracts were expressed in three different ways (Amarowicz and others 2004):

1. Initially, the rate of β-carotene bleaching (R) was calculated according to first-order kinetics as shown:

$$R = \ln (A_{t=0} / A_{t=t}) \times 1/t \qquad \text{(eq. 3.2)}$$

where $A_{t=0}$ is the initial absorbance (470 nm) of the emulsion at time 0, $A_{t=t}$ is the absorbance (470 nm) at 15, 30 and 45 min, and t is the time in min.

An average rate for each sample was calculated based on the R determined at t = 15, 30 and 45 min. The antioxidant activity (ANT) was calculated as the percent inhibition of the rate of β-carotene bleaching relative to the control using the equation:

$$\% \text{ ANT} = 100 \times (R_{control} - R_{sample}) / R_{control} \quad \text{(eq. 3.3)}$$

where $R_{control}$ and R_{sample} are the average bleaching rates of β-carotene in the emulsion without antioxidant and with extract, respectively.

2. The antioxidant activity was also expressed as the percent inhibition of coupled oxidation of β-carotene and linoleic acid against the BHA and control samples, based on absolute changes in absorbance measurements at two distinct points (60 and 120 min) during the assay rather than as an average rate. In the equation below, the results are normalized by using two extremes:

- the control, which should offer no protection against oxidation of the linoleic acid/β-carotene emulsion; therefore, the antioxidant activity is defined as 0% for this system, and
- the BHA control, which should offer essentially 100% protection against oxidation over the time course of the assay. The antioxidant activities of the SWE, ethanolic and hot water extracts were expressed as:

$$\%AA = 100 \times [\, 1 - (A_E^{t=0} - A_E^{t=t}) / \{(A_C^{t=0} - A_C^{t=t}) + (A_{BHA}^{t=0} - A_{BHA}^{t=t})\}] \quad \text{(eq. 3.4)}$$

where AA is the antioxidant activity, $A_E^{t=0}$ is the absorbance (470 nm) of the extract in question at 0 min, $A_E^{t=t}$ is the absorbance (470 nm) of the extract at t=60 or 120 min, $A_W^{t=0}$ is the absorbance (470 nm) of the control sample at 0 min, $A_W^{t=t}$ is the absorbance (470 nm) of the control sample at t=60 or 120 min, $A_{BHA}^{t=0}$ is the absorbance (470 nm) of the synthetic antioxidant control sample at 0 min, and $A_{BHA}^{t=t}$ is the absorbance (470 nm) of the synthetic antioxidant control sample at t=60 or 120 min.

3.4.4 Radical-scavenging activity (RSA) assay

The capacity of extracts to scavenge the 'stable' free radical 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) was monitored using the method of Stratil and others (2006), with slight changes. Extracts (0.000–0.666 mg) were dissolved in 4 mL of ethanol and added to an ethanolic solution of $DPPH^{\bullet}$ (1 mM, 0.5 mL). After vortexing the mixture for 15 s, it was left to stand at room temperature for 30 min. The absorbance of the resulting solution was read spectrophotometrically (Ultraspec 2000 spectrophotometer) at 515 nm. An ethanolic solution of $DPPH^{\bullet}$ that was decayed, and hence which no longer exhibited a purple colour (*i.e.*, 2 mg of BHA dissolved in 4 mL of ethanol with 0.5 mL of the $DPPH^{\bullet}$ solution added), was chosen for background correction instead of pure ethanol. Sample calculations are provided in Appendix D. The radical scavenging activity (RSA) was calculated as the percentage of $DPPH^{\bullet}$ discoloration using the equation below:

$$\% \text{ RSA} = 100 \times (1 - A_E/A_D) \qquad \text{(eq. 3.5)}$$

where A_E is the absorbance of the solution when an extract has been added at a particular level, and A_D is the absorbance of the $DPPH^{\bullet}$ solution with nothing added.

3.4.5 Reducing power assay

In order to determine the reducing power of the extracts (Amarowicz and others 2004), each extract (0.2–1.0 mg) was dissolved in 1.0 mL of deionized water to which 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% (w/v) potassium ferricyanide solution were added. The mixture was placed in a water bath at 50°C for 20 min. Following this, 2.5 mL of a 10% (w/v) trichloroacetic acid solution was added. A

2.5-mL aliquot was combined with 2.5 mL of deionized water and 0.5 mL of 0.1% (w/v) ferric chloride solution. Absorbance of the reaction mixture was recorded at 700 nm using a spectrophotometer (Ultraspec 2000 spectrophotometer). Sample calculations are provided in Appendix E.

3.4.6 Bulk stripped oil model system

This method was adopted from Madhujith and others (2004) with slight modifications. The dried canola extracts at 200 ppm and BHA at 200 ppm were applied to stripped corn oil (Fisher Scientific, Toronto, ON) to examine their antioxidative activity. The dried extracts and BHA were mixed with a minimum amount of absolute ethanol in an ultrasonic water bath and then were added to the oil (5 g), with vortexing for 10 min. A control sample contained the same amount of ethanol without any extract or BHA. Each sample was stored separately in a forced-air oven at 60°C over a 20-day period in small, open, glass containers for Schaal oven stability studies. Samples of each treatment were removed at 0, 2, 4, 8, 12, 16 and 20 days, and analyzed by a conjugated diene (CD) test (Pegg 2005). Chemical analysis of the oils subjected to accelerated oxidation consisted of determination of conjugated dienes (CD) at 234 nm (Ultraspec 2000 spectrophotometer). Sample calculations are provided in Appendix F. The following equations were used to calculate the CD value:

$$C_{CD} = A_{234} / (\varepsilon \times l) \qquad \text{(eq. 3.6)}$$

where C_{CD} is the CD concentration in mmol/mL, A_{234} is the absorbance of the solution at 234 nm, ε is the molar absorptivity (*i.e.*, the extinction coefficient) of linoleic acid hydroperoxide (2.525×10^4 $M^{-1}cm^{-1}$), and l is the path length of the cuvette in cm (1 cm);

$$\text{CD value} = [C_{CD} \times (2.5 \times 10^4)] / W$$

where 2.5×10^4 is a factor that includes 25 mL of 2,2,4-trimethylpentane used to dissolve the oil sample as well as a unit conversion (1000 µmol/mmol) so that the content of CDs can be expressed in µmol, and W is the weight of the sample in gram.

After calculation of the CD values of samples, the results were expressed as extinction values using the following equation:

$$E^{1\%}_{1cm} = A_\lambda / (C_L \times l) \qquad \text{(eq. 3.7)}$$

where E is the extinction value, A_λ is the absorbance measured at 234 nm for CDs, C_L is the concentration of lipid solution in g/100 mL, and l is the path length of the cuvette in cm (1 cm).

3.5 Statistical treatment

All measurements were replicated a minimum of three times on each batch of canola meal. A Primer of Biostatistic program version 3.01 (McGraw-Hill Inc., New York, NY) was used for data analysis. One-way analysis of variance (ANOVA) followed by Bonferroni t-tests was performed to assess and compare the influence of different extraction methods/conditions on antioxidant capacity, protein content and extract yields of crude extracts (significance level $P < 0.05$). The same software was used to determine correlation coefficients between different antioxidant capacity assays [significance level of 5% ($P < 0.05$)].

4.0 RESULTS AND DISCUSSION

4.1 Composition of canola meal samples

Results from analysis conducted by POS Pilot Plant Corporation, Saskatoon, SK, on the composition of three batches of commercial canola meal received from ADM, Lloydminster, SK, on May 2005 (batch 1), January 2006 (batch 2) and April 2006 (batch 3) are presented in Table 4.1. The three batches of meal exhibited similar contents of crude protein, crude fat, ash, moisture and carbohydrate. Carbohydrate (by difference) and protein represented 40.7-41.8% and 36.4-36.8% of the meals on a 10% moisture basis, respectively (values calculated from as-is moisture values in Table 4.1). Meal samples contained 4.0-4.8% crude fat and 6.8-8.6% ash on a 10% moisture basis. Total phenolics content represented 1.7-1.8% of the meal on a 10% moisture basis.

According to the Canola Council of Canada, Canadian canola meal typically contains approximately 36.0% crude protein, 3.5% crude fat, 6.1% ash, 12% crude fibre and 33% total dietary fibre on a 10% moisture basis (Anonymous 2001). Carbohydrate content (by difference) would typically be 44.4% on a 10% moisture basis. The crude fat content of Canadian canola meal is relatively high compared to the 1-2% crude fat in canola meals produced in European or Asian countries. This is due to adding canola gums back to canola meal at level of 1-2% in Canada (Anonymous 2001). The composition of each of the three batches of canola meal used in this study was similar to that reported by the Canola Council of Canada (Anonymous 2001). Therefore, it was concluded that the canola meal samples extracted in this study had compositions similar

Table 4.1 – Composition[a] of three batches of commercial canola meal (as-is moisture basis)

	Batch 1[b]	***Batch 2[c]***	***Batch 3[d]***
Crude Protein (N x 6.25), %	36.3 ± 0.42	36.4 ± 0.35	36.9 ± 0.37
Crude fat, %	3.9 ± 0.11	4.8 ± 0.17	4.4 ± 0.14
Ash, %	7.5 ± 0.17	6.8 ± 0.12	8.7 ± 0.15
Moisture, %	11.3 ± 0.22	10.6 ± 0.25	8.8 ± 0.19
Carbohydrate (by difference), %	41.0 ± 0.92	41.4 ± 0.89	41.2 ± 0.85
Total phenolics content (mg sinapic acid equivalents per gram of meal)	17.31 ± 0.07	17.32 ± 0.09	17.77 ± 0.08

[a]Values are means of at least three determinations ± standard deviation.
[b]Sample received in May 2005 and analyzed in October 2005.
[c]Sample received in January 2006 and analyzed in February 2006.
[d]Sample received in April 2006 and analyzed in April 2006.

to that of typical canola meal, and that the extraction results obtained in this study could be extrapolated to canola meal in general.

4.2 Extraction of antioxidants from canola meal

4.2.1 Extract yields

Results from extraction of three batches of canola meal are presented in Table 4.2. Subcritical water extraction at 160°C produced the highest extract yields (0.45-0.48 g/g of meal). Sequential SWE resulted in lower yields (0.36-0.37 g/g of meal) than did SWE at 160°C, but higher yields than did SWE at 135°C (0.27 g/g of meal) or at 110°C (0.20 g/g of meal). The extract yields obtained with hot water (80°C) extraction (0.19-0.21 g/g meal) were similar to that obtained with SWE at 110°C. Ethanol (95%) extraction produced the lowest extract yields (0.14-0.15 g/g meal), due to the low solubility of protein in ethanol (Table 4.3).

As the extraction temperature was increased, so too was the extract yield from SWE. As a polar fluid, water normally dissolves polar compounds much more readily than it does nonpolar compounds. Higher temperatures reduce the polarity of water, thus increasing its ability to solvate nonpolar compounds. For example, when the temperature of water is increased from 25°C to 200°C, its dielectric constant decreases from 79 to 35, a value equivalent to that of methanol (35) and similar to that of ethanol (24) (Cacace and Mazza 2006). The dielectric constant of water at 80°C, 110°C and 160°C is 81, 53 and 42, respectively (Lide 2001). Consequently, the ability of water to dissolve less polar compounds is enhanced at higher temperatures since the polarity of water, itself, is lowered. Raising the temperature of water also reduces its surface tension and viscosity, which increases the diffusion rate and the rate of mass transfer during

Table 4.2 – Extract yields (g/g meal)[a] obtained from subcritical water, hot water and ethanolic extraction of three batches[b] of canola meal

Extracts	*Batch 1*	*Batch 2*	*Batch 3*
SWE 160[c]	0.45 ± 0.01a	0.48 ± 0.02a	0.46 ± 0.03a
SWE 135[c]	0.27 ± 0.03c	0.27 ± 0.03c	0.27 ± 0.03c
SWE 110[c]	0.20 ± 0.01d	0.20 ± 0.02d	0.20 ± 0.04d
SWE Sequential[d]	0.36 ± 0.03b	0.37 ± 0.03b	0.36 ± 0.02b
Hot water[e]	0.21 ± 0.01d	0.20 ± 0.00d	0.19 ± 0.01d
Ethanolic[f]	0.15 ± 0.00e	0.14 ± 0.00e	0.15 ± 0.00e

[a]Values are means of at least three determinations ± standard deviation and are expressed as grams of freeze-dried extract per gram of meal, both on an as-is moisture basis. Means followed by the same letter are not significantly different ($P > 0.05$).
[b]Samples of canola meal were obtained from ADM, Lloydminster, SK, on May 2005 (batch 1), January 2006 (batch 2) and April 2006 (batch 3).
[c]Subcritical water extraction at 160, 135 or 110°C for 30 min at 6.89 MPa (1000 psi).
[d]Subcritical water extraction at 110, 135 and 160°C for 15 min at each temperature at 6.89 MPa (1000 psi).
[e]Hot water extraction at 80°C for 30 min.
[f]Refluxed with 95% ethanol for 30 min.

Table 4.3 – Crude protein concentrations (%)[a] of freeze-dried extracts from subcritical water, hot water and ethanolic extraction of canola meal[b]

Extracts	*Canola Batch #*	*Crude Protein (N x 6.25), %*
SWE 160[c]	2 and 3	35.03 ± 1.20a
SWE 135[c]	2 and 3	27.27 ± 0.39b
SWE 110[c]	1	18.78 ± 0.75c
SWE Sequential[d]	2 and 3	34.18 ± 1.32a
Hot water[e]	2 and 3	17.57 ± 0.65c
Ethanolic[f]	2 and 3	6.77 ± 0.29d

[a]Values are means of at least four determinations ± standard deviation and are expressed on an as-is moisture basis. Means followed by the same letter are not significantly different ($P > 0.05$).
[b]Samples of canola meal were obtained from ADM, Lloydminster, SK, on May 2005 (batch 1), January 2006 (batch 2) and April 2006 (batch 3).
[c]Subcritical water extraction at 160, 135 or 110^{o}C for 30 min at 6.89 MPa (1000 psi).
[d]Subcritical water extraction at 110, 135 and 160^{o}C for 15 min at each temperature at 6.89 MPa (1000 psi).
[e]Hot water extraction at 80^{o}C for 30 min.
[f]Refluxed with 95% ethanol for 30 min.

extraction (Ramos and others 2002). These effects of increased temperature would all lead to higher extract yields at higher SWE temperatures. Ibáñez and others (2003) showed that at 25°C, rosmanol was the major component of an SWE extract, accounting for >50% of its total composition. When the temperature was increased from 25°C to 200°C, an increase in the extraction ability of subcritical water toward less polar compounds such as carnosic acid and carnosol was observed. A similar trend was reported for SWE of phenolic compounds from flax shive (Kim and Mazza 2006), antioxidant compounds from oregano (Rodríguez-Meizoso and others 2006), polyphenolic compounds (catechins and proanthocyanidins) from winery by-products (García-Marino and others 2006), anthraquinones from *Morinda citrifolia* (Pongnaravane and others 2006), lignans, proteins and carbohydrates from flaxeed meal (Ho and others 2007), and saponins from cow cockle seed (Güçlü-Üstündağ and others 2007).

4.2.2 Crude protein contents of freeze-dried extracts

Crude protein concentrations in extracts obtained by SWE and ethanolic and hot water extraction of canola meal are presented in Table 4.3. Extracts from SWE at 160^{o}C and sequential SWE had the highest crude protein contents (35.0% and 34.2%, respectively). Extracts from SWE at 135^{o}C were lower in crude protein (27.3%) than were extracts from SWE at 160^{o}C and sequential SWE, but were higher in crude protein than were extracts from SWE at 110^{o}C or hot water extracts, which had similar crude protein concentrations (18.8% and 17.6%, respectively). Ethanolic extracts were the lowest in crude protein (6.8%). The ranking of the extraction methods was the same for both extract yield and the concentration of crude protein in the extract (SWE 160^{o}C >

SWE sequential > SWE 135°C > SWE 110°C = hot water > ethanolic) (Tables 4.2 and 4.3). It is well known that increasing the temperature and decreasing the dielectric constant of water can lower the energy required to disrupt solute-matrix interactions and also can reduce its polarity by weakening hydrogen bonds (Ho and others 2007). Cacace and Mazza (2006) and Ho and others (2007) reported a continuous increase in the concentrations of protein and carbohydrate in extracts obtained from flaxseed by SWE as the extraction temperature was increased over the range of 100-160°C.

4.3 Effects of temperature, pressure, time and pH on SWE of canola meal

Batch 2 of canola meal was chosen arbitrarily for use in this study. The total phenolics and TEAC assays were employed in this study since each has been used frequently to determine the total phenolics content and the total antioxidant capacity, respectively, of many food samples such as fruits, vegetables and spices (Stratil and others 2006; Huang and others 2006; Ragaee and others 2006; Liyana-Pathirana and Shahidi 2006; Prior and others 2005).

4.3.1 Effect of extraction temperature

The effect of SWE temperature on the total phenolics contents and antioxidant activities (TEAC assay) of extracts is shown in Table 4.4. Subcritical water extraction at 110°C or 135°C yielded extracts with the highest total phenolics contents (35.1 and 34.1 mg sinapic acid equivalents/g extract, respectively). Subcritical water extraction at 110°C yielded an extract with the highest antioxidant capacity (2.9 μmoles Trolox equivalents/g extract). Subcritical water extraction at 160°C and sequential SWE

Table 4.4 – Effect of extraction temperature[a] on the total phenolics content and the TEAC value of freeze-dried extracts from subcritical water extraction (SWE) of canola meal[b] at 6.89 MPa (1000 psi) for 30 min

Extraction Temperature	*Extract Yield (g/g meal)*	*Total content of phenolic compounds (mg sinapic acid equivalents/ g extract)*	*Total content of phenolic compounds (mg sinapic acid equivalents/g meal)*	*TEAC value (µmoles Trolox equivalents/ g extract)*	*TEAC value (µmoles Trolox equivalents/ g meal)*
160°C	0.48 ± 0.02a	31.82 ± 0.32b	15.27 ± 0.15a	2.68 ± 0.06b	1.29 ± 0.02a
135°C	0.27 ± 0.03c	34.14 ± 0.78a	9.21 ± 0.09d	2.71 ± 0.05b	0.74 ± 0.01c
110°C	0.20 ± 0.01d	35.19 ± 0.48a	7.03 ± 0.09e	2.97 ± 0.09a	0.59 ± 0.02d
SWE Sequential[c]	0.37 ± 0.03b	31.75 ± 0.41b	11.73 ± 0.11b	2.73 ± 0.05b	1.01 ± 0.02b

[a]Values are means of at least three determinations ± standard deviation and are expressed on an as-is moisture basis. Means within a column followed by the same letter are not significantly different ($P > 0.05$).
[b]Batch 2 of canola meal obtained from ADM, Lloydminster, SK, on January 2006 was chosen arbitrarily for use in this study.
[c]Subcritical water extraction at 110, 135 and 160°C for 15 min at each temperature at 6.89 MPa (1000 psi).

yielded similar results, *i.e.,* extracts with the lowest total phenolics contents and antioxidant capacities on a per gram of extract basis. The total phenolics contents of extracts obtained by SWE at 110°C or 135°C were similar on a per gram of extract basis. This was due to the semi-polar nature of antioxidant compounds (Figure 2.6) in canola meal. Naczk and others (1998) reported that sinapic acid was the predominant phenolic acid in canola. They also determined that sinapic acid represented 70-85% of the total free phenolic acids in canola; the remainder included small quantities of *p*-hydroxybenzoic, vanillic, gentisic, protocatechuic, syringic, *p*-coumaric, *cis*- and *trans*-ferulic, caffeic and chlorogenic acids in the free form. Since these compounds are neither very polar nor nonpolar, they can be extracted by moderately polar solvents, *e.g.*, SWE at 110°C or 135°C. The total phenolics contents and the TEAC values of extracts obtained by SWE at temperatures higher than 110°C showed decreases (Table 4.4), which may be due to the degradation of phenolics and other antioxidant compounds at higher temperatures.

On a per gram of meal basis, SWE at 160°C was superior with respect to both the total phenolics content and the antioxidant capacity of the extract. Both SWE at 135°C and sequential SWE resulted in higher total phenolics contents and antioxidant capacities in extracts on a per gram of meal basis than did SWE at 110°C. Subcritical water extraction at 110°C yielded extracts with the highest total phenolics contents and TEAC values on a per gram of extract basis (Table 4.4), hence SWE at 110°C was employed in subsequent studies. Subcritical water extraction at 160°C resulted in the highest extract yields and the highest total phenolics contents and TEAC values on a per gram of meal basis (Table 4.4), hence SWE at 160°C was employed in subsequent studies.

4.3.2 Effect of extraction pressure

This experiment was conducted at 110°C only, as operational problems were experienced with extraction at 160°C and 3.44 MPa (500 psi). Extraction pressure had no effect on the yield, total phenolics content or antioxidant capacity of extracts obtained by SWE at 110°C (Table 4.5). These results are in agreement with those of Alaya and Castro (2001) who reported that pressures ranging from 1.0 to 5.1 MPa (145-725 psi) had no influence on the chromatograms obtained for oil extracted from ground oregano by SWE. The pressure of the system needs only to be high enough to keep the water in the liquid state at any particular SWE temperature (Ramos and others 2002).

4.3.3 Effect of extraction time

The effect of extraction time (10 to 40 min) on the yield, total phenolics content and antioxidant activity of extracts obtained by SWE at 6.89 MPa (1000 psi) and at temperatures of 110°C and 160°C is shown in Tables 4.6 and 4.7. At either 110°C or 160°C, no increase in extract yield, total phenolics content or antioxidant activity was observed at extraction times longer than 25-30 min. Accordingly, an extraction time of 30 min was employed in subsequent experiments. Extract yield, total phenolics content and antioxidant activity on a per gram of meal basis were higher at 160°C. In contrast, total phenolics content and antioxidant activity on a per gram of extract basis were higher at 110°C.

At 160°C, the TEAC value was highest for extracts obtained between 15 and 30 min of extraction, whereas the total phenolics contents of extracts did not increase beyond 10 min of extraction (Table 4.7). Güçlü-Üstündağ and others (2007) reported that 60.2 wt% of total saponins was extracted from ground cow cockle seeds in the first

Table 4.5 – Effect of extraction pressure[a] on the total phenolics content and the TEAC value of freeze-dried extracts from subcritical water extraction (SWE) of canola meal[b] at 110°C for 30 min

Extraction Pressure	*Extract Yield (g/g meal)*	*Total content of phenolic compounds (mg sinapic acid equivalents/g extract)*	*Total content of phenolic compounds (mg sinapic acid equivalents/g meal)*	*TEAC value (µmoles Trolox equivalents/g extract)*	*TEAC value (µmoles Trolox equivalents/g meal)*
6.89 MPa (1000 psi)	0.20 ± 0.01a	35.19 ± 0.48a	7.03 ± 0.09a	2.97 ± 0.07a	0.59 ± 0.03a
5.17 MPa (750 psi)	0.20 ± 0.02a	35.98 ± 0.41a	7.19 ± 0.08a	3.03 ± 0.08a	0.61 ± 0.02a
3.44 MPa (500 psi)	0.20 ± 0.00a	36.15 ± 0.21a	7.23 ± 0.04a	3.04 ± 0.07a	0.61 ± 0.02a

[a]Values are means of at least three determinations ± standard deviation and are expressed on an as-is moisture basis. Means within a column followed by the same letter are not significantly different ($P > 0.05$).
[b]Batch 2 of canola meal obtained from ADM, Lloydminster, SK, on January 2006 was chosen arbitrarily for use in this study.

Table 4.6 – Effect of extraction time[a] on the total phenolics content and the TEAC value of freeze-dried extracts from subcritical water extraction (SWE) of canola meal[b] at 110°C and 6.89 MPa (1000 psi)

Extraction Time	*Extract Yield (g/g meal)*	*Total content of phenolic compounds (mg sinapic acid equivalents/g extract)*	*Total content of phenolic compounds (mg sinapic acid equivalents/ g meal)*	*TEAC value (µmoles Trolox equivalents/g extract)*	*TEAC value (µmoles Trolox equivalents/g meal)*
10 min	0.11 ± 0.00c	30.46 ± 0.12c	3.35 ± 0.01d	2.82 ± 0.06b	0.31 ± 0.01e
15 min	0.14 ± 0.01b	34.44 ± 0.68b	4.82 ± 0.08c	3.02 ± 0.04a	0.42 ± 0.01d
20 min	0.17 ± 0.00a	34.46 ± 0.12b	5.85 ± 0.02b	3.01 ± 0.03a	0.51 ± 0.01c
25 min	0.20 ± 0.00a	35.75 ± 0.71a	7.15 ± 0.14a	2.96 ± 0.04a	0.59 ± 0.01b
30 min	0.20 ± 0.02a	35.98 ± 0.41a	7.19 ± 0.08a	3.03 ± 0.04a	0.61 ± 0.02ab
35 min	0.21 ± 0.02a	34.94 ± 0.12a	7.33 ± 0.03a	3.02 ± 0.03a	0.64 ± 0.01a
40 min	0.21 ± 0.00a	34.85 ± 0.71a	7.31 ± 0.14a	3.05 ± 0.04 a	0.64 ± 0.02a

[a]Values are means of at least three determinations ± standard deviation and are expressed on an as-is moisture basis. Means within a column followed by the same letter are not significantly different ($P > 0.05$).
[b]Batch 2 of canola meal obtained from ADM, Lloydminster, SK, on January 2006 was chosen arbitrarily for use in this study.

Table 4.7 – Effect of extraction time[a] on the total phenolics content and the TEAC value of freeze-dried extracts from subcritical water extraction (SWE) of canola meal[b] at 160°C and 6.89 MPa (1000 psi)

Extraction Time	*Extract Yield (g/g meal)*	*Total content of phenolic compounds (mg sinapic acid equivalents/g extract)*	*Total content of phenolic compounds (mg sinapic acid equivalents/ g meal)*	*TEAC value (µmoles Trolox equivalents/g extract)*	*TEAC value (µmoles Trolox equivalents/g meal)*
10 min	0.26 ± 0.03d	31.06 ± 0.12a	8.07 ± 0.03e	2.37 ± 0.10c	0.62 ± 0.03d
15 min	0.34 ± 0.01c	31.24 ± 0.68a	10.62 ± 0.23d	2.79 ± 0.04a	0.95 ± 0.01c
20 min	0.35 ± 0.01c	31.46 ± 0.12a	11.01 ± 0.04c	2.83 ± 0.03a	0.99 ± 0.01c
25 min	0.42 ± 0.02b	30.75 ± 0.71a	12.91 ± 0.29b	2.67 ± 0.07ab	1.12 ± 0.03b
30 min	0.48 ± 0.02a	31.82 ± 0.32a	15.27 ± 0.15a	2.68 ± 0.05ab	1.29 ± 0.02a
35 min	0.49 ± 0.01a	30.94 ± 0.12a	15.16 ± 0.06a	2.55 ± 0.07bc	1.25 ± 0.03a
40 min	0.51 ± 0.02a	30.85 ± 0.51a	15.53 ± 0.26a	2.52 ± 0.06bc	1.28 ± 0.02a

[a]Values are means of at least three determinations ± standard deviation and are expressed on an as-is moisture basis. Means within a column followed by the same letter are not significantly different ($P > 0.05$).

[b]Batch 2 of canola meal obtained from ADM, Lloydminster, SK, on January 2006 was chosen arbitrarily for use in this study.

15 min at 175°C, whereas only 33.2 wt% was recovered in 3 h at 125°C. The high TEAC values of extracts obtained between 15 and 30 min of extraction while the total phenolics contents of extracts was steady after 10 min of extraction, suggests that phenolic compounds may not be the solely responsible for the antioxidant capacities of extracts obtained by SWE at 110°C or 160°C. It has been reported that canola contains tocopherols in addition to phenolic compounds (Hall 2001; Warner and Mounts 1990). The TEAC values of extracts from SWE at 160°C after 35 or 40 min of extraction were lower than those obtained after 15-30 min of extraction, possibly due to degradation of antioxidant compounds at 160°C at longer extraction times.

4.3.4 Effect of pH and ionic strength

The purpose of this study was to attempt to extract more antioxidant compounds and lesser amount of undesirable compounds such as proteins that may influence the antioxidant capacity of extracts. It has been reported that proteins interfere with free and bound phenolics in extracts and, therefore, may reduce the antioxidant capacity (Naczk and others 1998).

The effect of SWE pH and ionic strength on the yield, total phenolics content and antioxidant capacity of extracts is shown in Table 4.8. Canola meal was extracted at 110°C and 6.89 MPa (1000 psi) for 15 min using phosphoric acid/monosodium phosphate/disodium phosphate solution (0.2 M) adjusted to pH 2 or 4 or phosphate buffer (0.2 M) at pH 6 or 8. The pH of the system was measured at two points, namely before and after the extraction column, to confirm the pH of the system. Extract yields (0.30-0.41 g/g meal) from the buffered extractions, irrespective of pH, were higher than the yield achieved without buffer (0.14 g/g meal). The highest yields were obtained at

Table 4.8 – Effect of pH[a] on the total phenolics content and the TEAC value of freeze-dried extracts from subcritical water extraction (SWE) of canola meal[b] at 110°C for 15 min at 6.89 MPa (1000psi)

Extraction pH[c]	*Extract Yield (g/g meal)*	*Total content of phenolic compounds (mg sinapic acid equivalents/g extract)*	*Total content of phenolic compounds (mg sinapic acid equivalents/g meal)*	*TEAC value (µmoles Trolox equivalents/g extract)*	*TEAC value (µmoles Trolox equivalents/g meal)*
pH 2	0.41 ± 0.00a	10.33 ± 0.16c	4.23 ± 0.07b	1.46 ± 0.06d	0.60 ± 0.03a
pH 4	0.30 ± 0.02c	16.14 ± 0.16b	4.84 ± 0.05a	1.60 ± 0.04c	0.48 ± 0.02cd
pH 6	0.31 ± 0.02c	10.85 ± 1.01c	3.36 ± 0.34d	1.59 ± 0.04c	0.49 ± 0.02c
pH 8	0.36 ± 0.01b	10.42 ± 0.42c	3.75 ± 0.14c	1.78 ± 0.04b	0.64 ± 0.02a
no buffer[d]	0.14 ± 0.01d	34.44 ± 0.68a	4.82 ± 0.08a	3.02 ± 0.04a	0.42 ± 0.01d

[a]Values are means of at least three determinations ± standard deviation and are expressed on an as-is moisture basis. Means within a column followed by the same letter are not significantly different (P > 0.05).
[b]Batch 2 of canola meal was obtained from ADM, Lloydminster, SK, on January 2006 was chosen arbitrarily for use in this study.
[c] Phosphoric acid/monosodiumphosphate/disodium phosphate solution (0.2 M) adjusted at pH 2 or 4 or phosphate buffer (0.2 M) at pH 6 or 8
[d] Subcritical water with pH 6.8-7.0

pH 2 and pH 8 (0.41 and 0.36 g/g meal, respectively) and the lowest yields at pH 4 and pH 6 (0.30 and 0.31 g/g meal, respectively). The decrease in the extract yield at pH 4 and 6 was due to the effect of pH on the extraction of protein and carbohydrates. The lowest solubility of protein in canola meal was found to occur at pH 5 (Pedroche and others 2004). The effect of pH and ionic strength on the solubility of globular proteins is well known (Anonymous 1997; Ghodsvali and others 2005). Ho and others (2007) stated that pH was the major factor affecting SWE of protein from flaxseed meal, with a maximum recovery of protein at pH 9. They found that more carbohydrate was extracted in acidic or neutral water, and that pH, in general, was the least significant factor among other independent variables for recovery of lignans. Ghodsvali and others (2005) reported that extraction pH had a large effect on the yield of protein extracted from Iranian canola meal and that the extractability of protein was maximized at pH 12. Klockeman and others (1997) reported that the solubility of canola meal protein in the presence of 1 M NaCl at pH 2-8 was greater than at 0.1 M NaCl in deionized water. They observed a decrease in protein solubility at pH 4-6 in the presence of 1 M NaCl.

The total phenolics contents of extracts obtained at pH 2-8, on a per gram of extract basis (10.33-16.14 mg sinapic acid equivalents/g extract), were lower than that of the extract obtained without buffer (34.44 mg sinapic acid equivalents/g extract). Extracts obtained at pH 4 or without buffer exhibited higher total phenolics contents on a per gram of meal basis (4.84 and 4.82 mg sinapic acid equivalents/g meal, respectively) than did extracts obtained at pH 2, 6 or 8 (4.23, 3.36 and 3.75 mg sinapic acid equivalents/g meal, respectively). Extracts obtained at pH 2 and pH 8 exhibited the highest antioxidant capacities on a per gram of meal basis. The antioxidant capacity of the extract obtained without buffer (0.42 μmoles Trolox equivalents/g extract) was lower

than that of the extract obtained at pH 6 (0.49 µmoles Trolox equivalents/g extract), but similar to that of the extract obtained at pH 4 (0.48 µmoles Trolox equivalents/g extract). Lindeboom and Wanasundara (2007) observed higher phenolics contents in extracts from *Sinapis alba* seed at pHs above 9 and below 5 using Tris-HCl buffer. Comparing the total phenolics content and the TEAC value for extracts (Table 4.8), such as the extract obtained by SWE at pH 8 which exhibited the lowest phenolics content (on a per gram of extract basis) but the highest TEAC value (on a per gram of extract basis), demonstrades again that phenolic compounds may not be the only compounds responsible for the antioxidant capacity of extracts from canola meal.

4.4 Evaluation of total phenolics contents and antioxidant properties of crude extracts

4.4.1 Determination of total phenolics content

Under basic conditions, Folin & Ciocalteu's phenol (FC) reagent (yellow colour) reacts with phenolic compounds and, consequently, a phenolate anion is formed by dissociation of a phenolic proton. This sequence of reversible one- or two-electron reduction reactions leads to blue-coloured compounds being formed between phenolate and FC reagent (Huang and others 2005; Prior and others 2005).

The total phenolics contents of meals used in this study ranged from 17.2-17.8 mg sinapic acid equivalents per gram of meal (Table 4.1). These results are in good agreement with published results showing that the total pohenolics content of canola meal is up to 20.4 mg sinapic acid equivalents per gram of meal (Anonymous 2001; Naczk and others 1998; Naczk and others 2002). Hence, it may be concluded that SWE

at 160°C was the most efficient method/condition among other methods/ conditions to extract phenolic compounds from canola meal.

The total phenolics contents of extracts obtained by SWE, ethanolic extraction or hot water extraction of three batches of canola meal are shown in Table 4.9. The three batches of canola yielded similar results.

The total phenolics contents of the various extracts ranged from 28.0-70.9 mg sinapic acid equivalents per gram of extract, or 5.7-16.3 mg sinapic acid equivalents per gram of meal. Naczk and others (2005) reported the total phenolics content of canola hull to be 15 to 136 mg sinapic acid equivalents per gram of extract. The total phenolics contents, on a per gram of extract basis, were highest in ethanolic extracts (52.2-70.9 mg sinapic acid equivalents per gram of extract). Subcritical water extraction at 110°C yielded extracts higher in total phenolics (35.1-41.4 mg sinapic acid equivalents per gram of extract) than did SWE at 160°C (31.8-36.2 mg sinapic acid equivalents per gram of extract) or hot water extraction (28.0-30.4 mg sinapic acid equivalents per gram of extract). This may be due to degradation of phenolic compounds at 160°C, and also to the semi-polar nature of the phenolic compounds in canola meal (Figure 2.6) that would render them more soluble in subcritical water at 110°C than at 160°C. Cacace and Mazza (2006) reported that on a dry weight basis, the most concentrated extracts from flaxseed in terms of total phenolic compounds and protein were obtained by SWE at 140°C rather than at 160°C.

On a per gram of meal basis, extracts from SWE at 160°C had the highest total phenolics content (15.4-16.3 mg sinapic acid equivalents and 89-91% of total phenolics content per gram of meal). These results are in agreement with those of Naczk and others (1998) who determined the total phenolics content of canola meal to be 15.4-18.4

Table 4.9 – Total phenolics contents[a] of extracts obtained by subcritical water, hot water and ethanolic extraction from three batches of canola meal[b]

Sample	*Canola Batch #*	*Extract Yield (g/ g meal)*	*Total content of phenolic compounds (mg sinapic acid equivalents/g extract)*	*Total content of phenolic compounds (mg sinapic acid equivalents/g meal)*
SWE 160[c]	1	0.45 ± 0.01a	33.54 ± 0.37fg	15.42 ± 0.18a
SWE 110[c]	1	0.20 ± 0.01b	36.27 ± 0.45e	7.25 ± 0.09d
Hot water[d]	1	0.21 ± 0.01b	28.04 ± 0.28i	5.88 ± 0.05e
Ethanolic[e]	1	0.15 ± 0.00c	52.22 ± 0.70c	7.83 ± 0.09cd
SWE 160	2	0.48 ± 0.02a	31.82 ± 0.32gh	15.27 ± 0.15a
SWE 110	2	0.20 ± 0.02b	35.19 ± 0.48ef	7.03 ± 0.09d
Hot water	2	0.20 ± 0.00b	28.03 ± 0.41i	5.60 ± 0.07e
Ethanolic	2	0.14 ± 0.00c	70.90 ± 1.25a	9.90 ± 0.13b
SWE 160	3	0.46 ± 0.03a	36.23 ± 0.47e	16.30 ± 0.23a
SWE 110	3	0.20 ± 0.04b	41.46 ± 0.35d	8.29 ± 0.07c
Hot water	3	0.19 ± 0.01b	30.49 ± 0.67h	5.79 ± 0.11e
Ethanolic	3	0.15 ± 0.00c	66.70 ± 1.01b	10.00 ± 0.01b

[a]Values are means of at least three determinations ± standard deviation and are expressed on an as-is moisture basis. Means within a column followed by the same letter are not significantly different ($P > 0.05$).
[b]Samples of canola meal were obtained from ADM, Lloydminster, SK, on May 2005 (batch 1), January 2006 (batch 2) and April 2006 (batch 3).
[c]Subcritical water extraction at 160, 135 or 110°C for 30 min at 6.89 MPa (1000 psi).
[d]Hot water extraction at 80°C for 30 min.
[e]Refluxed with 95% ethanol for 30 min.

mg/g on a moisture-free basis. Ethanolic extracts exhibited higher total phenolics contents (7.8-10.0 mg sinapic acid equivalents per gram of meal) (45-56% of total phenolics content of meal) than did extracts from hot water exaction or SWE at 110^{o}C (5.6-5.9 mg and 7.0-8.3 mg sinapic acid equivalents per gram of meal, respectively) (32-33% and 40-47% of total phenolics contents of meal, respectively), with the exception of batch 1 of canola meal where ethanolic extracts and extracts from SWE at 110^{o}C had similar total phenolics contents on a per gram of meal basis (7.8 mg and 7.2 mg sinapic acid equivalents per gram of meal, respectively). Extracts from SWE at 110^{o}C were higher in total phenolics content than were hot water extracts in all cases.

The total phenolics assay is routinely used as it is simple, sensitive and precise (Stratil and others 2006; Huang and others 2006; Ragaee and others 2006; Liyana-Pathirana and Shahidi 2006; Prior and others 2005). The total phenolics assay actually measures the reducing capacity of a sample (Singleton and others 1999). Numerous publications reported excellent linear correlations between "total phenolic profiles" and "antioxidant capacity" (Huang and others 2005). It should be noted, however, that the total phenolics assay may not be a suitable candidate for measuring the antioxidant capacity of a particular sample. Wanasundara and others (1995) fractionated canola meal and concluded that the total phenolics content was not the critical factor in determining antioxidant activity. A fraction, which had a lower total phenolics content, showed greater antioxidant activity than those fractions with higher phenolics contents.

4.4.2 Trolox equivalent antioxidant capacity (TEAC) assay

In this assay, peroxyl radicals or other oxidants oxidize ABTS to its radical cation, $ABTS^{\bullet+}$ (intense blue colour). The antioxidant capacities of test compounds are

determined by measuring decreases in the blue colour as a result of reaction between the $ABTS^{\bullet+}$ radical and the antioxidant compounds in the sample (Re and others 1999).

The TEAC values of extracts obtained by SWE, ethanolic extraction or hot waterextraction of three batches of canola meal are presented in Table 4.10. On a per gram of extract basis, ethanolic extracts from the three batches of canola meal demonstrated the highest antioxidant capacities (3.62-4.42 µmoles Trolox equivalents per gram of extract). Extracts from SWE at 110°C or 160°C exhibited similar antioxidant capacities (2.63-3.14 µmoles and 2.56-2.85 µmoles Trolox equivalents per gram of extract), with the exception of batch 3 where extracts from SWE at 110°C (3.14 µmoles Trolox equivalents per gram of extract) exhibited higher TEAC values than did extracts from SWE at 160°C (2.85 µmoles Trolox equivalents per gram of extract).

On a per gram of meal basis, extracts from SWE at 160°C had the highest TEAC values (1.29-1.31 µmoles Trolox equivalents per gram of meal), which were approximately twice those of ethanolic extracts and extracts from SWE at 110°C (0.53-0.63 µmoles and 0.54-0.66 µmoles Trolox equivalents per gram of meal, respectively). Hot water extracts had the lowest antioxidant capacities (0.45-0.51 µmoles Trolox equivalents per gram of meal).

The TEAC values of extracts followed the same trend as did results for total phenolics contents (Tables 4.9 and 4.10). Amarowicz and others (2004) stated that total phenolics results can not be expressed as the antioxidant capacity of extracts. There is controversy over whether antioxidant capacity assays measure only phenols, or phenols plus reducing agents plus metal chelators (Prior and others 2005). Data from this study showed a high correlation ($r = 0.975$, $P<0.05$) between total phenolics contents and TEAC assay results (Table 4.15). It can be concluded that in the present study, at least,

Table 4.10 – Trolox equivalent antioxidant capacities (TEAC) values[a] of extracts obtained by subcritical water, hot water and ethanolic extraction from three batches of canola meal[b]

Sample	*Canola Batch #*	*Extract Yield (g/ g meal)*	*TEAC value (µmoles Trolox equivalents/g extract)*	*TEAC value (µmoles Trolox equivalents/g meal)*
SWE 160[c]	1	0.45 ± 0.01a	2.56 ± 0.13ef	1.15 ± 0.06b
SWE 110[c]	1	0.20 ± 0.01b	2.63 ± 0.10ef	0.53 ± 0.02ef
Hot water[d]	1	0.21 ± 0.01b	2.14 ± 0.09g	0.45 ± 0.01f
Ethanolic[e]	1	0.15 ± 0.00c	3.62 ± 0.07b	0.54 ± 0.02de
SWE 160	2	0.48 ± 0.02a	2.68 ± 0.05def	1.29 ± 0.02a
SWE 110	2	0.20 ± 0.02b	2.97 ± 0.07cd	0.59 ± 0.02cd
Hot water	2	0.20 ± 0.00b	2.40 ± 0.05fg	0.48 ± 0.01f
Ethanolic	2	0.14 ± 0.00c	4.12 ± 0.14a	0.58 ± 0.02cd
SWE 160	3	0.46 ± 0.03a	2.85 ± 0.09de	1.31 ± 0.05a
SWE 110	3	0.20 ± 0.04b	3.14 ± 0.08c	0.63 ± 0.02cd
Hot water	3	0.19 ± 0.01b	2.70 ± 0.14def	0.51 ± 0.04f
Ethanolic	3	0.15 ± 0.00c	4.42 ± 0.09a	0.66 ± 0.01c

[a]Values are means of at least three determinations ± standard deviation and are expressed on an as-is moisture basis. Means within a column followed by the same letter are not significantly different ($P > 0.05$).
[b]Samples of canola meal were obtained from ADM, Lloydminster, SK, on May 2005 (batch 1), January 2006 (batch 2) and April 2006 (batch 3).
[c]Subcritical water extraction at 160, 135 or 110°C for 30 min at 6.89 MPa (1000 psi).
[d]Hot water extraction at 80°C for 30 min.
[e]Refluxed with 95% ethanol for 30 min.

the majority of compounds that exhibited antioxidant capacity in the extracts were phenolic compounds.

The TEAC assay has been used frequently to determine the total antioxidant capacities of many food samples, such as fruits, vegetables and spices (Stratil and others 2006; Huang and others 2006; Ragaee and others 2006; Liyana-Pathirana and Shahidi 2006; Prior and others 2005). The TEAC assay is a fairly simple and quick method for determining the antioxidant capacity of a sample, and it does not require special instrumentation or reaction conditions. However, 12-16 hours is required to generate the $ABTS^{\bullet+}$ radical prior to performing the TEAC assay.

4.4.3 β-Carotene-linoleic acid (linoleate) assay

Oxidation results in the bleaching of carotenoids (Huang and others 2005). Oxidation is induced by light, heat or by peroxyl radicals (Ursini and others 1998). Classical antioxidants that can donate hydrogen atoms to quench radicals can prevent or reduce decolorization of carotenoids (Burda and Oleszek 2001).

Heat-induced oxidation of an aqueous emulsion system of β-carotene and linoleic acid was employed as an antioxidant activity assay for SWE, hot water and ethanolic extracts from each batch of canola meal and for different concentrations of BHA (Figures 4.1-4.4 and Table 4.11). All data is reported on a grams per gram of extract basis. Similar results were obtained for the three batches of canola meal.

The average rate (at 15, 30 and 45 min) of β-carotene bleaching is presented as %ANT (Table 4.11). Ethanolic extracts exhibited significantly higher antioxidant activities (92-95%) than did extracts from SWE at 110°C or 160°C (85-87% or 82-86%, respectively) and hot water extracts (76-83%). Hot water extracts exhibited the lowest

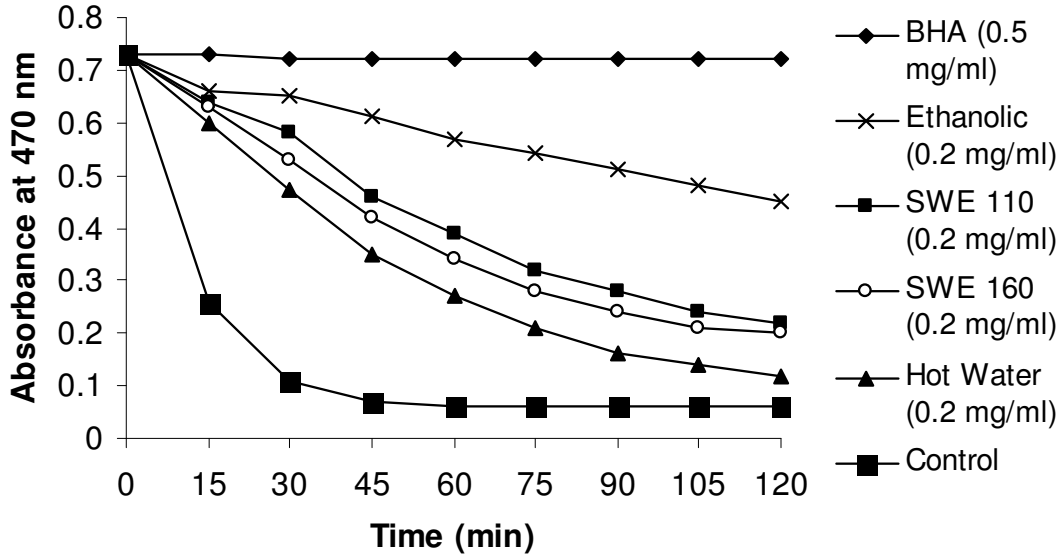


Figure 4.1 – Antioxidant activities of extracts obtained by subcritical water, ethanolic and hot water extraction of canola meal from batch 1 as assessed by the coupled oxidation of β-carotene and linoleic acid over 120 min. Abbreviations: SWE 160 and 110, subcritical water extraction at 160°C and 110°C for 30 min at 6.89 MPa (1000 psi); hot water, extraction at 80°C for 30 min; ethanolic, refluxed in 95% ethanol for 30 min; BHA, butylated hydroxyanisole; batch 1, canola meal received from ADM, Lloydminster, SK, on May 2005. Values are means of at least three determinations. Standard deviations were within 0.01 absorbance unit.

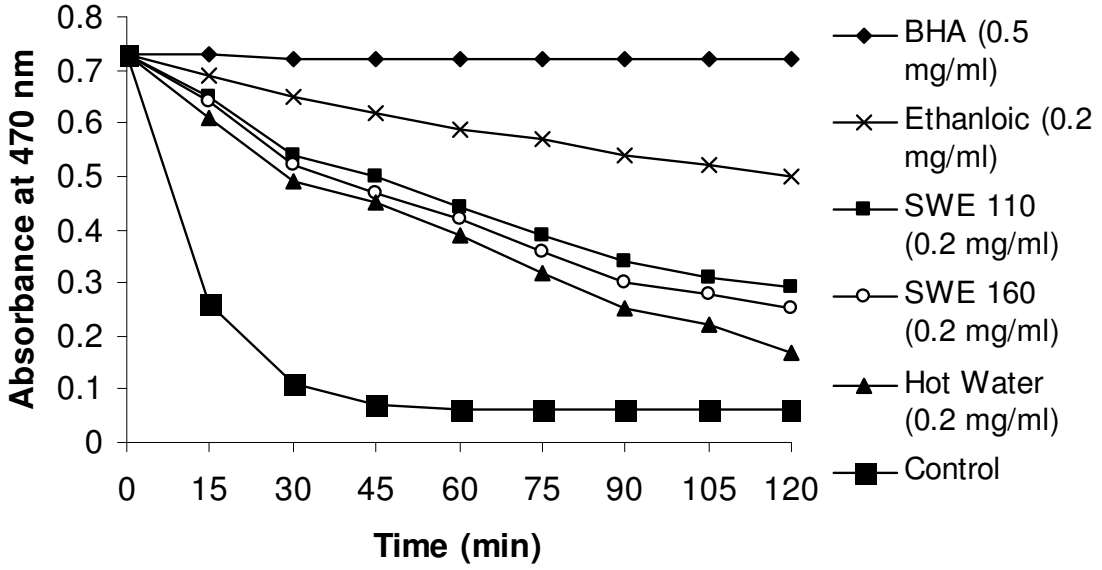


Figure 4.2 – Antioxidant activities of extracts obtained by subcritical water, ethanolic and hot water extraction of canola meal from batch 2 as assessed by the coupled oxidation of β-carotene and linoleic acid over 120 min. Abbreviations: SWE 160 and 110, subcritical water extraction at 160°C and 110°C for 30 min at 6.89 MPa (1000 psi); hot water, extraction at 80°C for 30 min; ethanolic, refluxed in 95% ethanol for 30 min; BHA, butylated hydroxyanisole; batch 2, canola meal received from ADM, Lloydminster, SK, on January 2006. Values are means of at least three determinations. Standard deviations were within 0.01 absorbance unit.

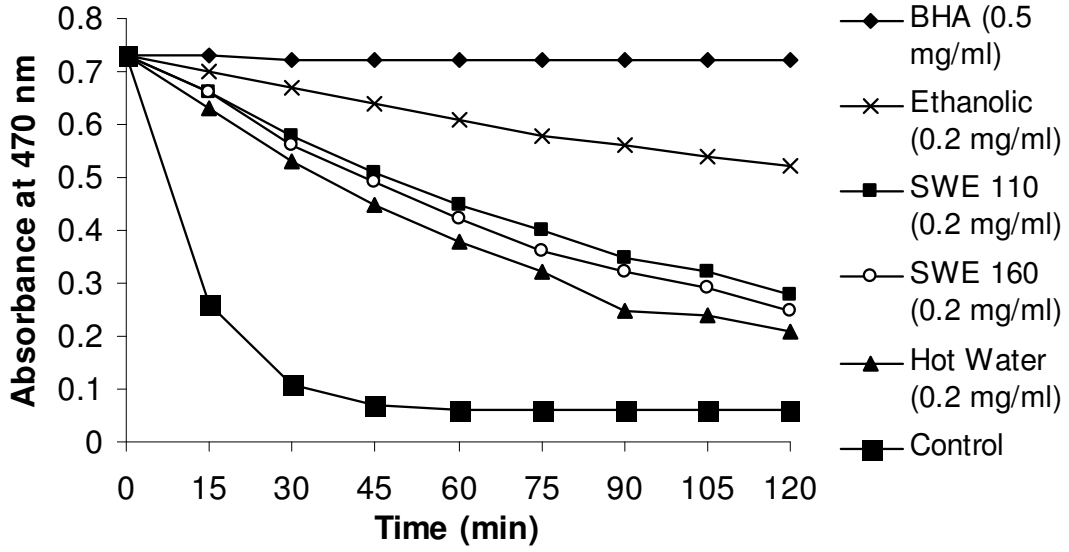


Figure 4.3 – Antioxidant activities of extracts obtained by subcritical water, ethanolic and hot water extraction of canola meal from batch 3 as assessed by the coupled oxidation of β-carotene and linoleic acid over 120 min. Abbreviations: SWE 160 and 110, subcritical water extraction at 160°C and 110°C for 30 min at 6.89 MPa (1000 psi); hot water, extraction at 80°C for 30 min; ethanolic, refluxed in 95% ethanol for 30 min; BHA, butylated hydroxyanisole; batch 3, canola meal received from ADM, Lloydminster, SK, on April 2006. Values are means of at least three determinations. Standard deviations were within 0.01 absorbance unit.

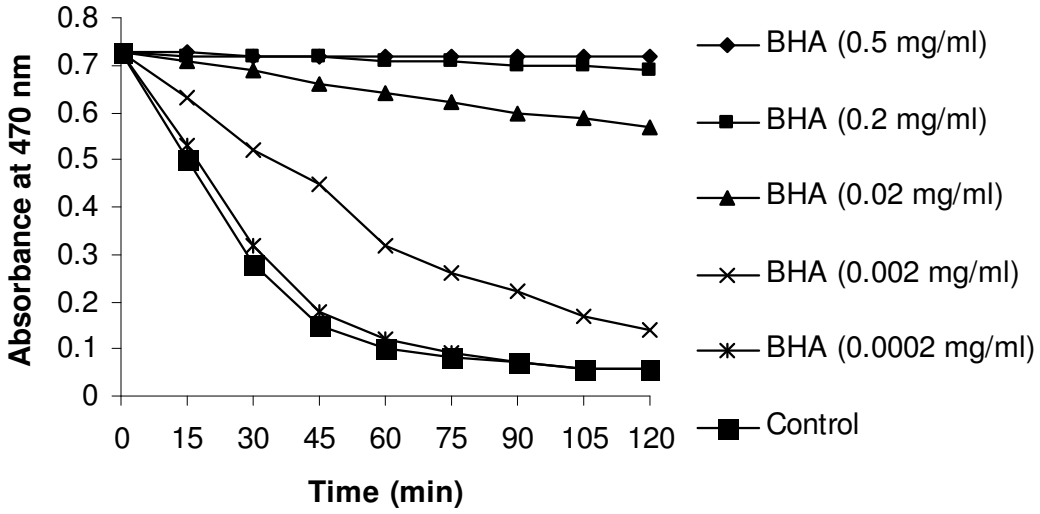


Figure 4.4 – Antioxidant activity of BHA at various concentrations as assessed by the coupled oxidation of β-carotene and linoleic acid over 120 min. Abbreviations: BHA, butylated hydroxyanisole. Values are means of at least three determinations. Standard deviations were within 0.01 absorbance unit.

Table 4.11 – Antioxidant activities of extracts from subcritical water, BHA, hot water and ethanolic extracts of canola meal, and of BHA, in an aqueous emulsion system of β-carotene and linoleic acid [a]

Sample	*Canola Batch #*	*% ANT*	*% AA t=60*	*% AA t=120*
SWE 160 (0.2 mg/ml)	1	82.19 ± 1.35ef	42.65 ± 1.31h	22.06 ± 0.12h
SWE 110 (0.2 mg/ml)	1	85.49 ± 2.49def	50.00 ± 2.01fg	25.00 ± 0.09h
Hot water (0.2 mg/ml)	1	76.04 ± 2.36g	32.35 ± 1.70i	10.29 ± 0.76k
Ethanolic (0.2 mg/ml)	1	92.08 ± 1.87bc	76.47 ± 1.89d	58.82 ± 0.35e
SWE 160 (0.2 mg/ml)	2	83.77 ± 1.03def	54.41 ± 1.37ef	29.41 ± 0.48g
SWE 110 (0.2 mg/ml)	2	85.76 ± 1.91de	57.35 ± 1.26e	35.29 ± 0.89f
Hot water (0.2 mg/ml)	2	80.43 ± 0.67fg	50.00 ± 0.24fg	17.65 ± 0.17i
Ethanolic (0.2 mg/ml)	2	93.88 ± 2.21ab	79.41 ± 2.01cd	66.18 ± 0.36d
SWE 160 (0.2 mg/ml)	3	86.73 ± 1.32cde	54.41 ± 1.05ef	29.41 ± 0.65g
SWE 110 (0.2 mg/ml)	3	87.85 ± 2.14cd	58.82 ± 1.57e	33.82 ± 0.35f
Hot water (0.2 mg/ml)	3	83.02 ± 1.27def	48.53 ± 0.97g	23.53 ± 0.05h
Ethanolic (0.2 mg/ml)	3	95.34 ± 2.76ab	82.35 ± 2.34bc	69.12 ± 0.89c
BHA (0.2 mg/ml)	-	98.17 ± 0.98a	96.88 ± 0.87a	94.12 ± 0.81a
BHA (0.02 mg/ml)	-	93.53 ± 0.91ab	85.94 ± 0.76b	76.47 ± 0.69b
BHA (0.002 mg/ml)	-	65.47 ± 0.72h	35.94 ± 0.56i	13.24 ± 0.22j
BHA (0.0002 mg/ml)	-	13.42 ± 0.23i	4.69 ± 0.04j	1.47 ± 0.04m

[a]Abbreviations: SWE 160 and 110, subcritical water extraction at 160°C and 110°C for 30 min at 6.89 MPa (1000 psi); hot water, extraction at 80°C for 30 min; ethanolic, refluxed in 95% ethanol for 30 min; BHA, butylated hydroxyanisole; %ANT, antioxidant activity based on average rate of β-carotene bleaching at 15, 30 and 45 min; AA, antioxidant activity of extract at t=60 min or t=120 min; batch 1, 2 and 3, canola meal received from ADM, Lloydminster, SK, received on May 2006, January 2006 and April 2006, respectively. Values are means of at least three determinations ± standard deviation. Values in the same column followed by the same letter are not significantly different (P > 0.05).

%ANT values, similar to or slightly lower than those of extracts from SWE at 110°C and 160°C of batches 2 and 3 (85-87% and 83-86%, respectively). Ethanolic extracts had %ANT values similar to those of BHA at a concentration of 0.02 mg/ml (93%). Hot water and SWE extracts had higher %ANT values than did BHA at a concentration of 0.002 mg/ml (65%), but lower than BHA at a concentration of 0.02 mg/ml.

Comparing %AA values after 60 min of incubation to corresponding %ANT values, the antioxidant activity of SWE extracts decreased from 85% to 52%, and that of ethanolic extracts decreased from 93% to 79%. At the same incubation time, extracts from SWE at 110°C and 160°C exhibited similar results (57-59% and 54%, respectively), except for batch 1 where extracts from SWE at 110°C (50%) showed higher activity than did extracts from SWE 160°C (42%). Ethanolic extracts exhibited the highest activity (76-82%). The lowest antioxidant activity was observed with hot water extracts (32-50%). The same scenario was observed after 120 min of incubation, with the exception of extracts from SWE. Extracts from SWE at 110°C exhibited higher antioxidant activity (25-35%) after 120 min than did extracts from SWE at 160°C (22-29%), with the exception of batch 1 where extracts from SWE at 110°C and 160°C exhibited similar antioxidant activities (22% and 25%, respectively). Ethanolic extracts exhibited the smallest decrease in antioxidant activity after a 120-min period (Figures 4.1-4.3). After 60 or 120 min incubation, all of the extracts exhibited higher antioxidant activities than did BHA at a concentration of 0.002 mg/ml (36% or 13%, after 60 or 120 min incubation, respectively), but lower activities than did BHA at a concentration of 0.02 mg/ml (86% or 76%, after 60 or 120 min incubation, respectively) (Table 4.11). Amarowicz and others (2000) reported that crude extracts of canola hulls exhibited antioxidant capacity as measured by the β-carotene-linoleic acid (linoleate) assay.

Data from the β-carotene-linoleic acid (linoleate) assay were presented as %ANT, %AA t=60 min and %AA t=120 (Table 4.11). Linear correlation coefficients (r) values between %ANT and %AA t=60, and between %ANT and %AA t=120, were 0.840 and 0.660, respectively ($P<0.05$). The correlation coefficient between %AA t=60 min and %AA t=120 was 0.952, $P<0.05$. Since the antioxidant activities of extracts between 60 min and 120 min of incubation were highly correlated, 60 min of incubation would have been sufficient for this test.

Beta-carotene bleaching requires two hours of measurement at 15-min intervals. In addition, emulsion preparation is critical which challenges the reproducibility of the method. Beta-carotene bleaching can occur by multiple pathways, so interpretation of results may be complicated (Prior and others 2005). In addition, there are no standard formats for expressing results, hence studies may utilize different methods for calculating inhibition kinetics (Burda and Oleszek 2001; Amarowicz and others 2004; Prior and others 2005).

4.4.4 Radical-scavenging activity (RSA) assay

The $DPPH^{\bullet}$ radical is a stable organic nitrogen radical, is commercially available and has a deep purple colour (Prior and others 2005). The RSA assay measures the reducing capacity of antioxidants toward $DPPH^{\bullet}$. Upon reduction, the colour of $DPPH^{\bullet}$ solution fades. Consequently, test compounds with high antioxidant activity result in a rapid decline in the absorbance of the $DPPH^{\bullet}$ solution (Amarowicz and others 2004).

Results are presented for the percent scavenging of $DPPH^{\bullet}$ by SWE, hot water and ethanolic extracts from three batches of canola meal (Figures 4.5-4.7 and Table

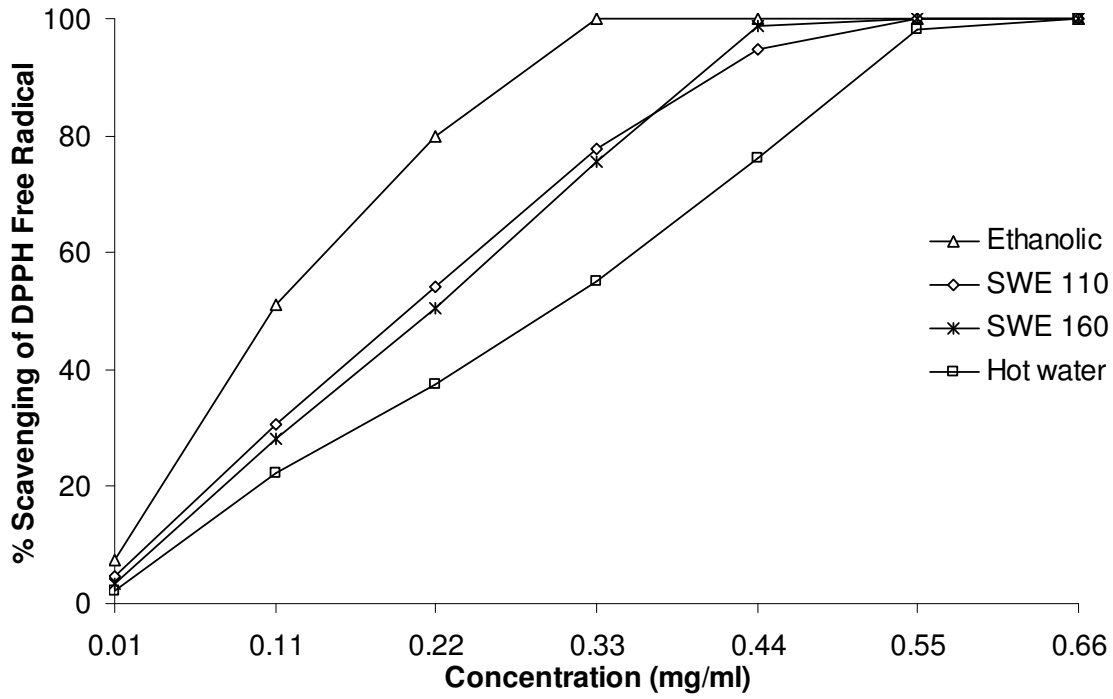


Figure 4.5 – Scavenging effect of extracts obtained by subcritical water, ethanolic and hot water extraction from batch 1 of canola meal at various concentrations on 2, 2-diphenyl-1-picrylhydrazyl free radical (DPPH•). Abbreviations: SWE 160 and 110, subcritical water extraction at 160°C and 110°C for 30 min at 6.89 MPa (1000 psi); hot water, extraction at 80°C for 30 min; ethanolic, refluxed in 95% ethanol for 30 min; batch 1, canola meal received from ADM, Lloydminster, SK, on May 2005. Values are means of at least three determinations. Standard deviations were within ± 3%.

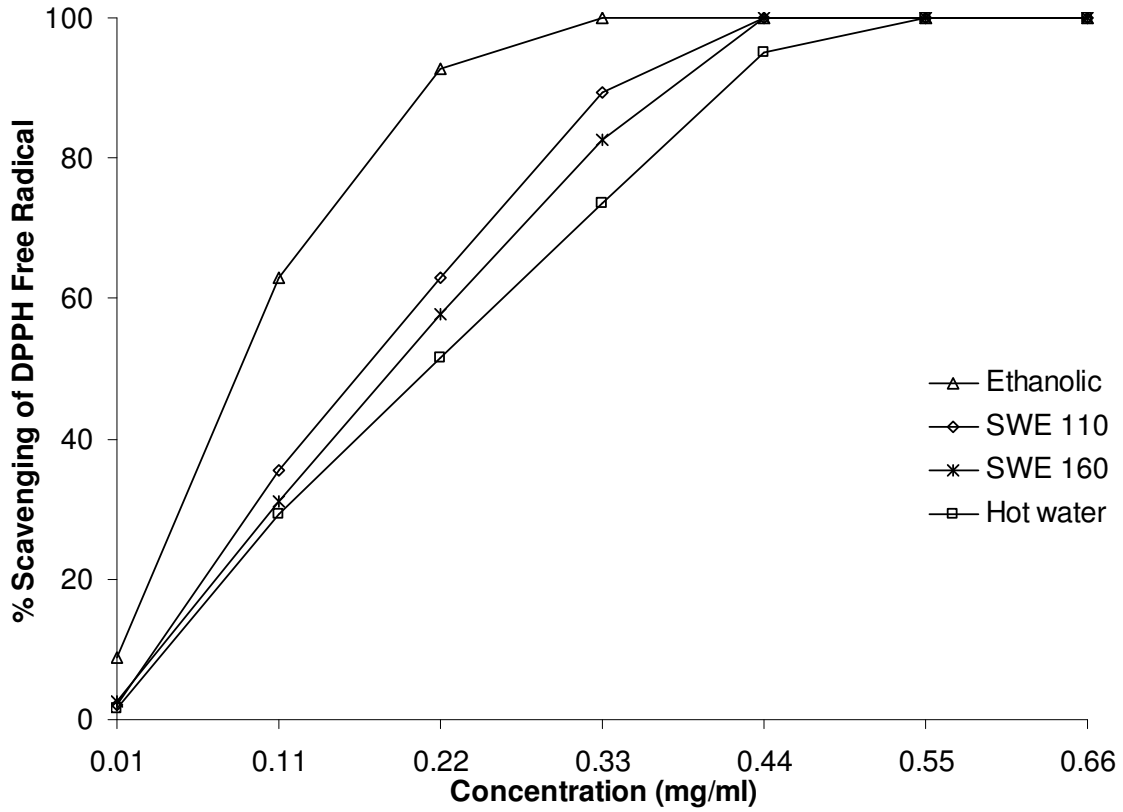


Figure 4.6 – Scavenging effect of extracts obtained by subcritical water, ethanolic and hot water extraction from batch 2 of canola meal at various concentrations on 2, 2-diphenyl-1-picrylhydrazyl free radical (DPPH•). Abbreviations: SWE 160 and 110, subcritical water extraction at 160°C and 110°C for 30 min at 6.89 MPa (1000 psi); hot water, extraction at 80°C for 30 min; ethanolic, refluxed in 95% ethanol for 30 min; batch 2, canola meal received from ADM, Lloydminster, SK, on January 2006. Values are means of at least three determinations. Standard deviations were within ± 3%.

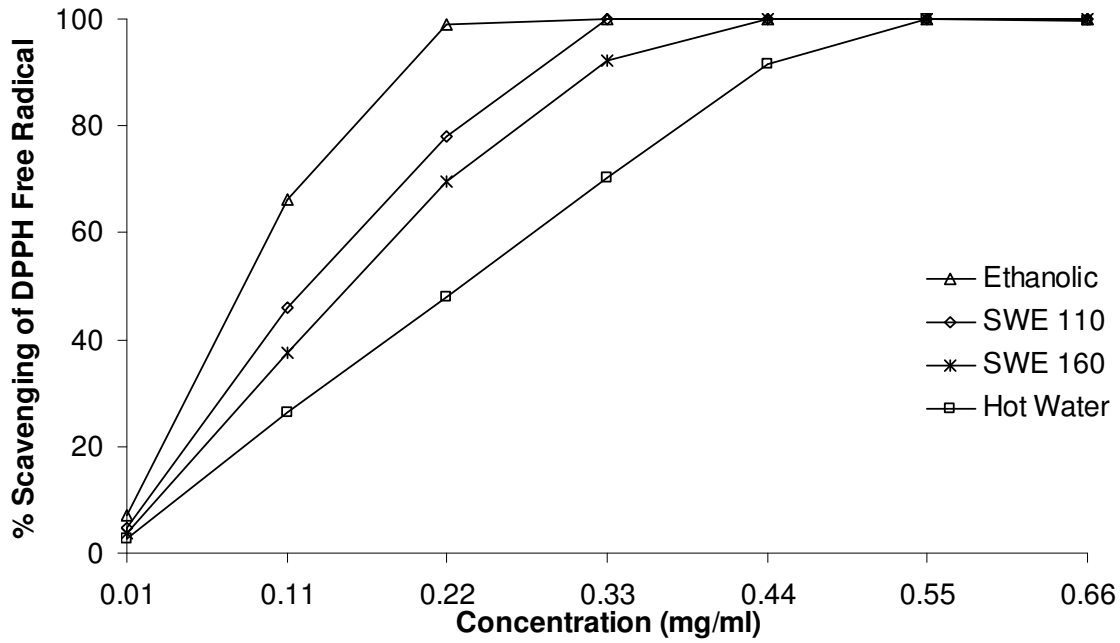


Figure 4.7 – Scavenging effect of extracts obtained by subcritical water, ethanolic and hot water extraction from batch 3 of canola meal at various concentrations on 2, 2-diphenyl-1-picrylhydrazyl free radical (DPPH•). Abbreviations: SWE 160 and 110, subcritical water extraction at 160^{o}C and 110^{o}C for 30 min at 6.89 MPa (1000 psi); hot water, extraction at 80^{o}C for 30 min; ethanolic, refluxed in 95% ethanol for 30 min; batch 3, canola meal received from ADM, Lloydminster, SK, on April 2006. Values are means of at least three determinations. Standard deviations were within ± 3%.

Table 4.12 – Slopes[a] of dose-response curves and concentrations[b] at 100% scavenging of 2, 2-diphenyl-1-picrylhydrazyl free radical (DPPH•) of extracts obtained by subcritical water, hot water and ethanolic extraction of canola meal[b]

Sample	*Canola Batch #*	*Slope (% Scavenging activity per mg/ml)*	*Concentration at 100% scavenging activity (mg/ml)*
SWE 160[c]	1	191.48 ± 5.74c	0.49 ± 0.01c
SWE 110[c]	1	186.10 ± 5.58c	0.49 ± 0.01c
Hot water[d]	1	159.32 ± 4.78d	0.61 ± 0.02a
Ethanolic[e]	1	298.70 ± 8.96a	0.31 ± 0.01e
SWE 160	2	228.82 ± 6.86b	0.42 ± 0.01d
SWE 110	2	230.82 ± 6.92b	0.40 ± 0.01d
Hot water	2	156.75 ± 4.70d	0.56 ± 0.02b
Ethanolic	2	299.96 ± 9.00a	0.29 ± 0.01e
SWE 160	3	231.73 ± 6.95b	0.39 ± 0.01d
SWE 110	3	301.82 ± 9.05a	0.31 ± 0.01e
Hot water	3	159.18 ± 4.78d	0.57 ± 0.02ab
Ethanolic	3	302.64 ± 9.08a	0.28 ± 0.01e

[a]Values are means of at least three determinations ± standard deviation and are expressed on an as-is moisture basis. Means within a column followed by the same letter are not significantly different ($P > 0.05$).
[b]Samples of canola meal were obtained from ADM, Lloydminster, SK, on May 2005 (batch 1), January 2006 (batch 2) and April 2006 (batch 3).
[c]Subcritical water extraction at 160 or 110°C for 30 min at 6.89 MPa (1000 psi).
[d]Hot water extraction at 80°C for 30 min.
[e]Refluxed in 95% ethanol for 30 min.

4.12). The figures show the dose-response curves for the radical-scavenging capacity of the various extracts. Each data point in Figures 4.5-4.7 is the ratio of the decrease in absorbance of $DPPH^{\bullet}$ solution containing canola meal extracts to the absorbance of the $DPPH^{\bullet}$ solution without extract (at 519 nm), expressed as a percentage, for any particular concentration of canola meal extract (Amarowicz and others 2004). Ethanolic extracts showed the highest radical-scavenging activity for all batches of canola meal, reaching 100% scavenging at extract concentrations of 0.28-0.31 mg/ml. Extracts from SWE at 110°C and 160°C showed similar radical-scavenging activity, with 100% scavenging of $DPPH^{\bullet}$ at the respective extract concentrations of 0.49 mg/ml and 0.49 mg/ml for batch 1, 0.42 mg/ml and 0.40 mg/ml for batch 2, and 0.39 mg/ml and 0.31 mg/ml for batch 3. Hot water extracts, which exhibited the lowest radical-scavenging activity, exhibited 100% scavenging at extract concentrations of 0.56-0.61 mg/ml. Amarowicz and others (2000) reprted that crude extracts of canola hulls possessed a "marked" scavenging effect on the $DPPH^{\bullet}$ radical.

Dose-response curves for ethanolic extracts exhibited the highest slopes (Table 4.12) for the three batches of canola meal (298-302 % scavenging activity per mg/ml). Extracts from SWE at 110°C and 160°C exhibited similar slopes within batches 1 (186 and 191% scavenging activity per mg/ml, respectively) and 2 (230 and 228% scavenging activity per mg/ml, respectively), but not among batches 1 and 2 of canola meal. In batch 3, the extract from SWE at 160°C exhibited a slope (231% scavenging activity per mg/ml) similar to those of extracts from SWE at 110°C and 160°C of batch 2, but not to that of the extract from SWE at 110°C of batch 3 (301% scavenging activity per mg/ml), which was similar to that of ethanolic extracts. Dose-response curves for hot water

extracts exhibited the lowest slopes for the three batches of canola meal (156-159% scavenging activity per mg/ml).

In the RSA assay, slopes from radical-scavenging activity curves were highly correlated ($r = 0.982$, $P<0.05$) with 100% scavenging activities of extracts (Table 4.12), showing that measuring either slope values or concentration at 100% scavenging activity values would have been sufficient to interpret the results from this test.

The DPPH test is simple and quick (within 30 min). The $DPPH^{\bullet}$ radical can be prepared in less than an hour which is very rapid compared to the $ABTS^{\bullet+}$ radical preparation (12-16 h). The DPPH test has been in widespread use in antioxidant capacity screening, probably due to the simplicity of the equipment required (Huang and others 2005). Sánchez-Moreno (2002) suggested the DPPH test as an easy and accurate method for measuring the antioxidant capacity of fruit and vegetable juices or extracts.

4.4.5 Reducing power assay

In the reducing power assay, the reductants (antioxidants) in the test compounds reduce the Fe^{3+}/ferricyanide complex [$FeCl_3/K_3Fe(CN)_6$] to the ferrous (Fe^{2+}) form (Chung and others 2002). Therefore, depending on the reducing power of the test compounds, the yellow colour of the test solution changes to various shades of green or blue (Amarowicz and others 2004).

The dose-response curves for reducing powers of extracts from SWE at 110°C or 160°C, hot water extraction and ethanolic extraction of three batches of canola meal are presented in Figures 4.8-4.10. The slopes of the dose-response reducing power curves are presented in Table 4.13. The dose-response curves for ethanolic extracts exhibited

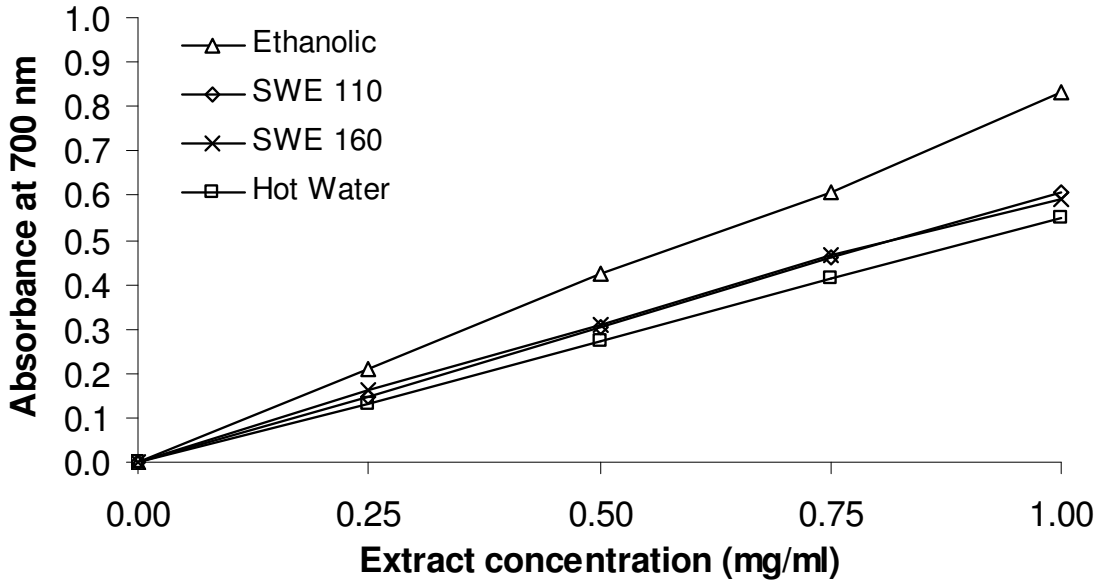


Figure 4.8 – Reducing powers of various concentrations of extracts obtained by subcritical water, ethanolic and hot water extraction of canola meal from batch 1. Abbreviations: SWE 160 and 110, subcritical water extraction at 160^{o}C and 110^{o}C for 30 min at 6.89 MPa (1000 psi); hot water, extraction at 80^{o}C for 30 min; ethanolic, refluxed in 95% ethanol for 30 min; batch 1, canola meal received from ADM, Lloydminster, SK, on May 2005. Values are means of at least three determinations. Standard deviations were within ± 0.02 absorbance unit.

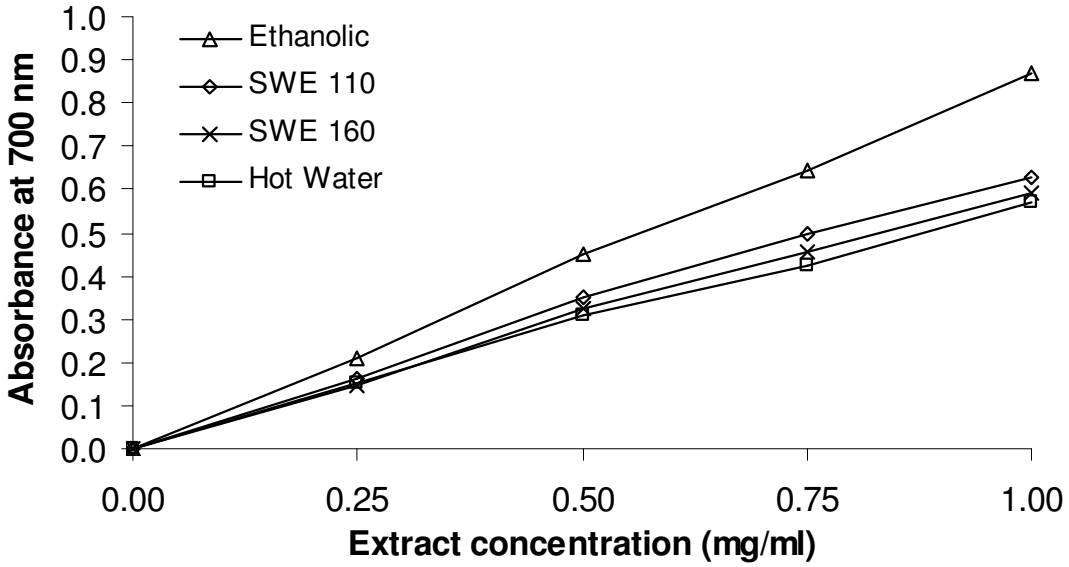


Figure 4.9 – Reducing powers of various concentrations of extracts obtained by subcritical water, ethanolic and hot water extraction of canola meal from batch 2. Abbreviations: SWE 160 and 110, subcritical water extraction at 160^{o}C and 110^{o}C for 30 min at 6.89 MPa (1000 psi); hot water, extraction at 80^{o}C for 30 min; ethanolic, refluxed in 95% ethanol for 30 min; batch 2, canola meal received from ADM, Lloydminster, SK, on January 2006. Values are means of at least three determinations. Standard deviations were within ± 0.02 absorbance unit.

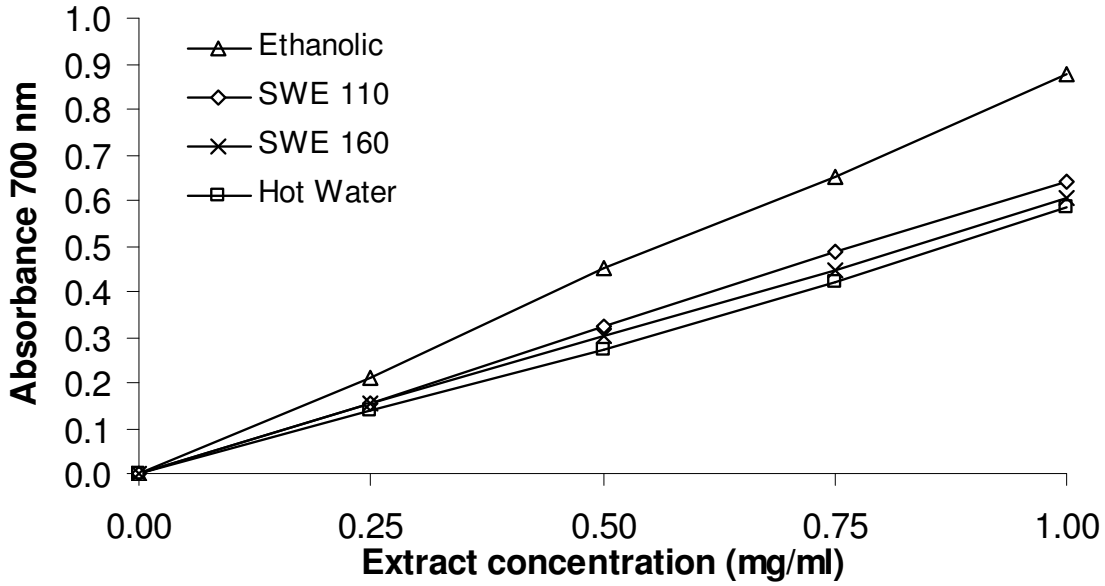


Figure 4.10 – Reducing powers of various concentrations of extracts obtained by subcritical water, ethanolic and hot water extraction of canola meal from batch 3. Abbreviations: SWE 160 and 110, subcritical water extraction at 160^{o}C and 110^{o}C for 30 min at 6.89 MPa (1000 psi); hot water, extraction at 80^{o}C for 30 min; ethanolic, refluxed in 95% ethanol for 30 min; batch 3, canola meal received from ADM, Lloydminster, SK, on April 2006. Values are means of at least three determinations. Standard deviations were within ± 0.02 absorbance unit.

Table 4.13 – Slopes[a] of dose-response reducing power curves of aqueous extracts from subcritical water, hot water and ethanolic extraction of canola meal[b]

Sample	*Canola Batch #*	*Slope (absorbance unit per mg/ml)*
SWE 160[c]	1	0.60 ± 0.02b
SWE 110[c]	1	0.61 ± 0.02b
Hot water[d]	1	0.55 ± 0.02b
Ethanolic[e]	1	0.83 ± 0.02a
SWE 160	2	0.60 ± 0.02b
SWE 110	2	0.64 ± 0.02b
Hot water	2	0.56 ± 0.02b
Ethanolic	2	0.87 ± 0.03a
SWE 160	3	0.60 ± 0.02b
SWE 110	3	0.65 ± 0.02b
Hot water	3	0.58 ± 0.02b
Ethanolic	3	0.88 ± 0.03a

[a]Values are means of at least three determinations ± standard deviation and are expressed on an as-is moisture basis. Means within a column followed by the same letter are not significantly different ($P > 0.05$).
[b]Samples of canola meal were obtained from ADM, Lloydminster, SK, on May 2005 (batch 1), January 2006 (batch 2) and April 2006 (batch 3).
[c]Subcritical water extraction at 160 or 110°C for 30 min at 6.89 MPa (1000 psi).
[d]Hot water extraction at 80°C for 30 min.
[e]Refluxed in 95% ethanol for 30 min.

higher slopes (0.83-0.88 absorbance unit per mg/ml) than did those of extracts from SWE at 110°C or 160°C (0.61-0.65 or 0.60 absorbance unit per mg/ml, respectively) or from hot water extraction (0.55-0.58 absorbance unit per mg/ml) of all batches of canola meal. The slopes of dose–response reducing power curves for hot water extracts were similar to those of extracts from SWE at 110°C or 160°C for all batches of canola meal, which might be due to the presence of the same reductants (*i.e.*, antioxidants) responsible for the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Amarowicz and others (2000) reported that crude extracts of canola hulls exhibited significant capacities to reduce the Fe^{3+}/ferricyanide complex to the ferrous form.

The reducing power assay is considered to be a sensitive method for the "semi-quantitative" determination of dilute concentrations of polyphenolics which participate in the redox reaction (Amarowicz and others 2004). The reducing power assay is easy and quick, but substantial solution preparation (see section 3.4.5) is required prior to the test.

4.4.6 Bulk stripped oil model system

The bulk stripped oil model system is based on monitoring the oxidative deterioration of marine oils or vegetable oils (Decker and others 2005). The presence of conjugated dienes (CD) in a PUFA can be interpreted as an indication of autoxidation of fatty acid moieties (Corongiu and Banni 1994). Thus, oil samples containing antioxidants exhibit a lower content of CDs compared to oil samples without antioxidant supplementation after a specific period.

Table 4.14 presents CD values at day 20, slopes of CD value curves and slopes of extinction value ($E^{1\%}_{1cm}$) curves over 20 days of storage derived from a stripped corn

Table 4.14 – CD values at day 20[a] and slopes[a] of conjugated diene (CD) value curves and extinction value ($E^{1\%}_{1cm}$) curves from the stripped corn oil model system in the presence of BHA and extracts from subcritical water, hot water and ethanolic extraction at temperatures of 110 and 160°C of canola meal[b] at a concentration of 200 ppm

Sample	*Canola Batch #*	*CD value at day 20 (µmol/g)*	*Slope from CD value (µmol/g per day)*	*Slope from $E^{1\%}_{1cm}$ ($E^{1\%}_{1cm}$ per day)*
SWE160[c]	1	46.99 ± 0.91b	0.87 ± 0.03de	0.48 ± 0.01d
SWE110[c]	1	47.12 ± 1.22b	0.83 ± 0.03e	0.47 ± 0.01de
Hot Water[d]	1	39.84 ± 1.27d	1.03 ± 0.03c	0.59 ± 0.02c
Ethanolic[e]	1	42.64 ± 1.53cd	0.96 ± 0.03cd	0.54 ± 0.02c
BHA[f]	1	30.15 ± 0.99e	1.31 ± 0.04b	0.76 ± 0.02b
Control	1	55.16 ± 1.34a	0.72 ± 0.02fg	0.41 ± 0.01f
SWE160	2	46.81 ± 1.00bc	0.86 ± 0.03e	0.48 ± 0.01d
SWE110	2	46.87 ± 1.62bc	0.83 ± 0.03e	0.48 ± 0.01d
Hot Water	2	39.02 ± 1.12d	1.03 ± 0.03c	0.59 ± 0.02c
Ethanolic	2	41.94 ± 1.42bd	0.96 ± 0.03cd	0.55 ± 0.02c
BHA	2	30.85 ± 1.05e	1.35 ± 0.04b	0.77 ± 0.02ab
Control	2	52.56 ± 1.12a	0.73 ± 0.02fg	0.42 ± 0.01ef
SWE160	3	46.81 ± 1.00bc	0.84 ± 0.03e	0.48 ± 0.01d
SWE110	3	47.92 ± 1.71b	0.81 ± 0.02ef	0.48 ± 0.01d
Hot Water	3	39.50 ± 1.77d	1.02 ± 0.03c	0.59 ± 0.02c
Ethanolic	3	42.64 ± 1.57cd	1.01 ± 0.03c	0.55 ± 0.02c
BHA	3	31.53 ± 0.99e	1.47 ± 0.04a	0.82 ± 0.02a
Control	3	54.96 ± 1.40a	0.68 ± 0.02g	0.40 ± 0.01f

[a]Values are means of at least three determinations ± standard deviation and are expressed on an as-is moisture basis. Means within a column followed by the same letter are not significantly different (P > 0.05).
[b]Samples of canola meal were obtained from ADM, Lloydminster, SK, on May 2005 (batch 1), January 2006 (batch 2) and April 2006 (batch 3).
[c]Subcritical water extraction at 160 or 110°C for 30 min at 6.89 MPa (1000 psi).
[d]Hot water extraction at 80°C for 30 min.
[e]Refluxed in 95% ethanol for 30 min.
[f]Butylated hydroxyanisole.

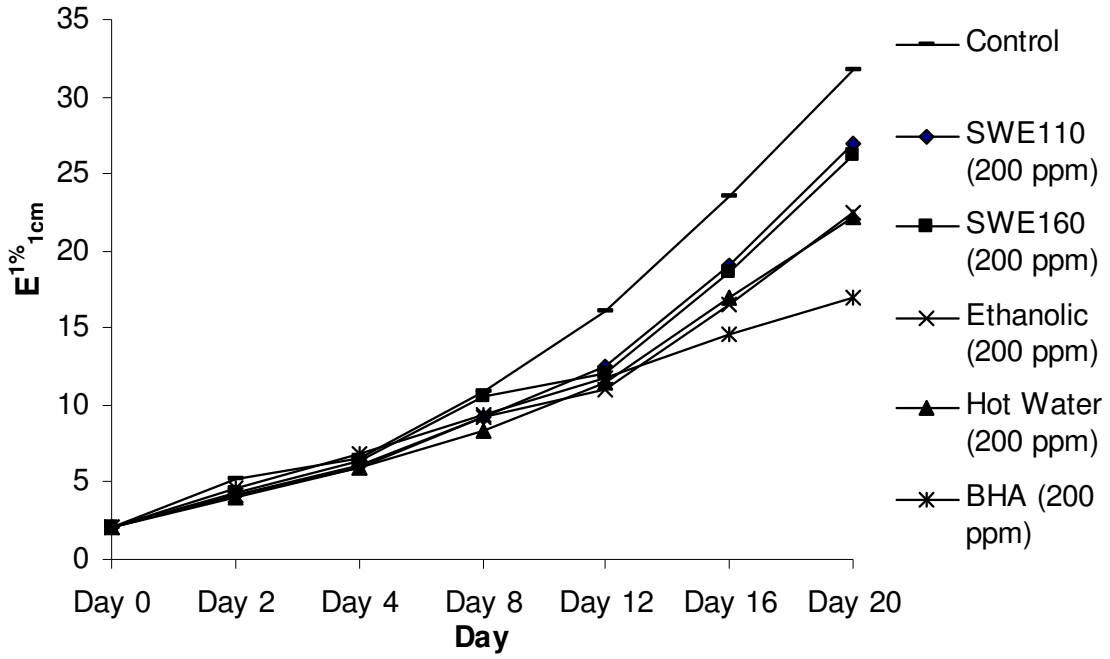


Figure 4.11 – Extinction values from the stripped corn oil model system over 20 days in the presence of canola meal extracts at 200 ppm obtained by hot water, ethanolic and subcritical water extraction of batch 1. Abbreviations: SWE 160 and 110, subcritical water extraction at 160°C and 110°C for 30 min at 6.89 MPa (1000 psi); hot water, extraction at 80°C for 30 min; ethanolic, refluxed in 95% ethanol for 30 min; batch 1, canola meal received from ADM, Lloydminster, SK, on May 2005; $E^{1\%}_{1cm,}$ extinction value. Values are means of at least three determinations. Standard deviations are within ± 3 $E^{1\%}_{1cm}$.

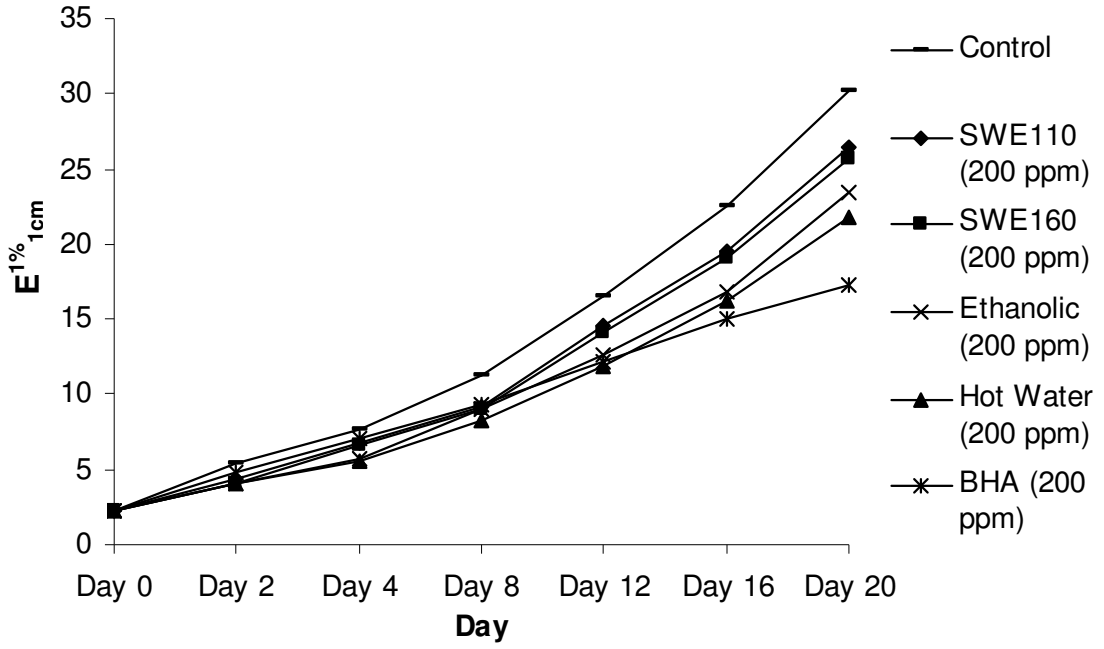


Figure 4.12 – Extinction values from the stripped corn oil model system over 20 days in the presence of canola meal extracts at 200 ppm obtained by hot water, ethanolic and subcritical water extraction of batch 2. Abbreviations: SWE 160 and 110, subcritical water extraction at 160°C and 110°C for 30 min at 6.89 MPa (1000 psi); hot water, extraction at 80°C for 30 min; ethanolic, refluxed in 95% ethanol for 30 min; batch 2, canola meal received from ADM, Lloydminster, SK, on January 2006; $E^{1\%}_{1cm,}$ extinction value. Values are means of at least three determinations. Standard deviations are within ± 3 $E^{1\%}_{1cm}$.

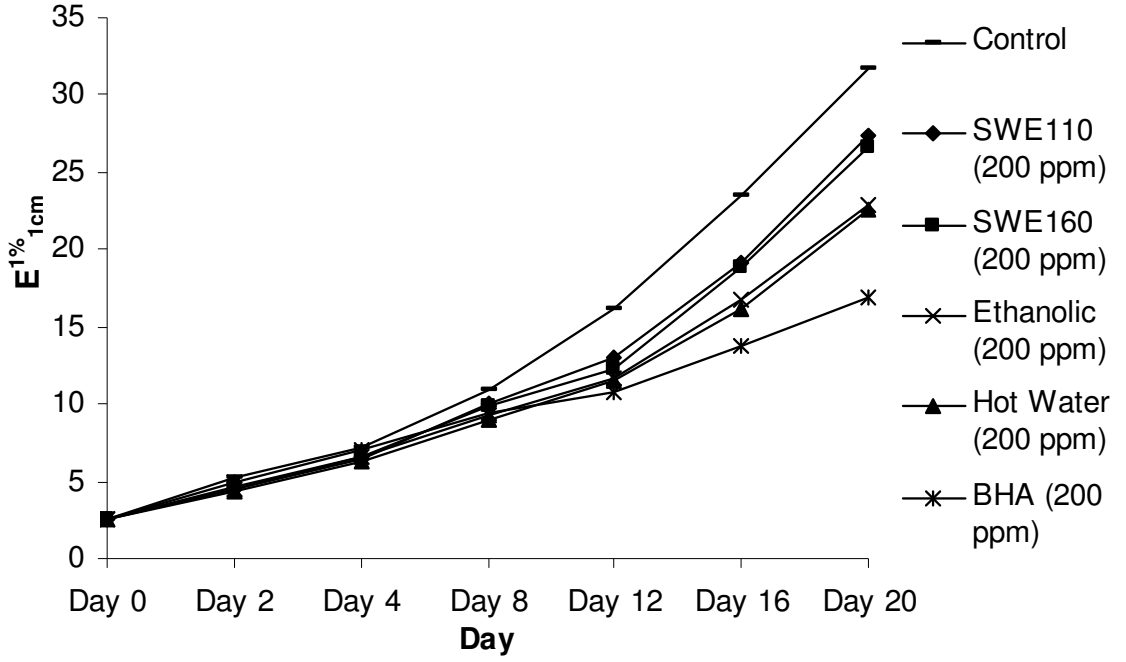


Figure 4.13 – Extinction values from the stripped corn oil model system over 20 days in the presence of canola meal extracts at 200 ppm obtained by hot water, ethanolic and subcritical water extraction of batch 3. Abbreviations: SWE 160 and 110, subcritical water extraction at 160°C and 110°C for 30 min at 6.89 MPa (1000 psi); hot water, extraction at 80°C for 30 min; ethanolic, refluxed in 95% ethanol for 30 min; batch 3, canola meal received from ADM, Lloydminster, SK, on April 2006; $E^{1\%}_{1cm,}$ extinction value. Values are means of at least three determinations. Standard deviations are within ± 3 $E^{1\%}_{1cm}$.

oil model system for SWE, hot water and ethanolic extracts from three batches of canola meal. Figures 4.11-4.13 show $E^{1\%}_{1cm}$ from extracts from SWE, hot water and hot water extractions of all batches of canola meal.

Extracts from SWE at 110°C and 160°C exhibited similar results with respect to CD values at day 20 (46.87-47.92 and 46.81-46.99 μmol/g, respectively) for the three batches of canola meal. Ethanolic extracts had CD values at day 20 (41.94-42.64 μmol/g) similar to those of hot water extracts (39.02-39.84 μmol/g). Control samples had the highest (52.56-55.16 μmol/g), and BHA samples (30.15-31.53 μmol/g) the lowest, CD values at day 20. Hot water and ethanolic extracts exhibited lower CD values than did extracts from SWE at 110°C and 160°C, but higher CD values than did BHA samples. Wanasundara and Shahidi (1994) reported that the antioxidant activity of a crude ethanolic extract of canola meal (500 and 1000 ppm) against the oxidation of canola oil was equivalent to that of TBHQ (200 ppm), and stronger than that of BHA (200 ppm), BHT (200 ppm) or BHA/BHT/monoglyceride citrate (MGC) (250 ppm) on a mass basis. The concentrations of crude extracts in oil used in this study were 200 ppm. Therefore, ethanolic extracts exhibited lower antioxidant activity against the oxidation of stripped corn oil than did BHA at 200 ppm.

Slopes from CD value curves and extinction value curves (Figures 4.11-4.13) exhibited trends similar to that observed for CD values at day 20. This was to be expected since these slope values were calculated from CD values obtained at 4, 8, 12, 16 and 20 days.

Slopes from 20-day CD value curves were highly correlated with extinction values ($r = 0.971$, $P<0.05$) and CD values ($r = 0.965$, $P<0.05$) of extracts on day 20. The extinction values of extracts were highly correlated with CD values of extracts on day 20

(r = 0.996, P<0.05). Thus, using CD values of extracts on day 20 would express the differences in antioxidant capacity between extracts without any need to prepare, or to calculate slopes from, CD values or extinction value curves.

The bulk stripped oil model system represents a simple method requiering no specialized instrumentation. However, oils need to be stripped of all minor oil constituents (to prevent any possible interference) before adding test compounds having antioxidant capability. In addition, it takes several days to complete this test.

4.4.7 Correlations between antioxidant assays

Linear correlation coefficients between the results of the various antioxidant tests are presented in Table 4.15. Significant correlations (P<0.05) were detected in all cases for the total phenolics assay, the TEAC assay, the β-carotene assay, the DPPH assay and the reducing power assay. Similar correlations were reported by Liyana-Pathirana and Shahidi (2006), Stratil and others (2006) and Amarowicz and others (2000). All antioxidant assays used in this study [either ET-based, HAT-based or mixed (section 2.3)] measured the radical scavenging capacity of primary antioxidants (section 2.1.1), with the exception of the bulk stripped oil assay (Huang and others 2005, Prior and others 2005). This accounts for the significant correlations between antioxidant tests, with the exception of the bulk stripped oil assay which measured the preventive antioxidant capacity of secondary antioxidants (section 2.1.1) with respect to the formation of hydroperoxides from polyunsaturated fatty acids in stripped oil (Gordon 2001b). Direct scavenging of free radicals is not involved in the bulk stripped oil assay (Decker and others 2005).

Table 4.15 - Correlation coefficients among the total phenolics content, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay, the Trolox equivalent antioxidant capacity (TEAC) assay, β-carotene-linoleic acid (linoleate) model assay, the reducing power assay and the bulk stripped oil model system (CD value) for extracts obtained by subcritical water extraction, hot water extraction and ethanolic extraction of canola meal

Tests	*Total phenolics content*	*TEAC value*	*β-carotene*	*DPPH*	*Reducing power*
TEAC value	0.971				
β-carotene	0.890	0.949			
DPPH	-0.812	-0.864	-0.902		
Reducing power	0.972	0.968	0.898	-0.822	
CD value	-0.079^{ns}	0.029^{ns}	0.164^{ns}	-0.104^{ns}	-0.128^{ns}

^{ns}not significant when $P < 0.05$ at level of 5% when n=12.

The TEAC assay is a simple and rapid test and does not require special instrumentation or reaction conditions. However, 12-16 hours is required to generate the $ABTS^{\bullet+}$ radical prior to performing the TEAC assay. This assay not only detected the difference in antioxidant capacity between extracts from SWE, ethanolic and hot water extraction but also between extracts from SWE at 110°C and 160°C. Therefore, the TEAC is recommended as the best choice of antioxidant capacity assay.

5.0 SUMMARY AND CONCLUSIONS

5.1 Summary

Organic solvents are commonly used for extractions. However, in recent years there has been a move toward the use of more "environmental-friendly" solvents. The objective of this study was to evaluate subcritical water extraction (SWE) as a means of effectively extracting natural antioxidants from commercial canola meal. Extractions were conducted using a home-built apparatus assembled at the Saskatoon Research Centre of Agriculture and Agri-Food Canada. Subcritical water extraction at temperatures of 110, 135 and 160°C was examined. The effects of temperature, pH, pressure and time on the efficiency of SWE of canola meal were investigated. Hot water (80°C) and ethanolic (95% ethanol) extractions were conducted for comparative purposes. The antioxidant capacities of extracts obtained from the three extraction methods were assessed by several antioxidant and radical-scavenging assays, namely the total phenolics assay (using Folin & Ciocalteu's phenol reagent), the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging method, the Trolox equivalent antioxidant capacity (TEAC) method, the β-carotene-linoleic acid (linoleate) model system, the reducing power assay and the stripped oil model system. The crude protein contents of extracts also were determined.

Three batches of canola meal were extracted in this study. Each exhibited a composition typical of that of commercial Canadian canola meal. In extracts obtained by SWE, the highest total phenolics contents and TEAC values on a per gram of extract

basis were observed at an extraction at temperature of 110^{o}C, and on a per gram of meal basis, an extraction temperature of 160^{o}C. Extraction pressure had no effect on the efficacy of SWE. Although using buffered water (pH 2-8) increased extract yields from SWE, it had adverse effects on the total phenolics contents and the TEAC values of extracts. At either 110^{o}C or 160^{o}C, no increase in extract yield, total phenolics content or antioxidant activity was observed at extraction times longer than 25-30 min. Extracts from SWE at 160°C exhibited higher yields and crude protein contents than did extracts from SWE 110°C or hot water extracts. Extracts from SWE at 110°C and hot water extracts exhibited similar yields and crude protein contents, and higher yields and crude protein contents than did ethanolic extracts. Similar results were obtained for extracts from the three batches of canola meal.

Antioxidant capacity measurements of extracts exhibited similar results for the three batches of canola meal. Ethanolic extracts exhibited significantly higher antioxidant capacities and total phenolics contents than did SWE or hot water extracts. The antioxidant capacity, as measured by the TEAC assay, and the total phenolics contents of extracts from SWE at 110°C were higher than those of extracts from SWE at 160°C. Extracts from SWE at 110°C and 160°C exhibited similar results for the β-carotene-linoleic acid (linoleate) and RSA assays. Hot water extracts exhibited the lowest antioxidant capacities and total phenolics contents, with the exception of the reducing power assay where similar values were obtained for hot water and SWE extracts. Extracts from SWE of canola meal at a concentration of 2.0 mg/ml exhibited higher antioxidant activities than did BHA at a concentration of 0.002 mg/ml in an aqueous emulsion system of β-carotene and linoleic acid. Hot water and ethanolic

extracts in the stripped corn oil system exhibited higher protection against oxidation than did extracts from SWE.

Results for the antioxidant capacity assays were significantly correlated, with the exception of results from the bulk stripped oil assay.

5.2 Conclusions

The hypothesis of this thesis was that SWE was a water-based extraction technology that may be able to extract natural antioxidants from defatted canola meal as effectively as ethanol extraction, and more effectively than conventional hot water extraction. The findings of this study partially reject this hypothesis, since ethanolic extraction was more efficient than SWE in extracting natural antioxidants from canola meal. Subcritical water extraction did extract natural antioxidants from canola meal more efficiently than did conventional hot water extraction.

Any of the antioxidant capacity assays employed in this study could have been employed to assess the antioxidant capacity of the canola meal extracts, with the exception of the bulk stripped oil system. The TEAC is recommended as the best choice of antioxidant capacity assay since the TEAC assay is a simple and rapid test and does not require special instrumentation or reaction conditions. However, 12-16 hours is required to generate the $ABTS^{\bullet+}$ radical prior to performing the TEAC assay.

The results of this study can be extrapolated to Canadian canola meal in general, due to the similarity in composition of the canola meal samples extracted in this study to that of typical Canadian canola meal.

6.0 FUTURE STUDIES

In any future studies derived from this study, the following research will provide a better understanding of SWE utilization with respect to the extracted material:

- Characterization and identification of antioxidant compounds in subcritical water extracts from canola meal
- Characterization and identification of proteins in subcritical water extracts from canola meal
- Purification of subcritical water extracts from canola meal
- Exploring applications for SWE extracts of canola meal
- Studying the effect of HCl (mixed with water as solvent) in extraction of tannins and lignans from canola meal by SWE

7.0 REFERENCES CITED

Aeschbach R, Rossi P. 1996. Novel approach for antioxidant extraction. In: Pokorný J, Yanishlieva N, Gordon M, editors. Antioxidants in food: practical application. Cambridge England: Woodhead Publishing Limited. P311-41.

Amarowicz R, Fornal J, Karamac M, Shahidi F. 2001. Antioxidant activity of extracts of phenolic compounds from rapeseed oil cakes. J Food Lipids 8:65-74.

Amarowicz R, Karamac M, Shahidi F. 2003. Antioxidant activity of phenolic fractions of lentil (*Lens culinaris*). J Food Lipids 10:1-10.

Amarowicz R, Naczk M, Shahidi F. 2000. Antioxidant activity of crude tannins of canola and rapeseed hulls. J Am Oil Chem Soc 77:957-61.

Amarowicz R, Naczk M, Shahidi F. 2000. Antioxidant activity of various fractions of non-tannin phenolics of canola hulls. J Agric Food Chem 48:2755-9.

Amarowicz R, Pegg RB, Rahimi-Moghaddam P, Barl B, Weil JA. 2004. Free-radical scavenging capacity and antioxidant activity of selected plant species from the Canadian prairies. Food Chem 84:551-62.

Amarowicz R, Raab B, Shahidi F. 2003. Antioxidant activity of phenolic fractions of rapeseed. J Food Lipids 10:51-62.

Anonymous. 2006. Beta-carotene-2D-skeletal. http://en.wikipedia.org/wiki/Image:Beta-carotene-2D-skeletal.png#filehistory (accessed October 26, 2007)

Anonymous. 2001. Canola meal nutrient composition. In: Canola meal feed industry guide. 3rd ed. P8-11.

Anonymous. 2000. Phosphate buffer calculation. http://www.columbia.edu/~scb2001/tools/phosphate/phosphate.html (accessed May 25, 2006)

Anonymous. 1997. The protein handbook. Lincoln NE: Isco, Inc. P67.

Arts MJTJ, Dallinga JS, Voss HP, Haenen GRMM, Bast A. 2004. A new approach to assess the total antioxidant capacity using the TEAC assay. Food Chem 88:567-70.

Awika JM, Rooney LW, Wu X, Prior RL, Cisneros-Zevallos L. 2003. Screening methods to measure antioxidant activity of sorghum (*Sorghum bicolor*) and sorghum products. J Agric Food Chem 51:6657-62.

Ayala RS, Castro MDL. 2001. Continuous subcritical water extraction as a useful tool for isolation of edible essential oils. Food Chem 75:109-13.

Banias C, Oreopoulou V, Thomopoulos CD. 1992. The effect of primary antioxidants and synergists on the activity of plant extracts in lard. J Am Oil Chem Soc 69:520-4.

Barlow SM. 1990. Toxicological aspects of antioxidants used as food additives. In: Hudson BJF, editor. Food antioxidants. New York: Elsevier. P253-307.

Baum B, Perun AL. 1962. Antioxidant efficiency versus structure. Soc Plast Eng Trans 2:250-7.

Bergeron C, Gafner S, Clausen E, Carrier DJ. 2005. Comparison of the chemical composition of extracts from *Scutellaria lateriflora* using accelerated solvent extraction and supercritical fluid extraction versus standard hot water or 70% ethanol extraction. J Agric Food Chem 53:3076-80.

Blokhina O, Virolainen E, Fagerstedt KV. 2003. Antioxidants, oxidative damage and oxygen deprivation stress: a review. Ann Botany 91:179-94.

Bondet V, Brand-Williams W, Berset C. 1997. Kinetics and mechanisms of antioxidant activity using the $DPPH^{\bullet}$ free radical method. Fat Sci Technol 30:609-15.

Brand-Williams W, Cuvelier ME, Berset C. 1995. Use of a free radical method to evaluate antioxidant activity. Fat Sci Technol 28:25-30.

Burda S, Oleszek W. 2001. Antioxidant and antiradical activities of flavonoids. J Agric Food Chem 49:2774-9.

Cacace JE, Mazza G. 2006. Pressurized low polarity water extraction of lignans from whole flaxseed. J Food Eng 77:1087-95.

Cai R, Hettiarachchy NS, Jalaluddin M. 2003. High performance liquid chromatography determination of phenolic constituents in 17 varieties of cowpeas. J Agric Food Chem 51:1623-7.

Cano A, Acosta M, Arnao MB. 2000. A method to measure antioxidant activity in organic media: application to lipophilic vitamins. Redox Rep 5:365-70.

Cano A, Alcaraz O, Acosta M, Arnao MB. 2002. On-line antioxidant activity determination: comparison of hydrophilic and lipophilic antioxidant activity using the $ABTS^{\bullet+}$ assay. Redox Rep 7:103-9.

Cano A, Hernández-Ruíz J, García-Cánovas F, Acosta M, Arnao MB. 1998. An end-point method for estimation of the total antioxidant activity in plant material. Phytochem Anal 9:196-202.

Carabias-Martínez R, Rodríguez-Gonzalo E, Revilla-Ruiz P, Hernández-Méndez J. 2005. Pressurized liquid extraction in the analysis of food and biological samples. J Chromatogr A 1089:1-17.

Chen PY, Tu YX, Wu CT, Jong TT, Chang CMJ. 2004. Continuous hot pressurized solvent extraction of 1,1-diphenyl-2-picrylhydrazyl free radical scavenging compounds from Taiwan yams (*Dioscorea alata*). J Agric Food Chem 52:1945-9.

Choi MPK, Chan KKC, Leung HW, Huie CW. 2003. Pressurized liquid extraction of active ingredients (ginsenosides) from medicinal plants using non-ionic surfactant solutions. J Chromatogr A 983:153-62.

Chung Y, Chang C, Chao W, Lin C, Chou S. 2002. Antioxidative activity and safety of the 50 ethanolic extract from red bean fermented by *Bacillus subtilis* IMR-NK1. J Agric Food Chem 50:2454-8.

Corongiu FP, Banni S. 1994. Detection of conjugated dienes by second derivative ultraviolet spectrophotometry. Methods Enzymol 233:303-10.

Crego A, Ibáñez E, García E, de Pablos RR, Señoráns FJ, Reglero G, Cifuentes A. 2004. Capillary electrophoresis separation of rosemary antioxidants from subcritical water extracts. Eur Food Res Technol 219:549-55.

Curren MSS, King JW. 2002. New sample preparation technique for the determination of avoparcin in pressurized hot water extracts from kidney samples. J Chromatogr A 954:41-9.

Cuvelier ME, Richard H, Berset C. 1992. Comparision of the antioxidative activity of some acid phenols structure activity relationship. Biosci Biotech Biochem 56:324-5.

Decker EA, Warner K, Richards MP, Shahidi F. 2005. Measuring antioxidant effectiveness in food. J Agric Food Chem 53:4303-10.

Duh PD, Yen GC. 1997. Antioxidant efficacy of methanolic extracts of peanut hulls in soybean and peanut oils. J Am Oil Chem Soc 74:745-8.

Folin O, Ciocalteu V. 1927. On tyrosine and tryptophane determinations in proteins. J Biol Chem 73:627-50.

García-Marino M, Rivas-Gonzalo JC, Ibáñez E, García-Moreno C. 2006. Recovery of catechins and proanthocyanidins from winery by-products using subcritical water extraction. Anal Chim Acta 563:44-50.

Ghodsvali A, Vosoughi Khodaparast MMH and Diosady LL. 2005. Preparation of canola protein materials using membrane technology and evaluation of meals functional properties. Food Res Int 38:223–31.

Gordon MH. 2001a. Measuring antioxidant activity. In: Pokorný J, Yanishlieva N, Gordon M, editors. Antioxidants in food: practical application. Cambridge England: Woodhead Publishing Limited. P73-84.

Gordon MH. 2001b. The development of oxidative rancidity in foods. In: Pokorný J, Yanishlieva N, Gordon M, editors. Antioxidants in food: practical application. Cambridge England: Woodhead Publishing Limited. P8-21.

Güçlü-Üstündağ Ö, Balsevich J, Mazza G. 2007. Pressurized low polarity water extraction of saponins from cow cockle seed. J Food Eng 80:619-30.

Hall C. 2001. Sources of natural antioxidants: oilseeds, nuts, cereals, legumes, animal products and microbial sources. In: Pokorný J, Yanishlieva N, Gordon M, editors. Antioxidants in food: practical applications. Cambridge England: Woodhead Publishing Limited. P159-209.

Halliwell B, Aeschbach R, Loliger J, Aruoma OI. 1995. The characterization of antioxidants. Food Chem Toxicol 33:601-17.

Herrero M, Arráez-Román D, Segura A, Kenndler E, Gius B, Raggi MA, Ibáñez E, Cifuentes A. 2005. Pressurized liquid extraction-capillary electrophoresis-mass spectrometry for the analysis of polar antioxidants in rosemary extracts. J Chromatogr A 1084:54-62.

Herrero M, Cifuentes A, Ibáñez E. 2006. Sub- and supercritical fluid extraction of functional ingredients from different natural sources: plants, food by-products, algae and microalgae: a review. Food Chem 98:136-48.

Herrero M, Martín-Álvarez PJ, Señoráns FJ, Cifuentes A, Ibáñez E. 2005. Optimization of accelerated solvent extraction of antioxidants from *Spirulina platensis* microalga. Food Chem 93:417-23.

Ho CL, Cacace J, Mazza G. 2007. Extraction of lignans, proteins and carbohydrates from flaxseed meal with pressurized low polarity water. Food Sci Technol 40:1637-47.

Huang D, Ou B, Prior RL. 2005. The chemistry behind antioxidant capacity assays. J Agric Food Chem 53:1841-56.

Ibáñez E, Herrero M, Martin-Álvarez PJ, Señoráns FJ, Reglero G, Cifuentes A. 2006. Accelerated solvent extraction: a new procedure to obtain functional ingredients from natural sources. In: Turner C, editor. Modern extraction techniques: food agricultural sample. Washington DC: American Chemical Society. P65-78.

Ibáñez E, Kubátová A, Señoráns FJ, Cavero S, Reglero G, Hawthorne SB. 2003. Subcritical water extraction of antioxidant compounds from rosemary plants. J Agric Food Chem 51:375-82.

Ishige K, Schubert D, Sagara Y. 2001. Flavonoids protect neuronal cells from oxidant stress by three distinct mechanisms. Free Radical Biol Med 30:433-46.

Jayasinghe C, Gotoh N, Aoki T, Wada S. 2003. Phenolics composition and antioxidant activity of sweet basil (*Ocimum basilicum* L.). J Agric Food Chem 51:4442-9.

Ju ZY, Howard LR. 2005. Subcritical water and sulfured water extraction of anthocyanins and other phenolics from dried red grape skin. J Food Sci 70:270-6.

Katsube N, Iwashita K, Tsushida T, Yamaki K, Kobori M. 2003. Induction of apoptosis in cancer cells by bilberry (*Vaccinium myrtillus*) and the anthocyanins. J Agric Food Chem 51:68-75.

Kim JW, Mazza G. 2006. Optimization of extraction of phenolic compounds from flax shives by pressurized low polarity water. J Agric Food Chem 54:7575-84.

King JW. 2006. Pressurized water extraction: resources and techniques for optimizing analytical applications. In: Turner C, editor. Modern extraction techniques: food agricultural sample. Washington, DC : American Chemical Society. P79-95.

Kitts DD, Hu C. 2005. Biological and chemical assessment of antioxidant activity of sugar-lysine model Maillard reaction products. Ann NY Acad Sci 1043:501-12.

Klockeman DM, Toledo R, Sims KA. 1997. Isolation and characterization of defatted canola meal protein. J Agric Food Chem, 45:3867-70.

Kozlowska H, Nowak H, Zadernowski R. 1988. Rapeseed hulls characteristics. Fat Sci Technol 90:216-9.

Krygier K, Sosulski F, Hogge L. 1982. Free, esterified, and insoluble bond phenolic acids. Composition of phenolic acids in rapeseed flour and hulls. J Agric Food Chem 30:334-6.

Kubátová A, Lagadec AJM, Miller DJ, Hawthorne SB. 2001. Selective extraction of oxygenates from savory and peppermint using subcritical water. Flavour Fragrance J 16:64-73.

Lacki K, Duvnjak Z. 1999. A method for the decrease of phenolic content in commercial canola meal using an enzyme preparation secreted by the white rot fungus *Trametes versicolor*. Biotechnol Bioeng 62:422-33.

Lachance PA, Nakat Z, Jeong WS. 2001. Antioxidants: an integrative approach. Nutr 17:835-8.

Lamm LJ, Yang Y. 2003. Off-line coupling of subcritical water extraction with subcritical water chromatography via a sorbent trap and thermal desorption. Anal Chem 75:2237-42.

Lemańska K, Szymusiak H, Tyrakowska B, Zieliński R, Soffers AEMF, Rietjens IMCM. 2001. The influence of pH on antioxidant properties and the mechanism of antioxidant action of hydroxyflavones. Free Radical Biol Med 31:869-81.

Li B, Yang Y, Gan Y, Eaton CD, He P, Jones AD. 2000. On-line coupling of subcritical water extraction with high-performance liquid chromatography via solid-phase trapping. J Chromatogr A 873:175-84.

Lide DR. 2001. Handbook of chemistry and physics. Boca Raton, FL: CRC Press. 81st ed. P8-121.

Lindeboom N, Wanasundara PKJPD. 2007. Interference of phenolic compounds in *Brassica napus*, *Brassica rapa* and *Sinapis alba* seed extracts with the Lowry protein assay. Food Chem 104: 30-8.

Liyana-Pathirana CM, Shahidi F. 2006. Antioxidant properties of commercial soft and hard winter wheats (*Triticum aestivum L.*) and their milling fractions. J Sci Food Agric 86:477-85.

López-Sebastián S, Ramos E, Ibáñez E, Bueno JM, Ballester L, Tabera J, Reglero G. 1998. Dearomatization of antioxidant rosemary extracts by treatment with supercritical carbon dioxide. J Agric Food Chem 46:13-9.

Madhujith T, Naczk M, Shahidi F. 2004. Antioxidant activity of common beans (*Phaseolus vulgaris* L.). J Food Lipids 11:220-33.

Maranz S, Wiesman Z, Garti N. 2003. Phenolic constituents of shea (*Vitellaria paradoxa*) kernels. J Agric Food Chem 51:6268-73.

Miller GJ, Quackenbush FW. 1957a. Antioxidant activity of phenols as related to effects of substituent groups. J Am Oil Chem Soc 34:404-7.

Miller GJ, Quackenbush FW. 1957b. A comparision of alkylated phenols as antioxidants for lard. J Am Oil Chem Soc 34:249-50.

Miller HE. 1971. A simplified method for the evaluation of antioxidants. J Am Oil Chem Soc 48:91.

Miller NJ, Rice-Evans C, Davies MJ, Gopinathan V, Milner A. 1993. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. Clin Sci 84:407-12.

Naczk M, Amarowicz R, Sullivan A, Shahidi F. 1998. Current research developments on polyphenolics of rapeseed. Food Chem 62:489-502.

Naczk M, Pegg RB, Zadernowski R, Shahidi F. 2005. Radical scavenging activity of canola hull phenolics. J Am Oil Chem Soc 82:255-60.

Naczk M, Pink J, Zadernowski R, Pink D. 2002. Multivariate model for the prediction of total phenolic acids in crude extracts of polyphenols from canola and rapeseed meals: a preliminary study. J Am Oil Chem Soc 79:759-62.

Naczk M, Shahidi F. 2006. Phenolics in cereals, fruits and vegetables: occurrence, extraction and analysis. J Pharm Biomed Anal 41:1523-42.

Naczk M, Shahidi F. 2004. Extraction and analysis of phenolics in food. J Chromatogr A 1054:95-111.

Naczk M, Wanasundara PKJPD, Shahidi F. 1992. Facile spectrophotomettric quantification method of sinapic acid in hexane-extracted and methanol ammonia water-treated mustard and rapeseed meals. J Agric Food Chem 40:444-8.

Naczk M, Zadernowski R, Shahidi F. 2003. Antioxidant capacity of crude phenolic extracts from canola hulls. J Am Chem Soc 226:63-7.

Nakamura Y, Tsuji S, Tonogai Y. 2003. Method for analysis of tannic acid and its metabolites in biological samples: Application to tannic acid metabolism in the rat. J Agric Food Chem 51:331-9.

Nijveldt RJ, van Nood E, van Hoorn DEC, Boelens PG, van Norren K, van Leeuwen PAM. 2001. Flavonoids: a review of probable mechanisms of action and potential applications. Am J Clin Nutr 74:418-25.

Nomura T, Kikuchi M, Kubodera A, Kawakami Y. 1997. Proton-donative antioxidant activity of fucoxanthin with 1,1-diphenyl-2-picrylhydrazyl (DPPH). Biochem Mol Biol Int 42:361-70.

Nowak H, Kujawa K, Zadernowski R, Roczniak B, Kozlowska H. 1992. Antioxidative and bactericidal properties of phenolic compounds in rapeseeds. Fat Sci Techol 94:149-52.

Odaci D, Timur S, Pazarlioglu N, Montereali MR, Vastarella W, Pilloton R, Telefoncu A. 2007. Determination of phenolic acids using *Trametes versicolor laccase*. Talanta 71:312-7.

Pedroche J, Yust MM, Lqari H, Girón-Calle J, Alaiz M, Vioque J, Millán F. 2004. *Brassica carinata* protein isolates: chemical composition, protein characterization and improvement of functional properties by protein hydrolysis. Food Chem 88:337-46.

Pegg RB. 2005. Lipid oxidation/stability. In: Wrolstad RE, Acree TE, Decker EA, Penner MH, Ried DS, Schwartz SJ, Shoemaker CF, Smith DM, Sporns P, editors. Handbook of food analytical chemistry. Hoboken, NJ.: John Wiley & Sons, Inc. P513-29.

Pink D, Naczk M, Baskin K, Shahidi F. 1994. Theoretical analysis of ultraviolet-visible spectra of various phenolic acid fractions of canola. J Agric Food Chem 42:1317-22.

Piñeiro Z, Palma M, Barroso CG. 2004. Determination of catechins by means of extraction with pressurized liquids. J Chromatogr A 1026:19-23.

Pokorný J, Korczak J. 2001. Preparation of natural antioxidants. In: Pokorný J, Yanishlieva N, Gordon M, editors. Antioxidants in food: practical application. Cambridge England: Woodhead Publishing Limited. P311-41.

Pongnaravane B, Goto M, Sasaki M, Anekpankul T, Pavasant P, Pavasant P, Shotipruk A. 2006. Extraction of anthraquinones from roots of Morinda citrifolia by pressurized hot water: Antioxidant activity of extracts. J Supercritical Fluids 37:390-6.

Porter WL, Black ED, Drolet AM. 1989. Use of polyamide oxidative fluorescence test on lipid emulsions: contrast in relative effectiveness of antioxidants in bulk versus dispersed systems. J Agric Food Chem 37:615-24
.

Pratt D, Hudson BJF. 1990. Natural antioxidants not exploited commercially. In: Hudson BJF, editor. Food antioxidants. New York: Elsevier. P171-91.

Prior RL, Wu XL, Schaich K. 2005. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. J Agric Food Chem 53:4290-302.

Ragaee S, Abdel-Aal EM, Noaman M. 2006. Antioxidant activity and nutrient composition of selected cereals for food use. Food Chem 98:32-8.

Ramos L, Kristenson EM, Brinkman UAT. 2002. Current use of pressurised liquid extraction and subcritical water extraction in environmental analysis. J Chromatogr A 975:3-29.

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biol Med 26:1231-7.

Rodríguez-Meizoso I, Marin FR, Herrero M, Señorans FJ, Reglero G, Cifuentes A, Ibáñez E. 2006. Subcritical water extraction of nutraceuticals with antioxidant activity from oregano. Chemical and functional characterization. J Pharm Biomed Anal 41:1560-5.

Rovio S, Hartonen K, Holm Y, Hiltunen R, Riekkola ML. 1999. Extraction of clove using pressurized hot water. Flavour Fragrance J 14:399-404.

Sánchez-Moreno C. 2002. Review: methods used to evaluate the free radical scavenging activity in foods and biological systems. Food Sci Technol Int 8:121-37.

Sartor V, Henderson PT, Schuster GB. 1999. Radical cation transport and reaction in RNA/DNA hybrid duplexes: effect of global structure on reactivity. J Am Chem Soc 121:11027-33.

Schantz MM. 2006. Pressurized liquid extraction in environmental analysis. Anal Bioanal Chem 386:1043-7.

Schwarz K, Bertelsen G, Nissen LR, Gardner PT, Heinonen MI, Hopia A, Huynh-Ba T, Lambelet P, McPhail D, Skibsted LH, Tijburg L. 2001. Investigation of plant extracts for the protection of processed foods against lipid oxidation. Comparison of antioxidant assays based on radical scavenging, lipid oxidation and analysis of the principal antioxidant compounds. Eur Food Res Techol 212:319-28.

Shahidi F. 2000. Antioxidant factors in plant foods and selected oilseeds. Biofactors 13:179-85.

Shahidi F, Wanasundara UN, Amarowicz R, Naczk M. 1995. Natural antioxidants from canola meal. Proceedings of the Ninth International Rapeseed Congress, Cambridge, U.K. 3:873-5.

Shahidi F, Naczk M. 1992. An overview of phenolics of canola and rapeseed: chemical, sensory and nutritional significance. J Am Oil Chem Soc 69:917-24.

Shahidi F, Naczk M. 1989. Effects of processing on the content of condensed tannins in rapeseed meals. J Food Sci 54:1082-3.

Shotipruk A, Kiatsongserm J, Pavassnt P, Goto M, Sasaki M. 2004. Pressurized hot water extraction of anthraquinones from the roots of *Morinda citrifolia*. Biotechnol Prog 20:1872-5.

Shan B, Cai YZ, Sun M, Corke H. 2005. Antioxidant capacity of 26 spice extracts and characterization of their phenolic constituents. J Agric Food Chem 53:7749-59.

Shi H, Noguchi N, Niki E. 2001. Introducing natural antioxidants. In: Pokorný J, Yanishlieva N, Gordon M, editors. Antioxidants in food: practical application. Cambridge England: Woodhead Publishing Limited. P147-58.

Singleton VL, Orthofer R, Lamuela- Raventós RM. 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Methods enzymol 299:152-78.

Smith RM. 2002. Extractions with superheated water. J Chromatogr A 975:31-46.

Stashenko EE, Cervantes M, Combariza Y, Fuentes H, Martínez JR. 1999. HRGC FID and HRGC MSD analysis of the secondary metabolites obtained by different extraction methods from *Lepechinia schiedeana*, and in vitro evaluation of its antioxidant activity. J High Res Chrommatogr 22:343-9.

Stratil P, Klejdus B, Kubáň V. 2006. Determination of total content of phenolic compounds and their antioxidant activity in vegetables. Evaluation of spectrophotometric methods. J Agric Food Chem 54:607-16.

Thiyam U, Kuhlmann A, Stöckmann H, Schwarz K. 2004. Prospects of rapeseed oil by-products with respect to antioxidative potential. Comptes Rendus Chimie 7:611-6.

Turner C. 2006. Overview of modern extraction techniques for food and agricultural samples. In: Turner C, editor. Modern extraction techniques: food agricultural sample. Washington, DC : American Chemical Society. P3-19.

Turner C, Turner P, Jacobson G, Almgren K, Waldebäck M, Sjöberg P, Karlsson EN, Markides KE. 2006. Subcritical water extraction and beta-glucosidase-catalyzed hydrolysis of quercetin glycosides in onion waste. Green Chem 8:949-59.

Ursini F, Zamburlini A, Cazzolato G, Maiorino M, Bon GB, Sevanian A. 1998. Postprandial plasma lipid hydroperoxides: a possible link between diet and atherosclerosis. Free Radical Biol Med 25:250-2.

van den Berg R, Haenen GRMM, van den Berg H, Bast A. 1999. Applicability of an improved Trolox equivalent antioxidant capacity (TEAC) assay for evaluation of antioxidant capacity measurements of mixtures. Food Chem 66:511-7.

Vekiari SA, Oreopoulou V, Tzia C, Thomopoulos CD. 1993. Oregano flavonoids as lipid antioxidants. J Am Oil Chem Soc 70:483-7.

Velioglu YS, Mazza G, Gao L, Oomah BD. 1998. Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. J Agric Food Chem 46:4113-7.

Vinson JA, Su X, Zubik L, Bose P. 2001. Phenol antioxidant quantity and quality in foods: fruits. J Agric Food Chem 49:5315-21.

Wanasundara UN, Amarowicz R, Shahidi F. 1994. Isolation and identification of an antioxidative components in canola meal. J Agric Food Chem 42:1285-90.

Wanasundara UN, Amarowicz R, Shahidi F. 1995. Partial characterization of natural antioxidants in canola meal. Food Res Int 28:525-30.

Wanasundara UN, Shahidi F. 1998. Antioxidant and prooxidant activity of green tea extracts in marine oils. Food Chem 63:335-42.

Wanasundara UN, Shahidi F. 1994. Canola extracts as an alternative natural antioxidant for canola oil. J Am Oil Chem Soc 71:817-22.

Wang CC, Chu CY, Chu KO, Choy KW, Khaw KS, Rogers MS, Pang CP. 2004. Trolox-equivalent antioxidant capacity assay versus oxygen radical absorbance capacity assay in plasma. Clin Chem 50:952-4.

Wang M, Simon JE, Aviles IF, He K, Zheng Q, Tadmor Y. 2003. Analysis of antioxidative phenolic compounds in artichoke (*Cynara scolymus* L.). J Agric Food Chem 51:601-8.

Warner K, Mounts TL. 1990. Analysis of tocopherols and phytosterols in vegetable oils by HPLC with evaporative light-scattering detection. J Am Oil Chem Soc 67:827-31.

Wright JS, Johnson ER, DiLabio GA. 2001. Predicting the activity of phenolic antioxidants: theoretical method, analysis of substituent effects, and application to major families of antioxidants. J Am Chem Soc 123:1173-83.

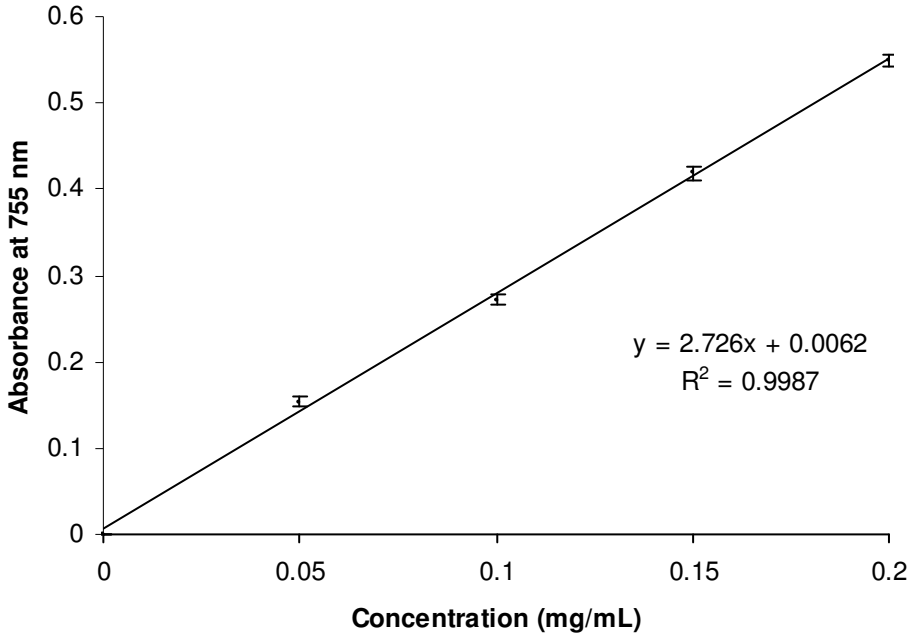
Yanishlieva N. 2001. Inhibiting oxidation. In: Pokorný J, Yanishlieva N, Gordon M, editors. Antioxidants in food: practical application. Cambridge England: Woodhead Publishing Limited. P22-70.

Yen GH, Chen HY. 1995. Antioxidant activity of various tea extracts in relation to their antimutagenicity. J Agric Food Chem 43:27-32.

8.0 APPENDICES

APPENDIX A: Total phenolics content: standard curve and calculations

The total phenolics content of extracts were estimated by a colorimetric assay (Amarowicz and others 2004). Sinapic acid was used as standard and results were calculated based on standard curves such as the one presented here (see section 3.4.1 for more details).



$$A_S = 2.726\ x + 0.0062$$

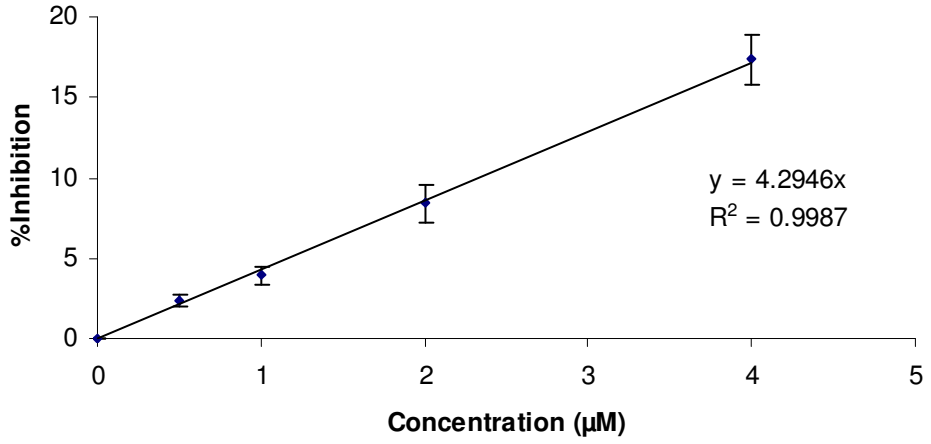
where A_S is the absorbance of the sample (or sinapic acid standard), x is the concentration of target compounds in the sample

Sample calculation:

when	$A_S = 0.410$ AU
then	$0.410 = 2.726\ x + 0.0062$
therefore	$x = 0.148$ mg/ml

APPENDIX B: Trolox equivalent antioxidant capacity (TEAC) assay: standard curve and calculations

The Trolox equivalent antioxidant capacity assay evaluates the capacity of a crude extract to scavenge 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^{\bullet+}$ radicals) (Liyana-Pathirana and Shahidi 2006; Arts and others 2004; Re and others 1999). Trolox was used as standard and results were calculated based on standard curves such as the one presented here (see section 3.4.2 for more details).



$$\% \text{ Inhibition} = [(A_C - A_S)/A_C] \times 100\%$$

$$\text{TEAC value} = \% \text{ Inhibition}/m$$

where A_C is the absorbance of the control at t = 6 min, A_S is the absorbance of the sample (or Trolox standard) at t = 6 min, and *m* is the slope of the standard curve

Sample calculation:

when $A_C = 0.690$ AU, $A_S = 0.608$ AU and $m = 4.2946\ \%\cdot(\mu\text{moles/litre})^{-1}$

then % Inhibition = (0.690 – 0.608)/0.690 x 100% = 11.9%

therefore TEAC value = $11.9\%/4.2946\ \%\cdot(\mu\text{mole/litre})^{-1} = 2.70\ \mu\text{moles/litre}$

APPENDIX C: β-Carotene-linoleic acid (linoleate) assay: calculations

The antioxidant capacities of extracts were estimated using a β-carotene-linoleic acid (linoleate) model system (Amarowicz and others 2004) (see section 3.4.3 for more details).

1. Initially, the rate of β-carotene bleaching (R) was calculated according to first-order kinetics as shown:

$$R = \ln (A_{t=0} / A_{t=t}) \times 1/ t$$

where $A_{t=0}$ is the initial absorbance (470 nm) of the emulsion at time 0, $A_{t=t}$ is the absorbance (470 nm) at 15, 30 and 45 min, and t is the time in minutes.

An average rate for each sample was calculated based on the Rs determined at t = 15, 30 and 45 min. The antioxidant activity (ANT) was calculated as the percent inhibition of the rate of β-carotene bleaching relative to the control using the equation:

$$\% \text{ ANT} = 100 \times (R_{\text{control}} - R_{\text{sample}}) / R_{\text{control}}$$

where R_{control} and R_{sample} are the average bleaching rates of β-carotene in the emulsion without antioxidant and with extract, respectively.

2. The antioxidant activity was also expressed as the percent inhibition of the coupled oxidation of β-carotene and linoleic acid against BHA and control samples, based on absolute changes in absorbance measurements at two distinct points (60 and 120 min) during the assay rather than as an average rate. In the equation below, the results are normalized by using two extremes:

- the control, which should offer no protection against oxidation of the linoleic acid/β-carotene emulsion; therefore, the antioxidant activity is defined as 0% for this system, and

- the BHA control, which should offer essentially 100% protection against oxidation over the time course of the assay. The antioxidant activities of the SWE, ethanolic and hot water extracts were expressed as:

$$\%AA = 100 \times [\, 1 - (A_E^{t=0} - A_E^{t=t}) / \{(A_C^{t=0} - A_C^{t=t}) + (A_{BHA}^{t=0} - A_{BHA}^{t=t})\}]$$

where AA is the antioxidant activity, $A_E^{t=0}$ is the absorbance (470 nm) of the extract in question at 0 min, $A_E^{t=t}$ is the absorbance (470 nm) of the extract at t=60 or 120 min, $A_W^{t=0}$ is the absorbance (470 nm) of the control sample at 0 min, $A_W^{t=t}$ is the absorbance (470 nm) of the control sample at t=60 or 120 min, $A_{BHA}^{t=0}$ is the absorbance (470 nm) of the synthetic antioxidant control sample at 0 min, and $A_{BHA}^{t=t}$ is the absorbance (470 nm) of the synthetic antioxidant control sample at t=60 or 120 min.

Sample calculation:

when Sample: $A_0 = 0.73$ AU, $A_{15} = 0.64$ AU, $A_{30} = 0.58$ AU and $A_{45} = 0.46$ AU

Control: $A_0 = 0.73$ AU, $A_{15} = 0.26$ AU, $A_{30} = 0.11$ AU and $A_{45} = 0.07$ AU

then Sample: $R_1 = \ln(0.73/0.64) \times 1/15 \text{ min} = 0.0087 \text{ min}^{-1}$,

$R_2 = \ln(0.73/0.58) \times 1/30 \text{ min} = 0.0076 \text{ min}^{-1}$,

$R_1 = \ln(0.73/0.46) \times 1/45 \text{ min} = 0.0102 \text{ min}^{-1}$ and

$R_{sample} = (R_1 + R_2 + R_3)/3 = 0.0089 \text{ min}^{-1}$

Control: $R_1 = \ln(0.73/0.26) \times 1/15 \text{ min} = 0.0688 \text{ min}^{-1}$,

$R_2 = \ln(0.73/0.11) \times 1/30 \text{ min} = 0.0630 \text{ min}^{-1}$,

$R_1 = \ln(0.73/0.07) \times 1/45 \text{ min} = 0.0521 \text{ min}^{-1}$ and

$R_{control} = (R_1 + R_2 + R_3)/3 = 0.0613 \text{ min}^{-1}$

therefore $\%\text{ ANT} = 100 \times (0.0613 \text{ min}^{-1} - 0.0089 \text{ min}^{-1}) / 0.0613 \text{ min}^{-1} = 85.49\%$

when $A_E^{t=0} = 0.73$ AU, $A_E^{t=60} = 0.39$ AU, $A_C^{t=0} = 0.73$ AU, $A_C^{t=60} = 0.06$ AU,

$A_{BHA}^{t=0}$ = 0.73 AU and $A_{BHA}^{t=60}$ = 0.72 AU

$A_E^{t=0}$ = 0.73 AU, $A_E^{t=120}$ = 0.22 AU, $A_C^{t=0}$ = 0.73 AU, $A_C^{t=120}$ = 0.06 AU,

$A_{BHA}^{t=0}$ = 0.73 AU and $A_{BHA}^{t=120}$ = 0.72 AU

then $\%AA_{t=60}$= 100 x [1 – (0.73 – 0.39) / {(0.73 – 0.06) + (0.73 – 0.72)}]

$\%AA_{t=60}$= 50%

$\%AA_{t=120}$= 100 x [1 – (0.73 – 0.22) / {(0.73 – 0.06) + (0.73 – 0.72)}]

$\%AA_{t=120}$= 25%

APPENDIX D: Radical-scavenging activity (RSA) assay: calculations

The capacity of extracts to scavenge the 'stable' free radical 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) was monitored using the method of Stratil and others (2006), with slight changes (see section 3.4.4 for more details).

$$\% \text{ RSA} = 100 \times (1 - A_E/A_D)$$

where A_E is the absorbance of the solution when an extract has been added at a particular level, and A_D is the absorbance of the $DPPH^{\bullet}$ solution with nothing added.

Sample calculation:

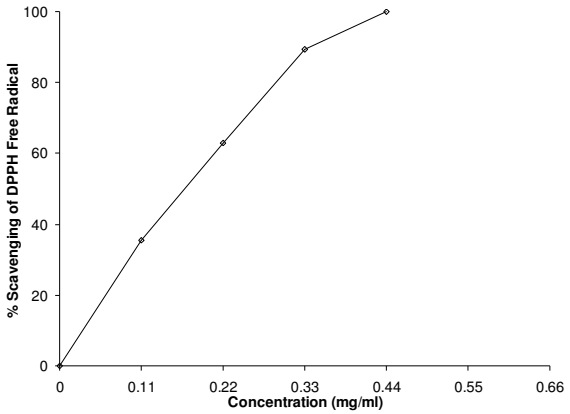
when $A_E = 0.716$ AU at a concentration of 0.11 mg/ml and $A_D = 1.11$ AU

then $\% \text{ RSA} = 100 \times (1 - 0.716/1.11) = 35.5\%$ at a concentration of 0.11 mg/ml

when

Concentration (mg/ml)	% Scavenging activity
0.11	35.50
0.22	63.01
0.33	89.42
0.44	100.0
Intercept[a]	6.80
Slope[a]	230.82% scavenging activity per $mg \cdot ml^{-1}$

[a] Slope and intercept were calculated from data presented in the table using Microsoft Excel 2000



then $y = 230.82\ \%\cdot(mg\cdot ml^{-1})^{-1}\ x + 6.80\%$

Slope = 230.82% scavenging activity per $mg\cdot ml^{-1}$

Concentration at 100% scavenging activity:

when $y = 230.82\ x + 6.80$

then $100\% = 230.82\ \%\cdot(mg\cdot ml^{-1})\ x + 6.80\%$

therefore $x = 0.40$ mg/ml

APPENDIX E: Reducing power assay: calculations

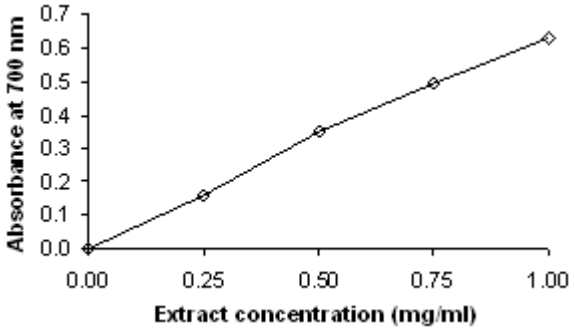
The reducing power of extracts were estimated using a reducing power assay (Amarowicz and others 2004) (see section 3.4.5 for more details).

Sample calculation:

when

Concentration (mg/ml)	Absorbance (AU)
0.25	0.16
0.50	0.35
0.75	0.49
1.00	0.62
Slope[a]	0.64 absorbance unit per $mg.ml^{-1}$

[a] Slope was calculated from data presented in the table using Microsoft Excel 2000



then $y = 0.64\ AU \cdot (mg \cdot ml^{-1})^{-1}x + b\ AU$ (b was not calculated)

Slope = 0.64 absorbance unit per $mg \cdot ml^{-1}$

APPENDIX F: Bulk stripped oil model system: calculations

The Bulk stripped oil model system was adopted from Madhujith and others (2004) with slight modifications (see section 3.4.6 for more details).

$$C_{CD} = A_{234} / (\varepsilon \times l)$$

where C_{CD} is the conjugated diene (CD) concentration in mmol/mL, A_{234} is the absorbance of the solution at 234 nm, ε is the molar absorptivity (*i.e.*, the extinction coefficient) of linoleic acid hydroperoxide (2.525×10^4 $M^{-1}cm^{-1}$), and l is the path length of the cuvette in cm (1 cm);

$$\text{CD value} = [C_{CD} \times (2.5 \times 10^4)] / W$$

where 2.5×10^4 is a factor that includes 25 mL of 2,2,4-trimethylpentane used to dissolve the oil sample as well as a unit conversion (1000 µmol/mmol) so that the content of CDs can be expressed in µmol, and W is the weight of the sample in grams.

After calculation of the CD values of samples, the results were expressed as extinction values using the following equation:

$$E^{1\%}_{1cm} = A_{\lambda} / (C_L \times l)$$

where $E^{1\%}_{1cm}$ is the extinction value, A_{λ} is the absorbance measured at 234 nm for CDs, C_L is the concentration of lipid solution in g/100 mL, and l is the path length of the cuvette in cm (1 cm).

Sample calculation:

when $A_{234} = 0.272$ at day 4

then $C_{CD} = 0.272 / (2.525 \times 10^4\ M^{-1}cm^{-1} \times 1\ cm) = 1.077 \times 10^{-5}$ mmoles/litre

when W= 0.011 g

then CD value = [1.077 × 10^{-5} × (2.5 × 10^{4})] / 0.011 = 24.48 µmoles/g

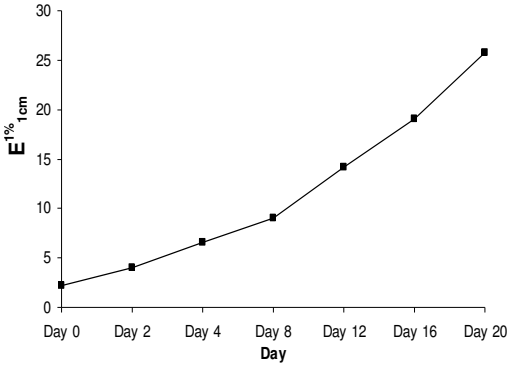
when A_{λ}= 0.272 and C_L= (4 × 0.011 g) = 0.044 g/100 ml

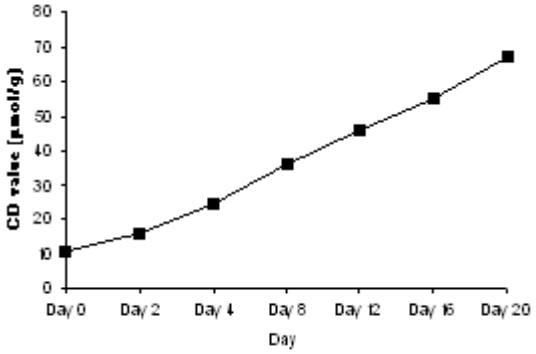
then $E1^{\%}_{1cm}$ = 0.272 / [0.044 (g/100 ml) × 1 cm] = 6.18

when

	Day 0	Day 2	Day 4	Day 8	Day 12	Day 16	Day 20	Slope[a]
CD value (µmoles/g)	11.12	15.84	24.48	36.23	45.90	54.90	66.87	0.36
$E^{1\%}_{1cm}$	2.19	4.05	6.18	9.02	14.17	19.08	25.76	0.86

[a] Slopes were calculated from presented data in the table using Microsoft Excel 2000





then $y = 0.36\ (\mu mol \cdot g^{-1}) \cdot day^{-1}\ x + b\ \mu mol \cdot g^{-1}$ (b was not calculated)

$Slope_{CD\ value} = 0.36\ \mu moles.g^{-1}$ per day

$y = 0.86\ (E^{1\%}_{1cm}) \cdot day^{-1}\ x + b\ E^{1\%}_{1cm}$ (b was not calculated)

$Slope_{E1\%1cm} = 0.86\ E^{1\%}_{1cm}$ per day