STRUCTURAL CHARACTERIZATION OF PHENOLIC COMPOUNDS IN CANOLA MEAL: IMPACT OF HIGH PRESSURE AND TEMPERATURE

By

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A Thesis Submitted to Faculty of Graduate Studies of the University of Manitoba

In Partial Fulfillment of the Requirements of Degree of

DOCTOR OF PHILOSOPHY

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Winnipeg, Manitoba

July 2021

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FORWARD

This thesis was prepared following a manuscript format. Four manuscripts contain most of the original work contributed by the author of this thesis, as demonstrated by his first authorship. A more detailed author contributions were outlined before each chapter. Manuscript I, was published in the Journal of Food Science. Manuscript II was published in the Journal of American Oil Chemists Society and manuscript III was published in the journal of LWT - Food Science and Technology in the special issue of Recovery, Enhancement and Functionalization of Value-added Bioactive Materials from Food Processing By-products. The abstract of the manuscript IV was published in the Journal of American Oil Chemists Society and while the complete manuscript was published in the Frontiers in Nutrition Journal under the section of Food Chemistry. The literature review of the thesis was published in a book chapter, Green Technology, Bioactive Compounds, Functionality, and Applications in Cold Pressed Oils.

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DEDICATION

I dedicate this document to my dearest mom Vajira and my dad Thompson, my sister Gayani, my wife Ramesha, and my little angel Riyana for their support, trust, motivation & being with me at both happy and sad moments of this journey.

I would also like to dedicate mt thesis in loving memory of my late supervisor Dr. Usha Thiyam-Holländer

Dedicated for the free education system of Sri Lanka

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// ACKNOWLEDGMENT

I would like to express my sincere gratitude and acknowledgment to my supervisor, late Dr. Usha Thiyam-Holländer, for introducing me to the interesting field of oilseeds, green technology, and thermal processing. Her continuous support and inspirational words with her philosophy of research helped me become an independent scientist. Dr. Thiyam-Holländer always provided various opportunities for me to attend conferences, seminars, webinars, and other learning resources, which expanded my interest and expertise in the field of thermal processing, green extraction, and oilseed chemistry. Unfortunately, just before my thesis defense Dr. Thiyam-Holländer passed away, and I'm very much grateful for all her support throughout my graduate studies career.

My sincere appreciation to my other mentor Dr. Michael Eskin for his continuous support and valuable feedback throughout my research program which helped to make my study a success. His expertise in food chemistry and oilseed chemistry helped me to expand my knowledge in oilseed processing. I would also like to appreciate his continuous support in my manuscript and thesis writing. His motivational words and his everlasting kindness in every meeting gave me hope when every time I was struggling both academically and in my personal life. I would also like to acknowledge Dr. Michael Eskin for taking the supervisory role upon passing away of my main supervisor Dr. Thiyam-Holländer and helping me throughout the hard times.

I would also like to gratefully acknowledge my co-supervisor Dr. Peter Eck or providing his valuable feedback for my thesis and taking over the supervisory role in the absence of late Dr. Thiyam- Holländer. His valuable feedbacks on my thesis made it a stronger one.

I would also like to acknowledge my other supervisory committee members Dr. Rotimi Aluko and Dr. Thomas Netticadan for providing their valuable feedback throughout my committee meetings and my research program. Their expertise opinions in diversified areas helped me to make my study a successful one.

I greatly acknowledge the financial support provided by the NSERC discovery grant and the Graduate Enhancement of Tri-Council Stipends (GETS) scholarship, to carry out my research program. I am very much thankful to Dr. Erika Zago for her insights on chemistry and natural product synthesis during her tenure as a post-doctoral researcher. Her expertise in thermal processing, instrumentation, and chemical synthesis helped me build my confidence in research. Special thanks and gratitude go to Dr. Helene Perreault and Ms. Emy Komatsu from the Department of chemistry for their tremendous support given to me during the mass spectrometry analysis and results interpretation. Their support on mass spectrometry helped me expand my research in a new direction. I would also like to thank Dr. Rashida Rabbani and Dr. Anas Mohamad for their statistical expertise on data analysis.

Also, I would like to thank my past and present lab members, Dr. Ayyappan Aachary, Jingbang Liang, Hui Xu, Jiaur Rahman, Afra Imran, Olamide Fadairo and Thu Min for their support throughout the lab work which made my research productive. My deepest gratitude goes to my parents, my sister and brother-in-law, my lovely wife, and my little angel Riyana for their love, care, and unrelenting support which always encouraged me to complete my higher studies and accomplish my goals. Especially for my lovely angel who received very little of my attention while this thesis was being prepared. Last but not least, I would like to thank my previous supervisor Dr. Vasantha Rupasinghe for introducing me to the wonderful world of antioxidants.

THESIS ABSTRACT

Canola is the major oilseed-crop of Canada. After extraction of the oil, a substantial amount of meal is generated. To-date, however, the meal has received little attention from both industry and researchers. Despite its nutrition and functional potential, utilization of canola meal has been limited by its high fiber content and antinutritional components. Nevertheless, it has a rich phenolic profile, that could be a valuable source of nutraceuticals. The presence of large amounts of sinapine and sinapic acid in canola meal and its conversion to canolol has received a lot of attention because of its powerful antioxidant and anticancer properties. What is desired, however, is an effective method for their extraction. The use of pressurized (1,500 psi) high-temperature processing is ideal for the extraction of phenolic compounds from such by-products including the meal. This study investigated the application of accelerated solvent extraction (ASE) as a method for extracting phenolic compounds from canola meal. Different extraction temperatures, solvents, and solvent concentrations were used to establish the optimum extraction conditions for major sinapates and other phenolic compounds. Changes in total phenolics, total flavonoids, and antioxidant capacity were quantified as a measure of extraction efficiency. The ASE method is an efficient method for extracting phenolic compounds from the meal. In addition to ASE, RapidOxy®100, an automated commercial instrument, was adapted as a solvent-free pre-treatment of canola meal prior to the ultrasonic extraction of phenolic compounds. It also proved to be a novel and versatile method for enhancing the extraction of phenolic compounds. Both techniques have the potential for the mainstream production of phenolic-rich extracts as a valuable source of natural bioactive compounds and nutraceuticals from underutilized agricultural by-products such as canola meal.

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LIST OF ABBREVIATIONS

MT - Metric Ton
T – Ton
$ - Dollar
µmol – Micro moles
g - Gram
ASE - Accelerated solvent extraction
PSE - Pressurized solvent extraction
MAE - Microwave aided extraction
SWE - Subcritical water extraction
UAE - Ultrasonic aided extraction
SFE - Supercritical fluid extraction
HPLC – High-performance liquid chromatography
LC-MS/MS - Liquid chromatography and mass spectrometry
KS - Kaempferol-3-*O*-*β*-sophoroside
KSS - Kaempferol 3-*O*-(2‴-*O*-sinapoyl-*β*-sophoroside)
TA - Thomasidiac acid
psi - Pound per square inch
^{o}C - Centigrade
N_2 - Nitrogen gas
® - Registered trademark
mmol - Millimoles
mg - Milligrams
gal - Gallons
UV - Ultraviolet
nm - Nanometer
kHz - Kilohertz
K - Kelvin
atm - Atmosphere(s)
Wcm^2 - Watt per square centimeter
W- Watt
Hz - Hertz
min - Minutes
kg – Kilogram
MSAE - Mega sonic assisted aqueous extraction
MHz - Megahertz
FFA - Free fatty acids
GHz - Gigahertz
PAH - Polycyclic aromatic hydrocarbons
NaOH - Sodium hydroxide
SFE - Supercritical fluid extraction
CO_2 - Carbon dioxide
CC - Column chromatography
DCCC - Droplet countercurrent chromatography
SPE - Solid-phase extraction
DPPH - 2,2-diphenyl-1-picrylhydrazyl

FRAP - Ferric reducing antioxidant power
v/v – Volume per volume
SAE - Sinapic acid equivalents
DM - Dry matter
FC - Folin-Ciocalteu
HCl - Hydrogen chloride
TPTZ - 2,4,6-tris-(2-pyridyl)-S-triazine
mm - Millimeter
mL - Milliliter
TPC - Total phenolic content
TFC - Total flavonoid content
µL - Microliter
mM - Millimoles per liter
R^2 - Coefficients of variance
µM - Micromoles per liter
QE - Quercetin equivalents
pH - Power of hydrogen
TE - Trolox equivalents
DAD - Diode array detection
Å - Angstrom
RP - Reverse phase
ANOVA - Analysis of variance
p - Probability
Fe^{3+} - Ferric ion
Fe^{2+} - Ferrous ion
K-4-G - Kaempferol 3-(2''-hydroxypropionylglucoside)-4'-glucoside
K-7-G - Kaempferol 3-[2''-glucosyl-6''-acetyl-galactoside] 7-glucoside
LC-MS/MS - Liquid chromatography with mass spectrometry and tandem mass spectrometry
LC **-** Liquid chromatography
ESI **-** Electrospray ionization
Q-TOF-MS - Quadrupole time of flight mass spectrometry
V **-** Voltage
eV **-** Electron volts
SP - Sinapine
SA - Sinapic acid
CL - Canolol
m/z **-** Mass to charge ratio
NMR - Nuclear magnetic resonance
BHA - Butylated-hydroxyanisole
BHT - Butylated-hydroxytoluene
TBHQ - Tert-butylhydroquinone
rpm - Revolutions per minute
TLC **-** Thin layer chromatography
MPa - Mega pascal
r **-** Regression coefficient

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

Canola meal, the major by-product of oil processing, is rich source of phenolic compounds. The production capacity of canola meal increases every year with an annual production of over 5,200 MT in 2019 (Canola Council of Canada, 2020). However, every year over 80% of the meal is exported, with over 4600 MT exported worldwide last year. According to the databases of the Canola Council of Canada, and Canadian International Merchandise Trade, the value of the exported quality canola meal in 2019 was only 350$ per ton (T) (Canola Council of Canada, 2020). However, its rich phenolic profile, well-balanced amino acid content, and good source of proteins (35-40%) makes it a far more valuable by-product (Alam et al., 2016; Matthäus et al., 2014).

The Canola Council of Canada has applied specific standards for both canola oil and canola meal. By definition, the meal should contain less than 30 μmol/g of aliphatic glucosinolates compounds to be introduced into the feed industry (Cai et al., 1999; Canola Council of Canada, 2020). Approximately 18 to 20% of the canola meal is composed of hulls (Meng and Slominski, 2005). This hull fraction is mostly fibre containing non-starch polysaccharides including pectic polysaccharides, arabinans, arabinogalactans, xyloglucans, galactomannans, and mannans (Meng and Slominski, 2005; Pustjens et al., 2013). These complex polysaccharides are often associated with plant secondary metabolites including flavonoids, phenolic acids, polymerized structures, and lignin compounds (Morley et al., 2013). To improve the nutritive value and reduce the impact of non-starch polysaccharides on canola meal, the plants were bred to produce low-fibre containing varieties its use in the animal feed industry is still limited (Khajali and Slominski, 2012). Even

though new varieties of canola were introduced to the market every year, the application of canola meal as a food ingredient has limited applicability due to its anti-nutritive factors including glucosinolates (Naczk et al. 1998).

The only successful commercial utilization of canola meal for human food is the production of functionally and nutritional valuable protein isolates from the meal by Burcon Nutraceutical Corporation (TSX: BU). These isolates have been branded under the names Supertein®, Purstein® and Nurstein®. A recent joint venture between Burcon and three veteran food executives established the Merit Functional Foods Corporation in 2019 with a new state-of-art facility opening up in Manitoba in 2021 for the production of both canola and pea proteins. The canola and pea protein blends are reported to be equivalent to or exceed the protein quality of dairy and meat and is designed to meet the growing demands of the plant protein market worldwide for utilization in foods and beverages. Consequently, the phenolic compounds in the meal represent an additional potential source of valuable underutilized components for use in the food and nutraceutical industries.

The most abundant phenolic compounds present in canola include sinapine and other sinapic acid derivatives (Jun et al., 2014; Nićiforović and Abramovič, 2014). Sinapine and sinapoyl glucose are the major sinapoyl esters of sinapic acid commonly found in canola meal (Nićiforović and Abramovič, 2014). Sinapine alone contributes to over 80% of the total phenolic content of canola meal whilst sinapic acid accounts for over 70% of the free phenolic acid content of the meal (Chen et al., 2014). In addition to sinapate derivatives, *p*-hydroxybenzoic, vanillic, gentisic, protocatechuic, syringic, *p*-coumaric, ferulic, caffeic, and chlorogenic acid are also present in the meal in minute amounts (Kozlowska et al., 1983). In addition to the major phenolic acids other

minor components including kaempferol derivatives are also present in the canola meal (Hald et al., 2019; Nandasiri et al., 2020). These kaempferol derivatives have been identified as the key bitter flavor molecules of the canola meal (Hald et al., 2019).

Sinapates and kaempferol derivatives contribute to both flavor and antioxidant activity in canola meal (Chen et al., 2014; Hald et al., 2019). Based on their antioxidant activity, sinapine, and sinapic acid have been identified as the most active antioxidant components present in canola meal (Chen et al., 2014). With the application of pressurized heat thermo-generative phenolic compounds including canolol are formed in an inert environment (Nandasiri et al., 2019). Furthermore, other parameters including the particle size of the meal, type of extraction solvent, extraction solvent polarity, and the extraction temperature may also contribute to the extraction efficiency of the phenolic compounds as well as their antioxidant activity.

Consequently, the removal of these phenolic compounds from the meal would be valuable by providing added-value components. Yet, extraction of these valuable phenolic compounds from the meal is often associated with the greater use of organic solvents and environmentally harmful chemicals (Chen et al., 2014). In addition, the extraction efficiency of the phenolic compounds also depends on the method of extraction used. To overcome these limitations, the application of novel and innovative extraction methods is required. Over the past years, many extraction techniques were introduced to the oil industry including microwave-aided extraction (MAE), subcritical water extraction (SWE), supercritical fluid extraction (SFE), ultrasonic aided extraction (UAE), and accelerated solvent extraction (ASE). Of these methods, accelerated solvent extraction (ASE) or pressurized high-temperature extraction (PSE) is one method that could be easily applied to the oil industry.

In comparison to other extraction techniques, ASE uses less solvents and shorter extraction times for obtaining higher yields of phenolic-rich extracts (Barros et al., 2013; Nandasiri et al., 2019). ASE is generally operated at high pressures above 1000 psi, and extraction temperatures over 100°C which facilitates the mass transfer and improves the solubility of the phenolic compounds (Barros et al., 2013; Li and Guo, 2016; Nandasiri et al., 2019). The high temperature (> 180°C) and pressure (> 1500 psi) applied in ASE facilitates the removal of the aglycone moieties attached to phenolic compounds by hydrolysis thereby increasing the extraction efficiency of the antioxidative phenolic molecules compared to conventional methods including ultrasound extraction (Li and Guo, 2016; Yang et al., 2015). The concurrent extraction of ASE further facilitates the structural transformations of sinapine to sinapic acid and canolol, at elevated temperatures (Li and Guo, 2016). Furthermore, pressurized temperature treatment of ASE enables the formation of other thermo generative phenolic compounds including lignans such as thomasidioic acid (TA). These unique features of ASE should facilitate the extraction of antioxidative phenolic compounds including sinapates and kaempferol derivatives with higher rate of efficiency thereby attenuating the bitter-flavor active phenolic compounds present in the meal (Nandasiri et al., 2020, 2019).

Identification and quantification of phenolic compounds by high performance liquid chromatography (HPLC) often requires standard chemicals. Up-to-date, many chromatographic methods have been published for measuring the phenolic compounds present in canola meal matrices (Luthria et al., 2004). The quantification of phenolic compounds via HPLC is limited to the availability of standards and by the similarity and overlapping of the UV-spectra of the different phenolic compounds (isomers) (Engels et al., 2012). Moreover, the minor components including flavor-active phenolic compounds such as hydroxycinnamic and kaempferol derivatives

with vinyl phenols have limitations in identification via HPLC due to their characteristic volatility (Wolfram et al., 2010). Therefore, a unique HPLC method determining both major and minor phenolic compounds present in canola meal is required. In contrast, the use of mass spectrometry (MS) and tandem mass spectrometry (MS/MS), with the aid of fragmentation patterns facilitates identification of these minor components and the flavor-active phenolic compounds with greater sensitivity and selectivity (Frolov et al., 2013). The identification and the quantification of these unique phenolic compounds could lead to their utilization by the nutraceutical industry.

The application of pressurized heat often requires special instruments including ASE and are often associated with higher capital costs. Therefore, there is a need for an economical and simple extraction techniques with a higher rate of precision for use by the oilseed processing industry. Furthermore, the use of extraction solvents has its own drawbacks particularly their impact on the environment (Chen et al., 2014). An alternative solvent-free pre-treatment capable of enhancing the extractability of the phenolics would be a great advantage for the pressurized temperature extraction process. Such a system was established by adapting the commercial RapidOxy® 100 system and compare to the ASE method. Comparatively RapidOxy® 100 uses less pressure (100 psi) compared to the ASE (1500 psi). However, the compact design, and the built-in pressurized heating chamber of RapidOxy® 100, creates the perfect environment for lower pressurized temperature pre-treatments. Hence, the application of lower pressurized temperature pre-treatment would provide the optimum pre-treatment conditions for the dry-heat extraction of the major canola sinapates, including sinapine, sinapic acid, and canolol in comparison to pressurized wet heat treatment (ASE).

Chapter 3 (the first manuscript) of my thesis examined the impact of pressurized high-temperature treatment of ASE on the antioxidant activity of canola meal extracts. Different extraction conditions including solvent type, solvent concentration, extraction temperature, and particle size were studied to better understand the impact of these extraction conditions on antioxidant activity. Previous studies by Li and Guo (2016) demonstrated that both antioxidant activity and the total phenolic content of canola meal extracts were affected by different extraction conditions. However, a complete understanding of all the aforementioned factors still remained to be fully investigated.

The lack of phenolic standards has limited the identification of phenolic compounds by high-performance liquid chromatography (HPLC) and determination of the structure-function relationship of phenolic compounds. However, application of liquid chromatography and mass spectrometry (LC-MS/MS), generates major and minor fragments, that helps to identify these phenolic compounds. Chapter 4 (the second manuscript) focused on the attenuation of the bitter flavor-active phenolic compounds. To-date, canola meal is underutilized and limited to animal feeds. Using ASE, I was able to improve the extraction of phenolic compounds including sinapine, sinapic acid, canolol, and kaempferol derivatives. The increased extractability of phenolic compounds attenuated the bitter flavor of the meal thereby improving it as a feed ingredient. The balanced amino-acid profile and, high digestibility of canola meal warrants it greater utilization in the feed industry.

Chapter 5 (the third manuscript) identified those flavor-active phenolic compounds some of which impart the bitter flavor of the meal. To-date, there is very limited information available on flavor-active phenolic compounds in canola by-products other than sinapine. Due to its

abundance, sinapine was considered as the major flavor-active phenolic compound present in canola by-products (Khattab et al., 2014; Thiyam et al., 2006). A recent sensory study conducted in Germany by Hald *et al*. (2019), however, demonstrated that kaempferol derivatives specifically, kaempferol 3-*O*-(2‴-*O*-sinapoyl-*β*-sophoroside), was the major bitter flavor-active phenolic compound present in the canola by-products. Identification and quantification of kaempferol 3-*O*-(2‴-*O*-sinapoyl-*β*-sophoroside) in canola meal would advance its value, while collectively increasing its applicability in the nutraceutical industry. Beside kaempferol-3-*O*-*β*-sophoroside and kaempferol 3-*O*-(2‴-*O*-sinapoyl-*β*-sophoroside), two other kaempferol derivatives were identified in the meal. These bitter flavor-active phenolic compounds appear to be impacted by different extraction conditions primarily temperature and solvent concentration. Other thermo-generative phenolic compounds, including lignin compound thomasidiac acid (TA), was also identified in the meal extracts with its concentration affected by extraction conditions.

Chapter 6 (the fourth manuscript) focused on the application of dry heat as a pre-treatment for the extraction of phenolic compounds. This differed from using wet extraction of phenolic compounds by ASE under high pressure (1500 psi), and high temperature (~180°C). The application of dry heat was conducted in a low pressurized (100 psi) environment by a simple modification of the commercial RapidOxy® 100 instrument using inert N_2 to determine its applicability on the industrial scale. Changes in the major sinapates were compared to the pressurized high-temperature wet extraction method. The replacement of air with nitrogen using the compact, intact, and portable design of RapidOxy® 100, with application for both solid and liquid samples, proved a novel dry pre-heat treatment for phenolic extraction.

1.2 Research Hypothesis and Expanded Objectives

The main research hypothesis of the current study was that pressurized heat treatments in an inert environment would increase the total phenolic content and the antioxidant activity of canola meal extracts.

The extractability of flavor-active phenolic compounds present in canola meal are impacted by many factors including high pressure, particle size, temperature, solvent type, and concentration. Hence, the overall objective of this study was to optimize conditions for the extraction of major phenolic compounds (sinapates and kaempferol derivatives) by high pressure and temperature treatments using accelerated solvent extraction (ASE) technique to obtain valuable phenol by-products from canola meal. To achieve this objective, a series experiments were conducted to address the following specific objectives:

1. To optimize the conditions for the accelerated solvent extraction (ASE) on the extractability of antioxidative phenolic compounds from canola meal

The traditional phenolic extraction methods are commonly associated with low extractability, lower recovery, and higher decomposition (Chen et al., 2014). Hence, alternative extraction methods are needed to increase both the extractability and recovery of phenolic compounds. Accelerated solvent extraction (ASE) has been reported to extract phenolic compounds from many matrices (Li and Guo, 2016; Nandasiri et al., 2019). Recent reports suggested that ASE could be a potential method for extraction of phenolic compounds from canola by-products including meal. However, application of ASE on extractability of phenolic compounds from canola meal has not been studied in detail considering factors including the

particle size, type of solvent, solvent concentration, and extraction temperature. Thus, the initial goal was to optimize the extraction conditions of ASE to extract thermo generative phenolic compounds including canolol, and to identify the extractability of major phenolic compounds present in canola meal extracts.

2. To evaluate the *in-vitro* antioxidant activity and to determine the optimum antioxidant activity of phenolic rich extracts *via* pressurized-heat treatment

Antioxidant capacity of oilseeds was reported to increase with the thermal treatments (Siger and Józefiak, 2016). The higher antioxidant capacity with higher processing temperatures in canola meal was closely associated with the production of canolol and other antioxidative phenolic compounds including sinapine and sinapic acid. Previous reports stated that production of canolol and its derivatives increases with higher temperatures (160°C) (Li and Guo, 2016; Nandasiri et al., 2019; Zago et al., 2015). Moreover, the application of pressurized heat via ASE favors the production of canolol and other thermo-generative antioxidative phenolic compounds including thomasidiac acid (TA). Nevertheless, a complete study on the changes in antioxidant activity of canola meal extracts was not reported. Different parameters including particle size, processing temperature, extraction solvent, solvent concentration was studied to understand the structure-based antioxidant activity of canola meal extracts in a pressurized wet-heat environment.

3. To develop a new HPLC-DAD method for identification and quantification of unique flavor-active phenolic compounds

Identification and quantification of phenolic compounds associated with oilseeds are often challenging due to the formation of novel derivatives of the phenolic compounds and volatile

phenols in pressurized temperature processing (Nandasiri et al., 2020). Furthermore, the application of pressure and temperature could impact the separation and stability of phenolic compounds in a HPLC system due to the formation of novel phenolic compounds (Harbaum-Piayda et al., 2010). Thus, a unique HPLC method with advanced separation is required to identify these major flavor-active phenolic compounds, novel thermo generative phenolic compounds, and phenolic derivatives of the pressurized temperature processing. Consequently, a modified HPLC method was developed to meet these requirements.

4. To determine and identify major flavor-active bitter tasting phenolic compounds using HPLC-MS/MS

Flavor-active phenolic compounds and volatile phenols are often difficult to identify by HPLC. Therefore, more advanced techniques, such tandem mass spectrometry is required for identifying the unique flavor-active phenolic compounds (Frolov et al., 2013). The lack of standard compounds, their similarity in UV-spectra, and retention times are major limitation for analysis by HPLC (Engels et al., 2012). As a result, the pressurized heat treatment on the structural transformation of major sinapates, kaempferol derivatives, and lignan compounds are yet to be examined. To achieve this, an HPLC method coupled with mass spectrometry and tandem mass spectrometry was developed to identify the major flavor-active phenolic compounds, including the sinapates and kaempferol derivatives, the thermo generative phenolic compounds and their degradation products in canola meal extracts.

5. To investigate the use of modified RapidOxy® 100 as a novel pressurized dry heat pre-treatment method for extraction of phenolic compounds compared to pressurized wet heat (ASE) method

The pressurized wet heat treatment (ASE) was used to extract thermo generative phenolic compounds including canolol. Thus, a solvent-free pressurized pre-heat treatment associated with improved extractability of the phenolic compounds holds promising advantages on value addition. The design of RapidOxy® 100 is ideal to study the optimum pre-heat treatment conditions for the dry-heat extraction of the major canola sinapates, sinapine, sinapic acid, and canolol at lower pressure conditions. Hence, the extractability of major sinapates and canolol at lower pressurized (100 psi) environment in comparison to pressurized heat treatment is yet to be investigated. The final objective was to understand the structure-based activity of major sinapates in a lower pressurized dry-heat environment with the application of RapidOxy® 100.

1.3 Thesis structure

The thesis is organized into four major sections including a general introduction, a literature review focusing on green extraction with main research findings as published manuscripts, and a general conclusion with future directions. Hence, there may be some possible overlaps among the chapters in introduction, materials, and methods.

PART 1: *General introduction*

Chapter 1: This chapter includes the introduction, research objectives and thesis overview, providing the background information, research hypothesis, main and specific objectives, and a summary of the major accomplishments of the thesis.

PART 2: *Literature review*

Chapter 2: This chapter recaps the recent literature on the major antioxidative phenolic compounds present in canola, current extraction techniques for the removal of phenolics from canola meal, the importance of green extraction in canola by-product industry, and applicability of green extraction for the production of canola by-products This chapter covers the research hypotheses that were established based on the literature review.

PART 3: *Consists of four research manuscripts for covering the research objectives. Each manuscript includes a specific introduction, materials and methods, results and discussion, and the specific conclusion for each study.*

Chapter 3: To examine the antioxidant activity and optimum extraction conditions (objective 1 and 2), different canola meal extractants obtained by ASE were assessed for their antioxidant capacity using *in-vitro* test tube assays

Chapter 4: To achieve the third objective, a new HPLC method was developed to identify the unique flavor-active phenolic compounds present in canola meal extracts focusing on the attenuation of the bitter flavor-active phenolic compounds (objective 3).

Chapter 5: To further identify other flavor-active bitter phenolic compounds in canola meal extracts, besides sinapates, HPLC was coupled with mass spectrometry (objective 4). The extracted flavor-active unique phenolic compounds identified including kaempferol derivatives as well as other thermo-generated phenolic compounds including TA, tentatively identified.

Chapter 6: The final objective (objective 5) used dry heat as a novel pre-treatment method for extracting the major sinapates in a lower pressured (100 psi) inert environment (N_2) with the RapidOxy® 100 equipment at different temperature settings (140, 160, and 180°C).

PART 4: *Conclusion and future directions*

Chapter 7: The conclusions presented are based on the major and novel findings obtained from the four studies, with future directions for further study.

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BRIDGE TO CHAPTER 2

In chapter 1, I have explained the background information related to canola meal and its importance towards the province of Manitoba. Further, the research objectives and thesis overview, research hypothesis, main and specific objectives, and a summary of the major accomplishments of the thesis were explained in the chapter 1. In the following chapter 2, in depth analysis of phenolic composition in oilseeds specifically in canola was determined. Different phenolic extraction methods with the emphasis on green technology, towards oilseed industry was explained in detail in the current chapter.

AUTHOR CONTRIBUTIONS FOR CHAPTER 2

Application of green technology on extraction of phenolic compounds in oilseeds (canola). **Nandasiri R**., Eskin N. A. M., Eck P., Thiyam-Holländer U. Cold Pressed Oils: Green Technology, Bioactive Compounds, Functionality, and Applications, 2020, 81-96. Ruchira Nandasiri wrote the first draft of the book chapter on green extraction and its applications on oilseed industry. Both Dr. Michael Eskin and Dr. Usha Thiyam-Holländer provided critical feedback on the book chapter with proof reading. Dr. Peter Eck did the final proof reading after editor's comments.

CHAPTER 2

LITERATURE REVIEW

APPLICATION OF GREEN TECHNOLOGY ON EXTRACTION OF PHENOLIC COMPOUNDS IN OILSEEDS (CANOLA)

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Published in Cold Pressed Oils: Green Technology, Bioactive Compounds, Functionality, and Applications, 2020, 81-96

2.1 Introduction

The production of oil from oilseeds has its origins in ancient times. Recent studies, however, have demonstrated that oilseeds could be a rich source of bioactive compounds with potential for use in pharmaceutical and cosmetic industries. Canola (*Brassica napus*), a relatively new crop, is the major oil crop of Canada, accounting for more than 20% of global production (Azargohar et al., 2013). Canola oil consists mainly of triglycerides and other minor compounds such as free phenolic acids (vanillic, ferulic, *p*-coumaric, chlorogenic, caffeic, etc.), esterified phenolic acids, free fatty acids, and proteins (Abuzaytoun & Shahidi, 2006; Alam et al., 2016; Alkan, Tokatli, & Ozen, 2012). During the past few years, researchers have revealed that esterified phenolic acids are the predominant phenolic compounds in canola oil, with sinapine being the most abundant (Cai, Arntfield, & Charlton, 1999).

Each year thousands of metric tons of defatted canola meal, the by-product of oil production, are underutilized and wasted by the industry (Casseus, 2009). In fact, defatted oilseed meal could be a valuable source of antioxidants, and a rich source for phenolic compounds (Alam et al., 2016; Chen, et al., 2014). Oilseeds are rich in plant secondary metabolites including hydroxycinnamic acids derivatives, glycosides, flavonoids, and lignans (Morley et al., 2013). Furthermore, with reference to canola meal, phenolic compounds such as sinapic acid esters (sinapine and glucopyransol sinapate) account for 90%-95% of the total phenolic compounds, while free sinapic acid accounts for less than 10% (Chen et al., 2014; Morley et al., 2013). Consequently, the rich phenolic content of oilseed meal, especially canola, has garnered considerable interest by researchers as a natural source of antioxidants and nutraceuticals (Chen et al., 2014).

Extraction of phenolic compounds from canola oil is carried out over an extended period using different extraction systems (Chen et al., 2014). Among them, the solvent extraction technique was extensively used, due to its simple design, relatively higher yield, and the availability of economically affordable apparatus (Luthria, 2006; Wanasundara & Shahidi, 1994). Yet this technique is associated with many drawbacks, including the high cost of unit operation and the excessive use of solvents (Luthria, 2006).

The above limitations encouraged food engineers and technologists to investigate alternative methods that were ecofriendly and green. High selectivity, short extraction time, lower solvent usage, energy competency, and environmental friendliness are all commonly attributed with green extraction which is highly applicable to the oilseed industry (Carabias-Martínez et al., 2005). Among the green extraction technologies, ultrasonic aided extraction (UAE), microwave-aided extraction (MAE), subcritical (SWE) and supercritical (SFE) extraction, as well as accelerated solvent extraction (ASE), have gained considerable attention from researchers due to their energy competency, eco-friendliness, and relatively higher yield. These common green extraction techniques can be readily applied in the oilseed industry to minimize the utilization of toxic solvents, extract phenolic compounds, and meet the current energy and solvent demanding conventional extraction methods with the focus on developing a better-quality final product.

2.2 Canola: A source of phenolic compounds

"Canola" belongs to the *Brassica* genus, which includes rutabaga, mustard, turnips, cauliflowers, cabbages, and broccoli (Casseus, 2009). The name canola originated from Canadian oil, low acid, used to describe a modified rapeseed variety containing a glucosinolate level of < 18mmol/g whole seeds, and < 1% erucic acid in oil (Obied et al., 2013). An ancient oilseed,

rapeseed was first introduced in Asia and Europe as a source of lamp oil and, later, as cooking oil. Afterward, its physical characteristics made it an important lubricating oil for steam engines for use in merchant ships and warships (Casseus, 2009). Rapeseed is not indigenous to Canada but was introduced by a Polish farmer who immigrated to Western Canada in the 1920s. Its subsequent conversion to canola involved two Canadian plant breeders, Baldur Stefannson and Keith Downey (Daun, Eskin, & Hickling, 2011). Together they changed the agricultural landscape in Canada, making canola the second major crop in Canada after wheat. Global Production now ranks canola varieties third among the world's oilseed crops, after soybean and palm (Carré & Pouzet, 2014). Canola has a high oil yield (40%) and is one of the richest sources of phenolic compounds compared to other oilseeds (**Table 2.1**) (Naczk et al., 1998).

Table 2.1 Phenolic Composition of Most Common Oilseed Products (Naczk et al., 1998)

Oilseed Product	g/kg dry basis
Peanut flour	0.63
Soybean meal	4.60
Rapeseed/ Canola flour	6.4 - 12.8
Canola meal	15.4- 18.4

2.2.1 Canola oil

Canola oil is composed of 6%-14% α-linolenic acid, 50%-65% oleic acid, and < 7% of saturated fatty acids (Ghazani & Marangoni, 2013; Gunstone, 2011). However, cold pressed canola oil contains a comparatively higher content of tocopherols (60-70mg/100g) and phytosterols. Generally, canola oil contains a ratio of 1:2 between α-: γ-tocopherols, and a ratio of 1:1 between free and esterified forms of phytosterols (Ghazani & Marangoni, 2013; Gunstone, 2011). It usually has relatively lower levels of saturated fats, and a ratio of two to one (2:1) for linoleic to linolenic acid (Xu & Diosady, 2012). Overall, the oil quality and fatty acid composition in canola make it ideal for human consumption. The replacement of *trans* rich hydrogenated canola oil with high oleic canola oil for industrial/commercial frying also provides a much healthier and stable oil for the consumer market (DeBonte et al., 2012; Ghazani & Marangoni, 2013).

2.2.2 Canola meal

Canola meal is the second largest protein meal produced in the world after soybean meal (Aachary, Thiyam-Hollander, & Eskin, 2015). It has a high fiber content, which can interfere with its digestibility (Azargohar et al., 2013). However, this problem has been mitigated with the production of genetically modified varieties with less than 2% erucic acid in its oil, and less than

30 µmol/g of one or any mix of the four recognized aliphatic glucosinolates (gluconapin, progoitrin, glucobrassicanapin, and napoleiferin) (Cai et al., 1999). Canola meal contains the common essential amino acids, with the levels of lysine, methionine, cysteine, threonine, and tryptophan being higher than those of the other cereal varieties (Azargohar et al., 2013; Cai et al., 1999).

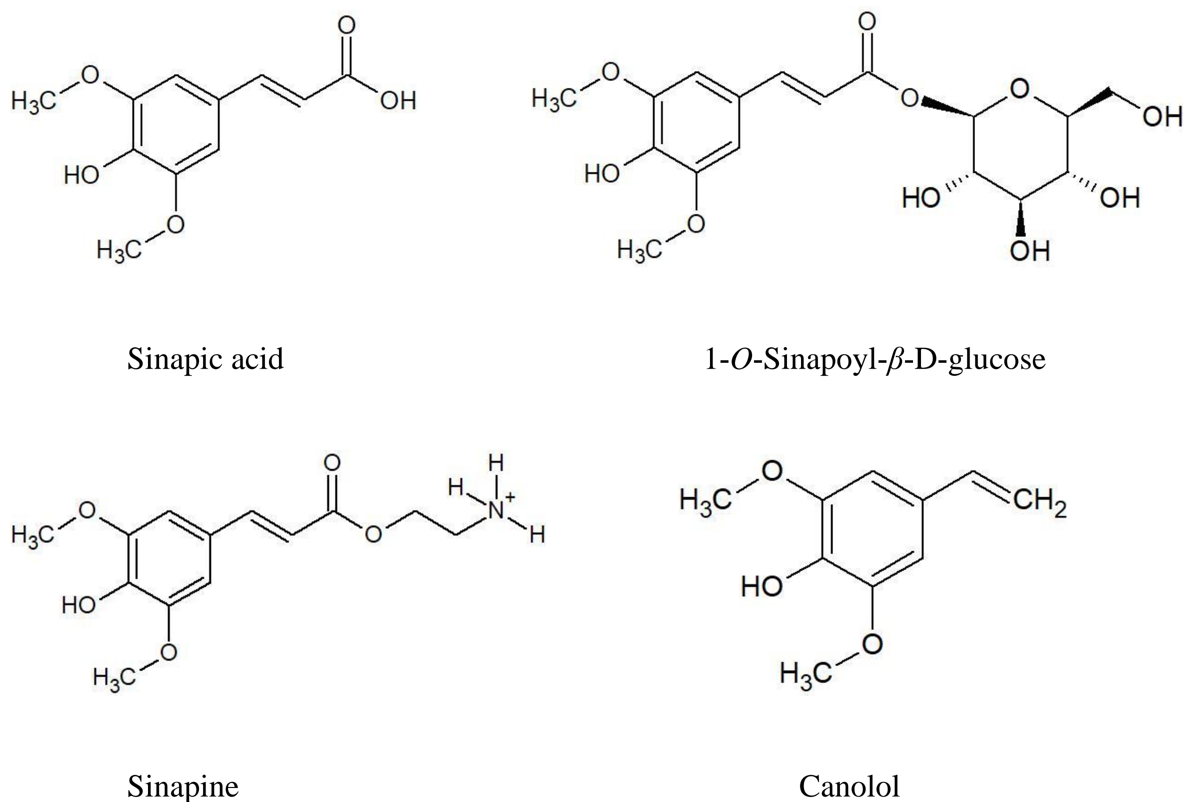
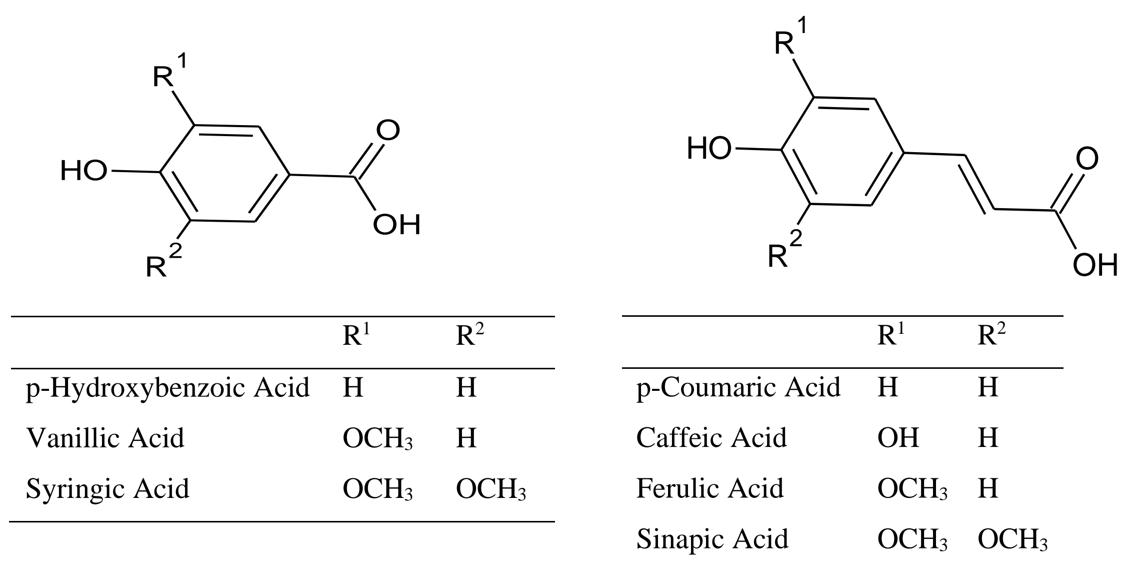


Figure 2.1 Chemical structures of sinapic acid and derivatives

2.2.3 Natural phenolic compounds of canola

Sinapic acid and its derivatives are dominant phenolic antioxidants in *Brassicaceae* species such as rapeseed, canola (*B. napus* L.), and mustard (*Brassica juncea* / *Sinapis alba*), and are well-known to be effective as synthetic antioxidants (Jun, Wiesenborn, & Kim, 2014; Nićiforović &

Abramovič, 2014). Sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid) can be found in both the free and esterified forms. Sinapine, the choline ester of sinapic acid, and sinapoyl glucose (1-*O*-*β*-D-glucopyranosyl sinapate), the sugar ester of sinapic acid, are two common sinapoyl esters found in oilseeds (Nićiforović & Abramovič, 2014). Sinapic acid itself comprises more than 73% of the free phenolic acids whilst sinapine (choline ester of sinapic acid), the key phenolic ester, constitutes around 80% of overall phenolic compounds (**Figure 2.1**) (Chen et al., 2014).



	R^1	R^2
p-Hydroxybenzoic Acid	H	H
Vanillic Acid	OCH_3	H
Syringic Acid	OCH_3	OCH_3

	R^1	R^2
p-Coumaric Acid	H	H
Caffeic Acid	OH	H
Ferulic Acid	OCH_3	H
Sinapic Acid	OCH_3	OCH_3

Figure 2.2 Structure of major phenolic acids in canola and rapeseed

Furthermore, decarboxylation of sinapic acid could occur throughout oilseed processing at relatively higher temperatures. It was subsequently found that “canolol,” a novel antioxidant compound (2,6-dimethoxy-4-vinyl phenol / vinylsyringol), could be formed by decarboxylation at high operating temperatures and pressures of oil extraction (Galano, Francisco-Márquez, & Alvarez-Idaboy, 2011). Moreover, *p*-hydroxybenzoic, vanillic, gentisic, protocatechuic, syringic, *p*-coumaric, ferulic, caffeic, and chlorogenic acids are some other common phenolic compounds

which are present in rapeseed, particularly in canola seed/meal (**Figure 2.2**) (Kozlowska et al., 1983).

The amount of phenolic compounds in canola extract depends on the extraction method used, suggesting that the extraction solvent and isolation steps should be examined to maximize the phenolic compounds in canola seed extracts (Jun et al., 2014). Thus, the solvent selection is the primary factor affecting the amount and the rate of antioxidants extracted by the conventional method of extraction (Hassas-Roudsari et al., 2009). However, most of the phenolic compounds extracted into the oil during the extraction process depreciate during the chemical refining, while a high proportion of unsaturated fatty acids makes them highly susceptible to oxidation (Chen et al., 2014).

2.3 Industrial approach toward mechanical and solvent extraction

As mentioned earlier, rapeseed and canola undergo two extraction processes: mechanical extraction followed by solvent extraction. During mechanical extraction, the oil is separated from the seed by mechanical pressure. There are two main techniques of extraction, batch hydraulic pressing and continuous mechanical pressing, which can be further subdivided into cold and hot pressing (Akpan, 2012). The major difference between cold and hot pressing is the application of temperature. In general, it is believed that cold pressed oils have a better ability to preserve the organoleptic properties compare to the hot-pressed oils. However, the recovery yield of the cold pressed oils is much lower than for the hot-pressed oils. In addition, hot pressed oils have a rich aroma and flavor and might contain some novel phenolic compounds, including canolol and its derivatives, which may be potent antioxidants (Galano et al., 2011). However, the major economic drawback of mechanical extraction is that the majority of the oil gets trapped during the process.

This bottleneck can be mitigated by solvent extraction (Carré & Pouzet, 2014). Solvent extraction depends mainly on the dispersion extraction procedure of oilseeds, where the expression "extraction" describes the ability of a solvent for the aggregation of fluid via liquid-solid components (Akpan, 2012). Solvent extraction has been carried out using many different organic solvents, although the primary one is hexane (Carré & Pouzet, 2014). Most industries used a combination of mechanical extraction and solvent extraction to obtain higher yields, followed by additional purification or refining steps to remove problematic minor components (**Figure 2.3**).

Due to the complexity of the extraction process, industries are currently facing many challenges including plant and ecological protection issues, organic compound volatility (0.2-2.0 gal/ton), elevated production costs (shipping, cargo, and dumping), and a massive quantity of organic solvent used and additional phases in processing. The pressed cake also requires additional processing steps including de-solemnization and toasting to remove excess solvents. However, these conditions may alter the nutritional quality of the meal due to the loss of thermo-labile components (Akpan, 2012).

2.3.1 Extraction of phenolic compounds from canola

Conventional solvent extraction was commonly used as a convenient method for the extraction of phenolic compounds from rapeseed meal or hulls in the early days (Amarowicz, Naczk, & Shahidi, 2000; Wanasundara & Shahidi, 1994). Various solvents, including methanol, ethanol, acetone, and ethyl acetate, with different proportions of water, have been extensively studied for extracting oilseeds including canola products (Wanasundara & Shahidi, 1994). Among the factors affecting the yield of phenolics were the oilseed cultivar, solvent type, extraction time, temperature, sample-to-solvent ratio, and the properties of the extracted materials (Stalikas, 2007).

In addition, extraction efficiency can also be affected by the chemical nature of target phytochemicals, extraction technique, particle size, and the existence of tampering materials such as hulls, seeds, and other impurities (Stalikas, 2007).

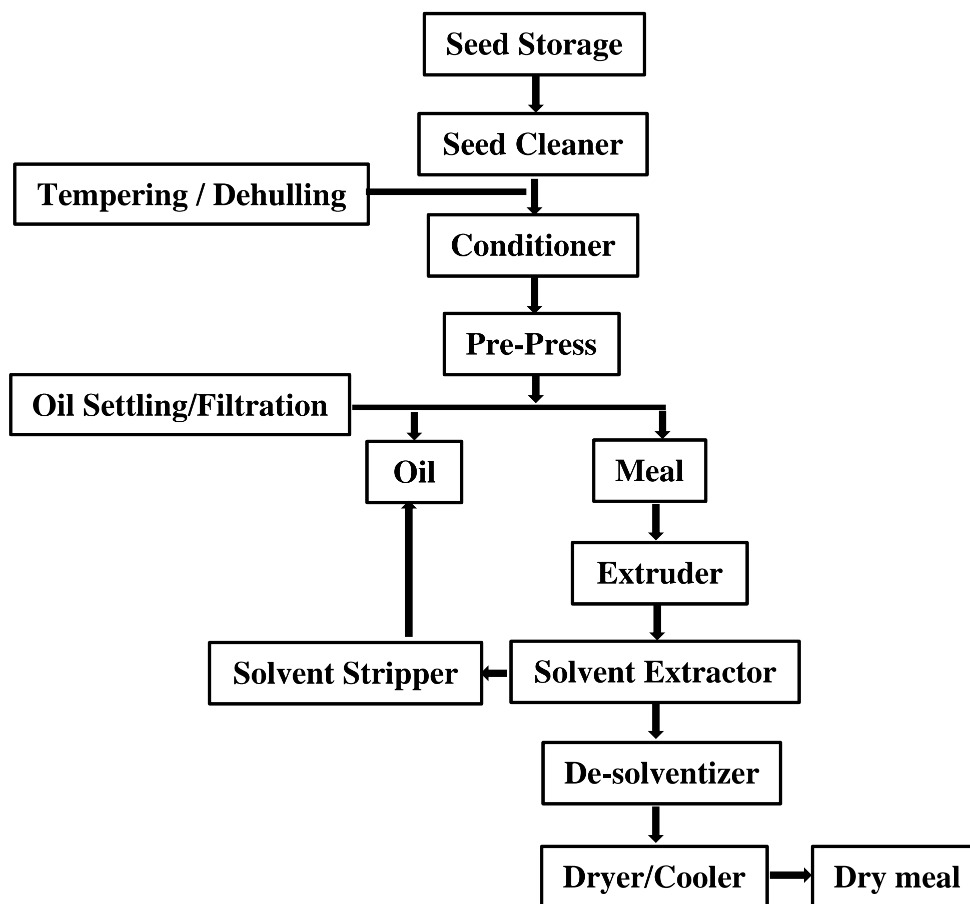


Figure 2.3 Oil manufacturing procedure from rapeseed/canola (Gunstone (2011)

The concentration of some phenolic compounds was shown to increase when extracted at high temperatures (Azargohar et al., 2013). This was especially true for canolol, which was formed from sinapic acid by decarboxylation (Galano et al., 2011). This phenomenon was confirmed by alterations in the UV spectrum, with the two main peaks for sinapic acid changing from 230 and 320 nm to three main peaks 210, 225, and 310 nm. Approximately 40% of sinapic acid was

reported to be transformed into novel elements during heat treatment (Cai et al., 1999). In addition, during the heating process, some new phenolic compounds were formed (**Figure 2.4**) (2,6-dimethoxyphenol, 4-ethylphenol, 2-methylphenol, phenol, etc.), fatty acids (e.g., oleic acid), phenolic acids (e.g., 4-hydroxybenzenesulfonic acid), ketonic compounds, and benzyl alcohol (Azargohar et al., 2013; Cai et al., 1999).

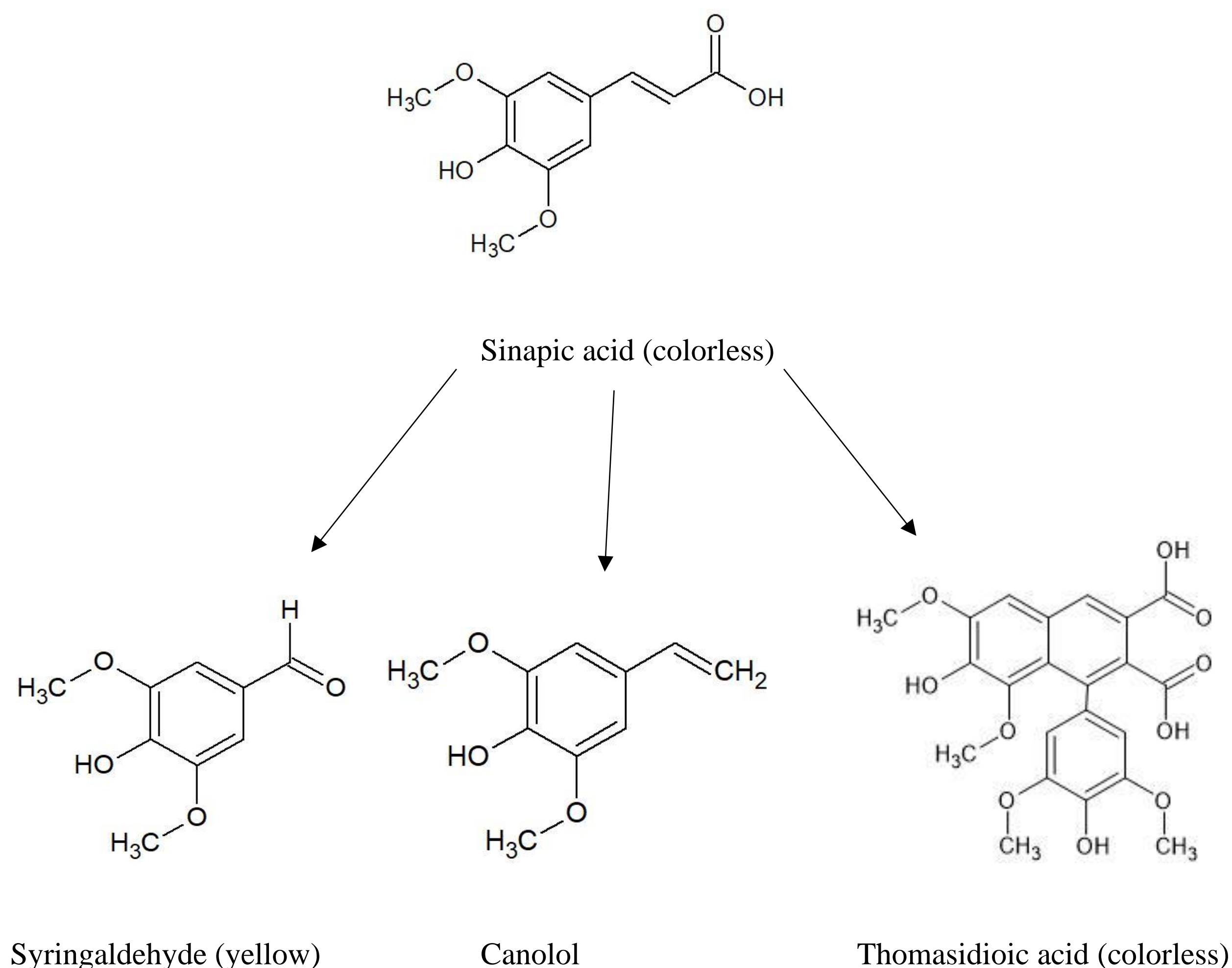


Figure 2.4 Heat-related novel phenolic compounds associated with rapeseed/canola

It is evident from the results that rapeseed and canola are rich sources of phenolic content and could be applied in the pharmaceutical and nutraceutical industry. However, the extraction of

these phenolic compounds has been mainly focused on solvent extraction using different solvents. The classical solvent extraction method is Soxhlet. This has been extensively employed to extract many compounds including phenolics from a solid matrix using a variety of different solvents. The preferential solvent extractants used alongside with Soxhlet are hexane, acetone, ethyl acetate, methanol, ethanol, propanol, and their combinations (Garcia-Salas et al., 2010). The Soxhlet/reflux extraction methods normally operate near the boiling point of the solvent employed. While this method is relatively simple, it is energy-demanding and uses a large amount of solvents (Luthria, 2006). Current environmental concerns demand a reduction in use of solvents and more green extraction methods. This has led to the development of more energy-efficient and eco-friendly green techniques that still produce high yields. Such processes also reduce the utilization of toxic solvents and develop better-quality products.

These advantages have been welcomed by the oilseed industry and encouraged by manufacturers and associates in food science and technology. Green techniques have thus gained attention from the food industry and been adopted by many industries due to its great applicability and environmental benefits. Green extraction technologies such as ultrasonic-aided extraction (UAE), microwave-aided extraction (MAE), supercritical fluid extraction (SFE), and subcritical water extraction (SWE) are receiving closer attention from the oilseeds industry.

2.4 Green technology

The use of new alternative nonconventional methodologies has gained considerable attention during the last decade. These novel techniques have been applied in the oilseed industry to minimize any detrimental changes in the nutritional quality and physicochemical and sensory properties of the extracted oils, while at the same time reducing the carbon footprint from fossil-

derived solvents (Koubaa et al., 2016). A general definition of green extraction is one in which there is a reduction in energy consumption and solvent use while obtaining sustainable natural products, with the guarantee of a safe and pure extract (Chemat, Vian, & Cravotto, 2012). Furthermore, this technique could be applied on a lab or industrial scale by improving its current operation, by the use of non-devoted equipment, and application of innovative operations using alternative solvents (Chemat et al., 2012). The basic concept of green technology is to develop and utilize procedures that minimize/eliminate the use of environmentally hazardous materials (Mustafa & Turner, 2011).

2.4.1 Principles of green extraction

The concept and principles of green extraction of natural products were presented by Chemat *et al*. (2012). This was in response to the urgent need to meet the competitiveness of the world market while at the same time protecting the environment. Rather than continuing to use the previous environmentally and energy-demanding methods, new and sustainable processes were desperately needed. The new green extraction methods required innovation that optimized the consumption of raw materials, solvents, and energy. The following six principles for the green extraction of natural products were presented and were designed to innovate not only processes but also all aspects of solid-liquid extraction.

Principle 1: Innovation for the selection of varieties and the use of renewable plant resources.

Principle 2. Use of alternative solvents and principally water and agro-solvents.

Principle 3: Reduce energy consumption by energy recovery and using innovative technologies.

Principle 4: Production of coproducts instead of waste to include the bio- and agro-refining industry.

Principle 5: Reduce unit operations and favor safe, robust, and controlled processes.

Principle 6: Aim for a nondenatured and biodegradable extract.

These six principles are considered essential to meet the demands for extracting natural products in the 21st century. Such guidelines presented by Chemat *et al*. (2012) were needed to safeguard both the environment and consumers, while increasing competition between industries with more eco-friendly, cost-effective, and innovative processes.

2.4.2 Green extraction techniques

2.4.2.1 Ultrasonic/ultrasound aided extraction (UAE)

Ultrasound/ultrasonic aided extraction (UAE) is one of the most common and widely used green extraction methods by industry. This technique usually uses higher frequencies over 20kHz under mechanic vibrations (Tiwari, 2015). The principle underlying this technique is acoustic cavitation, which involves a series of high-pressure (compression) and low-pressure (rarefaction) cycles (favoring penetration and transport) following each other at high frequency. This results in the formation of small bubbles which collapse rapidly, creating physical and chemical changes in the media that assist the transport phenomena displacing separation stability (Bogdanov, 2014). In addition, mechanic vibrations enable disruption and thinning of the cell membranes (**Figure 2.5**), creating a consistent mass diffusion of analytes from the solid matrix into the solvent (Takeuchi et al., 2009). Consequently, during the implosion of bubbles, temperature and pressure rise up to

5000K (4727°C) and 2000atm (29,392 psi), respectively, creating a momentum high enough to disrupt the cellular matrix (Bogdanov, 2014).

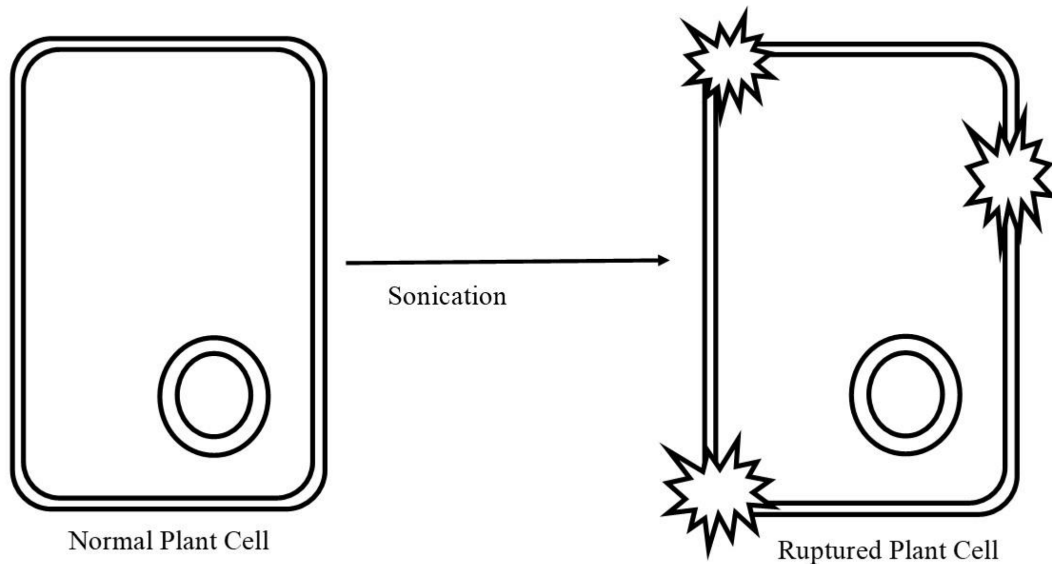


Figure 2.5 Schematic diagram of illustrating the effect of ultrasonic treatment on cells

The UAE technique can be further subdivided into two types depending on its intensity: low-intensity sonication (<1 Wcm^{-2}) and high-intensity sonication (10-1000 Wcm^{-2}). Low-intensity sonication (<1 Wcm^{-2}) is usually applied in process control as well as quality assurance whereas high-intensity sonication (10-1000 Wcm^{-2}) is usually used in the extraction process (Tiwari, 2015). This UAE technique is again subdivided into two types based on the mode of application: direct and indirect. Direct approaches use ultrasonic horns/probes to sonicate the sample. The probe usually gets immersed in the sample, reducing the barrier between the sample and the source in the direct method of application. Indirect applications involve an ultrasonic bath (Santos, Veggi, & Meireles, 2012). The major distinction between the two techniques is the

intensity of the ultrasonic waves produced, as the probe is able to generate 100 times higher intensity than the sonication bath (Santos et al., 2012).

Optimization of ultrasonic extraction conditions normally depends on the type of compounds and the properties of the matrix. The most common extraction conditions for the ultrasonic extraction of phenolic compounds include a sonication power of 90-150W, a frequency of 20-60Hz, a time of 2-30min and 1-5cycles (Santos-Buelga et al., 2012). An increase in extraction time, cycles, power, or frequency could aid the auto generation of heat, which could affect the degradation of thermally labile phenolic compounds (Santos-Buelga et al., 2012). The major advantages of the application of UAE include its ability to operate at ambient temperature and pressure. This method particularly favors the extraction of the more thermo-labile compounds. Thus, a reduction in extraction time and in use of solvents combined with its simple equipment design are all additional advantages associated with UAE (Capote & De Castro, 2006). However, obligatory filtration and rinsing, and inability to refurbish the solvents, are the main shortcomings of UAE. Other drawbacks associated with UAE include that the intensity of the ultrasonic waves generated may not be uniform (Kiani et al., 2011). This is evident by regions of high and low intensities identified using the foil test (**Figure 2.6**). High cavitation areas could be located by regions of the foil with holes (Kiani et al., 2011). The use of this technique is encouraged by the food industry, although it still has a number of limitations. Nevertheless, this technique has demonstrated promising results in seed oils by shortening the extraction time, reducing the solvent usage, and producing high value-added compounds in pilot- and lab-scale experiments.

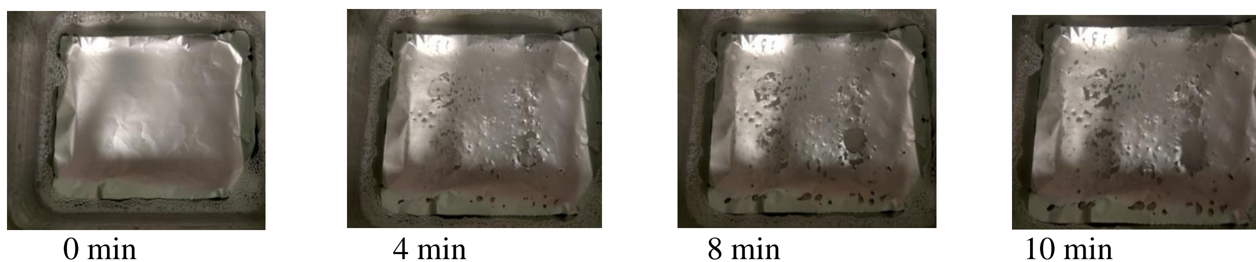


Figure 2.6 Foil test in ultrasonic water bath (VWR 250HT)

However, to date there are still a very limited number of studies conducted on a commercial scale to estimate the efficacy and effectiveness of the UAE technique. Nevertheless, a wide range of foods applications have been tested using this method including emulsification and homogenization (Kentish et al., 2008), crystallization (Gogate & Kabadi, 2009), and alteration of viscosity (Gallego-Juárez, Rodriguez, Acosta, & Riera, 2010; Jambrak et al., 2010). Furthermore, a very limited amount of work has been carried out to assess the economic benefit of UAE and the energy requirement per kg/ton of treated seeds compared to the more traditional techniques. A recent study by Perrier *et al*. (2017) found that combining ultrasound application with isopropanol appeared to be an alternative solution to hexane for obtaining higher oil recovery from rapeseed flakes.

2.4.2.2 Megasonic-assisted aqueous extraction (MSAE)

In recent years, ultrasonic/ultrasound aided extraction (USE) has shown promising results in industrial oil extractions, while the application of MSAE has been very limited. Megasonic separation is similar to USE but uses much higher frequencies (0.4-2MHz) (Gaber et al., 2019). During the application of MSAE, the acoustic field exerts Bjerknes radiation forces, which facilitate the movement of particles and droplets toward pressure nodal or anti-nodal planes based

on the density and compressibility of the particles (Gaber et al., 2019). This phenomenon results in the amalgamation of the droplets into a larger mass with lower density. In contrast, bubble microstreaming with the application of MSAE also contributes to the oil removal by aiding the action of cell-wall breaking enzyme activity or by facilitating the removal of oil from solid matter (Juliano et al., 2017). Other parameters including the level of frequency, ultrasound power, liquid to solid material ratio, duration of sonication, temperature of sonication, and solvent temperature also influence the effectiveness of MSAE (Gaber et al., 2019).

Application of the MSAE technique includes the industrial extraction of oil, the separation of fat from milk and whey, creaming enhancement (Juliano et al., 2011), and removal of residual oils from by-products such as canola meal (Gaber et al., 2019). A recent study by Gaber *et al.* (2019) found that MSAE improved oil quality by having very low free fatty acid (FFA) levels (0.36%) compared to regular industrial oil extractions (between 0.5% and 0.8%) (**Table 2.2**). Free fatty acids (FFA) are considered as one of the quality parameters of frying. Lower FFA levels are generally associated with deodorization at lower temperatures and inhibit the smoking of oils at lower temperature levels (Cmolik & Pokorny, 2000). Furthermore, MSAE produces oils with a lower chlorophyll content, which reduces the amount of bleaching clay needed for de-pigmentation. In addition, the MSAE oils are much lower in peroxides with less off-flavors and off-odors. These findings further support MSAE as a viable alternative method for oil extraction.

Table 2.2 Canola oil quality parameters in mega-sonic trial and industrial specifications (Gaber et al., 2019)

Oil quality Parameters	Units	Mega Sonic Assisted Extraction (MSAE)	Industrial Specification
Free Fatty Acid	g acid oleic, %	0.36	0.5 - 0.8
Chlorophyll	ppm	1.0	10 - 30
Peroxide	ppm	0.90	10.0
Phosphorous	ppm	32.0	70 - 100

2.4.2.3 Microwave-assisted extraction (MAE)

Microwave-assisted extraction (MAE) is a distinct technique commonly applied for the extraction of phenolic compounds as well as to improve the extraction efficiencies. Microwaves are characterized as nonionizing electromagnetic radiations in the range of frequency from 300MHz to 300GHz (Carré & Pouzet, 2014). MAE consists of two perpendicular oscillating electric and magnetic fields, and its heating principle is based on immediate consequences of the microwaves on polar molecules through an ionic transmission and dipole revolution mechanism (Krishnaswamy et al., 2013; Santos-Buelga et al., 2012) (**Figure 2.7**).

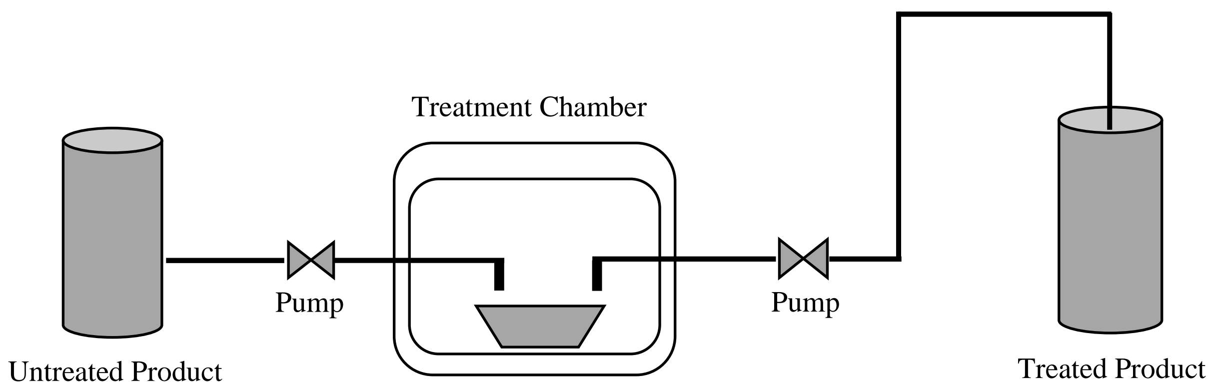


Figure 2.7 Schematic representation of continuous laboratory-scale microwave equipment

During the process of extracting, chemical compounds absorb microwaves approximately related to the solvent's dielectric constant. The dielectric constant and the level of microwave absorption have a positive correlation, providing relatively higher microwave energy with the increase in its frequency (Santos-Buelga et al., 2012). In addition, solvents with a higher dielectric constant appear to contain higher microwave-absorbing properties, which enhance the extraction yields (Santos-Buelga et al., 2012) (**Table 2.3**).

Table 2.3 Di-electric constant of common extractants at 20°C

Solvent	Dielectric constant (Fm^{-1})
Hexane	1.89
Toluene	2.4
Dichloromethane	8.9
Acetone	20.7
Ethanol	24.3
Water	78.5
Methanol	32.6

Thus, blending solvents extractants together could alter the extracting selectivity and the ability to interrelate with microwaves. With relatively higher microwave frequencies, solvent molecules fail to realign themselves and commence vibrating. For example, this phenomenon could lead to molecules vibrating at a rate of 4.9×10^{9} times per second at a frequency of 2.45GHz. This would further direct the development of thermal energy through friction and vibrations (Manadal, Mohan, & Siva, 2007). Thus, once the plant materials are exposed to microwaves, the water tends to evaporate and build up pressure in the cell matrix. The exertion of such forces ruptures the cell wall, thereby enabling better and more efficient extraction by the solvent (Veggi, Martinez, & Meireles, 2012).

Microwaves have been used for the extraction of anthocyanins from grapes (Liazid et al., 2011), phenolics from tomatoes (Li et al., 2012), flaxseed (Beejmohun et al., 2007), and grape seeds (Krishnaswamy et al., 2013). Reductions in the extraction time and energy-related costs are key advantages of this nonconventional technology. Furthermore, in comparison to the untreated samples, oil-aided extraction via microwave processing demonstrated comparable or elevated oil quality indicating the potential benefits to oilseed industry. In addition, this technique has proven to improve the shelf-life as well as the oxidative stability of the oils with promising results.

The key leverage of MAE is its fast extraction of analytes, together with the extraction of thermo-labile compounds (Santos-Buelga et al., 2012). Furthermore, the above is a rapid method of extraction that produces higher yields while lowering solvent consumption compared to conventional extraction techniques. However, few studies have been conducted so far to examine the performance of this technique on a small scale in uninterrupted mode.

2.4.2.4 Accelerated solvent extraction (ASE)

Most traditional extractions are based on solvent reflux techniques which require extensive time, a large amount of samples, and an excessive amount of solvents (Mustafa & Turner, 2011; Szydłowska-Czerniak & Tułodziecka, 2014). Another major drawback of traditional techniques is that they operate at atmospheric pressure, which prevents the operation temperature from exceeding the boiling point. This limits the mass transfer of the analytes across the matrix. This is markedly different for accelerated solvent extraction (ASE), which employs high pressure and high temperature to increase extraction efficiencies (Barros et al., 2013).

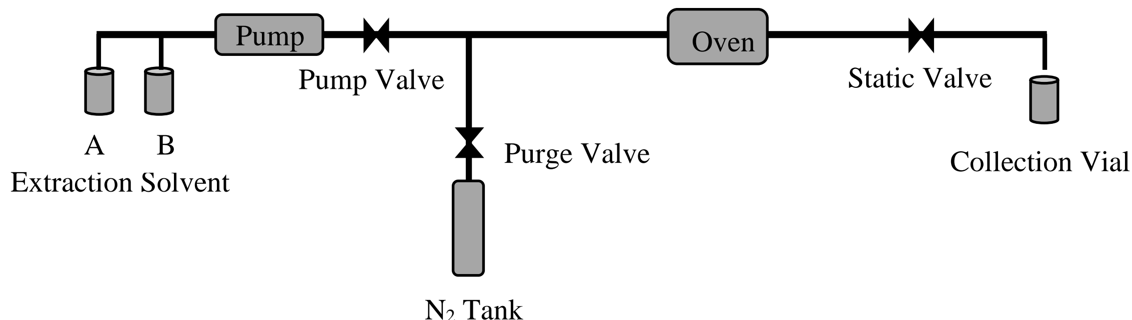


Figure 2.8 Schematic diagram of an ASE system

Dionex Corporation first introduced pressurized liquid extraction in 1995 and named this technique accelerated solvent extraction (ASE). It is also known as pressurized liquid extraction, pressurized solvent extraction, and enhanced solvent extraction (Mustafa & Turner, 2011). It generally operates at higher pressure conditions (~2000 psi), which allows the temperature of the extraction to exceed the flashpoint of the solvent, thereby enhancing the extraction kinetics of the matrix. During this process, stainless steel extraction cells filled with 1-50g of sample (solid/semisolid) are then extracted with organic solvents. Afterward, the extracted sample is flushed with clean solvent, followed by N_2 flush, and then collected in a glass bottle under nitrogen (**Figure 2.8**) (Ajila et al., 2011; Luthria et al., 2004).

The purging step with N_2 prevents any losses of extractants and facilitates the removal of solvents from the extraction cells and solvent tubes (Mustafa & Turner, 2011). The elevated temperature and pressure increase the mass transfer and reduce the viscosity of the solvent, facilitating easy diffusion into the sample matrix (Barros et al., 2013). The presence of moisture in the sample matrix often affects the extraction efficiency. Therefore, a hydro-matrix is often used to absorb water and moisture from the sample matrix to aid the extraction process (Mustafa & Turner, 2011). During the extraction process, dispersing agents are also often used to reduce the

cell volume and the usage of solvents. This method uses small amounts of samples and substantially fewer solvents and produces higher yields in a relatively short period of time (**Table 2.4**) (Luthria, 2006).

Table 2.4 Assessment of different extraction techniques

Technique	Average Time* (hours)	Solvent Usage* (mL)	Cost per Sample (USD)
Soxhlet	4 - 48	150 - 500	20 - 25
Automated Soxhlet	1 - 4	50 - 100	15 - 20
Sonication	0.5 - 1	150 - 200	17 - 23
Supercritical Fluid Extraction	0.5 - 2	5 - 50	15 - 20
Microwave	0.5 - 1	25 - 50	12 - 18
Accelerated Solvent Extraction	0.2 - 0.3	5 - 150	10 - 15

[*Based on 2000 samples/year Costs (USD)]

ASE 350 itself is fully automated and the extractor could be used with different solvents (Luthria, 2006). This could be applied for the selective extraction of the compound of interest with specific polarity. Furthermore, the use of extraction cells eliminates the additional filtration steps associated with extraction, since the matrix residue is preserved inside the extraction cell (Carabias-Martínez et al., 2005). In addition, the application of an inert atmosphere during the extraction facilitates the yield of readily oxidizable phenolic compounds (Santos-Buelga et al., 2012). In addition, the new model ASE 350 is equipped with both static and dynamic extraction techniques. This further allows the system to introduce fresh solvents during the extraction process (Mustafa & Turner, 2011). Furthermore, the static mode consists of one or more extraction cycles with replacement of solvents between the cycles, whereas in the dynamic mode the pump executes solvents at a constant flow rate to achieve maximum extraction efficiency (Mustafa & Turner,

2011). With static extraction, however, complete extraction might not occur due to the limited volume of extraction fluids, therefore, multiple static extraction cycles are required to achieve the complete extraction (Luthria, 2006). The common solvents used with ASE are methanol, ethanol, and acetone with different proportions of water during the extraction process. Research has indicated that extractions through ASE could be carried out with a multitude of solvents at various pH and temperatures (Barros et al., 2013). This technique could also use water as an extractant and is referred to as pressurized hot water extraction or superheated water extraction (Mustafa & Turner, 2011).

Thus, its use of higher temperatures could produce polymeric phenolic compounds with antioxidant properties. However, elevated temperatures associated with ASE also appears to improve the organoleptic properties of the oils extracted as well as aid in the formation of novel phenolic compounds with higher antioxidant properties such as canolol (Li & Guo, 2016a, 2016b). Additionally, the extraction efficiency of ASE could be improved using smaller particle size samples (grinding), drying (reduction of moisture), or incorporation of a desiccant. In addition, ASE could be operated with one or combinations of two organic solvents during the extraction process depending on the polarity of the compound of interest (Santos-Buelga et al., 2012). Other researchers found that acidification could yield better results in terms of flavonoids specifically anthocyanins in comparison with methanol alone (Ponmozhi et al., 2011). Furthermore, ASE has been employed in the extraction process of phenolic mixtures from grape pomace, rosemary, marjoram, oregano, essential oil from lamiaceae plant, and edible oil from different oilseeds (Hossain et al., 2011; Matthäus & Brühl, 2001; Rajha et al., 2014; Rodríguez-Solana et al., 2015). Low solvent use, speed and convenience, high yields, and the low extraction costs per sample makes ASE one of the best methods for green extraction (**Table 2.4**).

2.4.2.5 Subcritical water extraction (SWE)

Subcritical water extraction is one of the eco-friendliest techniques by using water as the extraction solvent to extract bioactive compounds, essential oils, and organic pollutants (Yu et al., 2015). This technique uses water at its subcritical stage where water is forced to gain the liquid state beyond its boiling point and below its supercritical point (374°C, 3205psi) (Plaza & Turner, 2015). This process is 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. It is a very promising “green” technique using water as the only extraction solvent (Li & Guo, 2016a, 2016b). The unique solvent properties of water make it ideal for extracting both polar and nonpolar compounds, depending on extraction temperature.

Generally, temperatures between 100°C and 374°C (as the critical point of water is 374°C at 3200 psi) are used together with pressure <3200psi, to maintain water in the liquid state (Li & Guo, 2016a, 2016b). Room temperature (25°C), however, has also been used for SWE. The major drawback with water as an extractant is its high polarity. Increasing the temperature of water (over 100°C) significantly changes the physical and chemical properties as well as the dielectric constant of water (Teo et al., 2010). In addition, higher temperatures would facilitate the disruption of hydrogen bonding thereby, making dispersion forces more dominant (Plaza & Turner, 2015). Hence, the ability of water to dissolve less polar compounds including flavanols at an elevated temperature makes it a favorable solvent for extraction compared to other conventional methods (Santos-Buelga et al., 2012).

Table 2.5 Chemical and physical properties of water at different temperatures and saturation pressures

Property	25°C 0.1 MPa	100°C 0.1 MPa	200°C 1.5 MPa	350°C 17 MPa
Dissociation Constant, K_w	1.0×10^{-14}	5.6×10^{-13}	4.9×10^{-12}	1.2×10^{-12}
pK_w	13.99	12.25	11.31	11.92
Dipole moment	1.85	1.85	1.85	1.85
Density (g/cm^3)	0.997	0.958	0.865	0.579
Relative Static Permittivity, ε_r	78.5	55.4	34.8	14.1

Further, with the increase in temperature permittivity, the thickness and surface tension of the water are reduced, leading to an increase in its diffusivity characteristics (Teo et al., 2010). This changes the dielectric constant of water from 78.5 at 25°C to 34.8 at 200°C, which is in close proximity to the dielectric constant of methanol (ε=33) (**Table 2.5**). By varying the temperature, water could be used to mimic a methanol–water mixture or ethanol-water mixtures or acetone (ε=21) (Plaza & Turner, 2015). This is a major advantage of using SWE, where water could be applied to impersonate different solvent systems. Additionally, it could help to reduce or replace different environmentally harmful solvents.

This method has been applied for environment protection to extract organic pollutant polycyclic aromatic hydrocarbons (PAH), to extract volatile compounds from plant materials, to identify the pesticide content from soil, to recover polyphenol compounds including catechins and proanthocyanidins in wine industry (García-Marino et al., 2006), to extract lignans and associated compounds from flaxseed meal (Kanmaz, 2014), and to extract canola meal phenolic compounds (Hassas-Roudsari et al., 2009).

A study conducted by Ibañez *et al*. (2003) found that SWE extracted most active antioxidant compounds including carnosol, rosmanol, carnosic acid, methyl carnosate, and flavonoids such as cirsimaritin and genkwanin. Kim and Mazza (2006) later reported that the extraction efficiency of SWE was improved at high temperatures and relatively higher NaOH concentrations. They further reported that extraction temperatures ranging from 140°C to 160°C extracted higher yields of lignans and other bioactive peptides (Cacace & Mazza, 2006). Moreover, García-Marino *et al*. (2006) demonstrated that SWE extracts had greater antioxidant activity compared to both ethanol extracts and ultrasound-assisted extracts (UAE) and were similar to Soxhlet extracts.

The higher capital required for operating/pressurized system, however, could be the major drawback of this application (Liang & Fan, 2013). Furthermore, the operation costs of SWE are relatively high compared to those for other extraction techniques. Nevertheless, the use of SWE for extracting antioxidants is a feasible option as the purity and extraction efficiency of the phenolic antioxidants will compensate for the higher extraction costs (Ramos, Kristenson, & Brinkman, 2002). Since water as an extraction material is cheap, renewable, and nontoxic, SWE has a huge potential for scaling up in the future.

Thus, the use of SWE in the extraction of antioxidants seems to be a feasible option since price will not be affected due to its purity and the extraction efficiency of the phenolic antioxidants (Ramos et al., 2002). Extraction of thermo-labile compounds is another problem associated with this technique. Nevertheless, the use of water as the extraction material is cheap, renewable, and nontoxic, and thus has a huge potential for scaling up in near future.

2.4.2.6 Supercritical fluid extraction (SFE)

Supercritical fluid extraction (SFE) is a distinct technique, which is quite similar to subcritical water extraction. This extraction technique has been extensively applied, including natural phenolic compounds. Interest in SFE has increased over time due to the high quality of the final product, the absence of toxic residues, and the preservation of the active ingredients (Pereira & Meireles, 2010). Thus, supercritical fluids are very useful for both the extraction of bio-actives and the removal of undesirable compounds. Furthermore, this method can be programmed (at a specific pressure and temperature) to extract specific compounds (Pereira & Meireles, 2010). In reality, any type of solvent can become a supercritical solvent. Thus, cost, toxicity, technical feasibility, and salvation power could determine the most appropriate solvent for exact function (Pereira & Meireles, 2010).

CO_2, however, is the most commonly used in SFE due to its relatively lower cost, availability, and nontoxic nature. In addition, propane, ethane, hexane, pentane, and butane are some other types of solvents that can also be applied in SFE as cosolvents. It has been shown that cosolvents increase the solubility in oil, making them very important in improving the efficacy of this process (Hussain, 2014). These cosolvents form hydrogen bonds with solutes of oil and increase the density of supercritical carbon dioxide, creating elevated solubility of the solvent in solutes of crude oil. Furthermore, the effect of cosolvents solubility followed the order of ethanol > methanol > propanol > acetone (Hussain, 2014). Of these, however, methanol and ethanol are the most commonly used cosolvents in SFE. Moreover, it was found that using cosolvents such as 10% ethanol improved the SFE extraction of the phenolic compounds (HadiNezhad, Rowland, & Hosseinian, 2015). A comparative study between SFE and conventional extraction showed that

while there was no difference between the total extraction of phenolic compounds, the concentrations of individual phenolic compounds were between four and 10 times greater using SFE (Pinelo et al., 2007). Other studies have found that prehydrolyzation of the seeds improved the solubility of the matrix which resulted in higher extraction yields (Comin, Temelli, & Saldaña, 2011). These researchers also found that combined mode of static/dynamic extraction (running and soaking in CO_2) further increased the overall extraction yield and shortened the total extraction time. These studies indicated that there are many important factors affecting extracting efficiency of the SFE process including the design, position of the solute, particle size, shape, and porosity of the solid material, moisture content of the solid material, solvent flow rate, temperature, and pressure (Pereira & Meireles, 2010).

2.5 Application of green technology for the canola industry

Plant secondary metabolites, including phenolic compounds, are a mixture of different classes. The complexity of the phenolics can affect their extraction efficiency and yield requiring additional steps to exclude unwanted substances when isolating specific phenolic compounds (Santos-Buelga et al., 2012). The most common methods of isolation include solid phase extraction (SPE), column chromatography (CC), and droplet countercurrent chromatography (DCCC) (Santos-Buelga et al., 2012). Coupling an isolation technique with green extraction could facilitate better extraction efficiencies for the oilseed industry. Thus, capital investment and a restricted range of applications make green extraction limiting. Yet many small-scale pilot versions have been applied by the oilseed industry for measuring the feasibility and the extraction efficiencies of green and eco-friendly extraction techniques. Canola/rapeseed, being major oilseeds, have been widely studied with respect to their applicability to green technology, particularly their phenolic compounds. For example, UAE extraction of rapeseed resulted in better extraction efficiency and higher total phenolic content compared to MAE (Szydłowska-Czerniak & Tułodziecka, 2014; Yang et al., 2013). These studies nevertheless confirmed that ability of both ultrasound or microwave technology to produce a phenolic antioxidant as a potential nutraceutical from canola by-products (Yang et al., 2013).

The application of SWE has also proven to be an effective technology for producing greener extracts compared to conventional methods of hydro-distillation (Khajenoori et al., 2009). Temperature, pressure, and solvent modifier intensities are some factors that affect the solubility of the phenolic compounds during the subcritical extraction process (Hussain, 2014). Application of SFE and SWE to rapeseed has both demonstrated that controlling the extraction parameters

could facilitate extraction of individual phenolic compounds, including sinapic acid (HadiNezhad et al., 2015).

Of the abovementioned techniques discussed so far, ASE is the most economical and eco-friendly green method for extraction of phenolic compounds from oilseeds. For instance, 10g of oilseed sample can be extracted in about 10-12min using the ASE system, compared to 24h using Soxhlet extraction (Luthria et al., 2004). Furthermore, elevated temperatures facilitate the release of targeted analytes by providing thermal energy to overcome interactions (hydrogen bonds, dipole attraction, etc.) (Galano et al., 2011). The ability of the phenolic compounds to withstand the higher temperatures makes it an effective method for their extraction from oilseeds.

2.6 Summary and conclusion

The above information indicates the importance of green technology toward the oilseed industry as well as its applications. The advantages of extraction of phenolic compounds over conventional methods make green extraction one of the most applicable methods of extraction of phenolic compounds on oilseeds. Furthermore, current interest in environmental conservation has gained attention in many fields including the research field. Many studies have been conducted to find and apply research techniques related to green technology in different applications. At the time of writing, the oilseed industry elutes a lot of waste chemicals as well as harmful substances in the process of oil refining. This has been a major issue until now and industry is still searching for an alternative method that could reduce the carbon footprint and aid in reducing production costs. The literature data presented in this review demonstrated that alternative methods including MAW, ASE, USE, SWE, and SFE could be of interest. However, the major disadvantage of all these alternative techniques is the high capital or investments associated with them compared to the conventional methods. Many instances the higher capital and operational costs have been mistaken with the novel techniques that cannot contend with traditional methods. Adding this to facts that the above techniques involve clean technology, we could expect green extraction to prosper in many fields, including the oilseed industry, in the near future.

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BRIDGE TO CHAPTER 3

In chapter 2, a detailed analysis of the literature related to different phenolic extraction conditions with the emphasis towards the canola industry was explained. Further, the importance of green extraction in canola industry was explained in detail. Antioxidant activity plays a key role in the food industry as a shelf-life enhancer and facilitates in oxidation reduction. Canola meal is rich in antioxidative phenolic compounds. Chapter 3 comprises the optimization of the extraction of antioxidative phenolic compounds using accelerated solvent extraction (ASE). Different canola meal extractants obtained by ASE was assessed for their antioxidant capacity using different *in-vitro* assays including FRAP, DPPH and metal ion chelation activity.

AUTHOR CONTRIBUTIONS FOR CHAPTER 3

Antioxidative polyphenols of canola meal extracted by high pressure: impact of temperature and solvents. **Nandasiri R.**, Eskin N. A. M., Thiyam-Holländer U. Journal of Food Science, 2019, 84 (11), 3117-3128. Ruchira Nandasiri designed the study, performed the experiments, conducted the statistical analysis, and wrote the first draft of the manuscript. Both Dr. Michael Eskin and Dr. Usha Thiyam-Holländer provided critical feedback on the manuscript with proof reading and assisted with the review comments. Dr. Usha Thiyam-Holländer obtained the funding.

CHAPTER 3

MANUSCRIPT I

ANTIOXIDATIVE POLYPHENOLS OF CANOLA MEAL EXTRACTED BY HIGH PRESSURE: IMPACT OF TEMPERATURE AND SOLVENTS

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Published in Journal of Food Science, 2019, 84 (11), 3117-3128.

3.1 Abstract

Canola meal, a by-product of oil-pressing, is a rich source of phenolic antioxidants. However, its use in the food and feed sector is still limited by the need for greener, sustainable, and more cost-effective extraction methods. This study used Accelerated Solvent Extraction (ASE) to enhance the extraction efficiency of the phenolic antioxidants. The high selectivity and short extraction time associated with ASE was ideal for obtaining high yields of these antioxidants. The structure-based activity of phenolic compounds may be influenced by the high pressure and temperature of the greener ASE process. The present study evaluated the effect of temperature (140, 160 & 180°C) and pressure (1500 psi) on the extraction and yield of phenolic compounds from canola meal as well as the solvent type (ethanol and methanol) and concentration (30%, 40%, 60% and 70% v/v). Antioxidant activity was determined by DPPH, FRAP, and Ion chelating activity. The highest yield of phenolic compounds was obtained with 70% methanol (20.72 ± 1.47 mg SAE/g DM) and 70% ethanol (24.71 ± 2.77 mg SAE/g DM) at 180°C temperatures. A similar trend was observed for the antioxidant activity of the extracts and their total flavonoid content. The structure-based antioxidant activity of the extracts examined increased with the increase in the percentage of the extracting solvent ($p > 0.05$). This study established ASE as an efficient green method for extracting phenolic compounds from canola meal, with potential application for the production of natural bioactive compounds from underutilized agricultural by-products.

Keywords - canola meal, accelerated solvent extraction (ASE), antioxidant activity, high temperature, and pressure

3.2 Introduction

Canola (*Brassica napus*), a major oil crop in Canada, accounts for more than 20% of the global production (Azargohar, Nanda, Rao, & Dalai, 2013). Furthermore, the phenolic content of canola is much greater than that of other oilseeds (Chen, Thiyam-Holländer, Barthet, & Aachary, 2014). Canola oil mainly consists of triglycerides, together with minor compounds such as free phenolic acids (vanillic, ferulic, *p*-coumaric, chlorogenic, caffeic, and so on), esterified phenolic acids, free fatty acids, proteins, and so on (Abuzaytoun & Shahidi, 2006; Alam et al., 2016; Alkan, Tokatli, & Ozen, 2011). Esterified phenolic acids are known to be the predominant phenolic compounds in canola meal, with sinapine being the most abundant (Cai, Arntfield, & Charlton, 1999). Each year, thousands of metric tons of defatted canola meal by-product are produced by the oil industry (Casseus, 2009). However, such defatted canola meal could be an invaluable source of natural antioxidants as well as phenolic compounds (Alam et al., 2016; Chen et al., 2014). These by-products could be potential nutraceuticals due to their strong antioxidant properties.

Consequently, different extraction systems were studied for their ability to extract these phenolic compounds (Chen et al., 2014). In many instances, these secondary metabolites and plant phenolic compounds are associated with the outer layers of the oilseeds, between lipoprotein bilayers and sometimes cell wall compounds, making these compounds difficult to remove via simple extraction systems (Corrales, Toepfl, Butz, Knorr, & Tauscher, 2008). The recent extraction of phenolic compounds by an accelerated solvent extraction system (ASE), using a combination of high temperature (>100 °C) and high pressure (1,500 psi), could provide a novel method for obtaining phenolic compounds from canola meals (Azargohar et al., 2013). ASE is an economical, environmentally friendly, and energy saving and yet effective and efficient technique that could benefit the food industry in many ways (Luthria, Vinjamoori, Noel, & Ezzell, 2004).

The use of high pressure and high temperature (>100 °C) during the extraction had many added advantages including the applicability of solid and semisolid samples as starting materials for extraction (Luthria et al., 2004).

High selectivity, short extraction time, and lower solvent usage provide the significant green technology advantages of the ASE method (Carabias-Martínez, Rodríguez-Gonzalo, Revilla-Ruiz, & Hernández-Méndez, 2005). The use of high pressure and temperature could also improve the solubility of the targeted phenolic compounds, the solute diffusion rate, and the mass transfer rate of the phenolic compounds, resulting in a relatively higher extraction efficiency (Li & Guo, 2016a). Its much shorter extraction time and the minimal use of green solvents makes it more attractive for use by the pharmaceutical and food industries in future years, by replacing many of the conventional extraction solvents and methods (Luthria et al., 2004). Up to date, there is a very limited amount of information available on the efficacy of ASE for specialized phenolic molecules. In addition, extraction parameters associated with ASE, such as solvent type, temperature, and concentration, are yet to be investigated. Furthermore, there is very limited information available related to the structure-based antioxidant capacity of ASE-derived canola extracts.

Based on the limited information, this study developed and optimized the method of ASE extraction and validated the structure-function-related antioxidant activity of canola phenolics. The results of the current study provide novel information on the structure-related antioxidant properties linked with ASE. To the best of our knowledge, this is the first report evaluating the antioxidant properties of canola meal extracts as affected by high pressure, temperature, and the type and concentration of the solvent extractants used.

3.3 Materials

Double expeller pressed canola meal (*B. napus* L.) with an oil content of 6% to 8% was used in this study. All the raw materials were obtained from the Viterra group, St. Agathe, Manitoba, Canada. Folin-Ciocalteu's (FC) reagent, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox, 97%), iron (II) chloride hexahydrate (98%), iron (III) chloride hexahydrate (97%), iron (II) sulphate heptahydrate (99%), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p-disulfonic acid monosodium salt hydrate (ferrozine, 97%), glacial acetic acid (99.8%), hydrogen chloride (HCl, 99%), sodium acetate, 2,4,6-tris-(2-pyridyl)-S-triazine (TPTZ >98%), L-ascorbic acid (>99%), sinapic acid (>97%), and 2,2-diphenyl-1 picrylhydrazyl (DPPH, 97%) were purchased from Fisher Scientific Canada Ltd. (Ottawa, ON, Canada). Quercetin hydrate (>95%) and 2-aminoethyldiphenyl borate (98%) were purchased from Acros (Mississauga, ON, Canada). All the extraction solvents, including methanol, ethanol, and hexane, were also purchased from Fisher Scientific Canada Ltd.

3.4 Methods

3.4.1 Sample preparation

Canola meal was cleaned and separated into two different samples based on the particle size. The cleaned meal was passed through a mesh (sieve sizes of 0.5 and 1.0 mm) of the sieve set (Ro-Tap Testing Sieve Shaker Model B, WS Tyler, Mentor, OH, USA) to obtain the two particle size samples. The particle size of the canola meal was confirmed via the Mastersizer 2000 (Malvern Instruments Ltd., Malvern, United Kingdom). Samples of both particle sizes (0.5 mm and 1.0 mm) were defatted using the Soxtec 2050 (Foss-Tecator, Foss North America, Eden

Prairie, MN, United States) to obtain the oil percentage of the samples (Khattab, Eskin, Aliani, & Thiyam, 2010). Sieved samples were kept at -20 °C until further analysis.

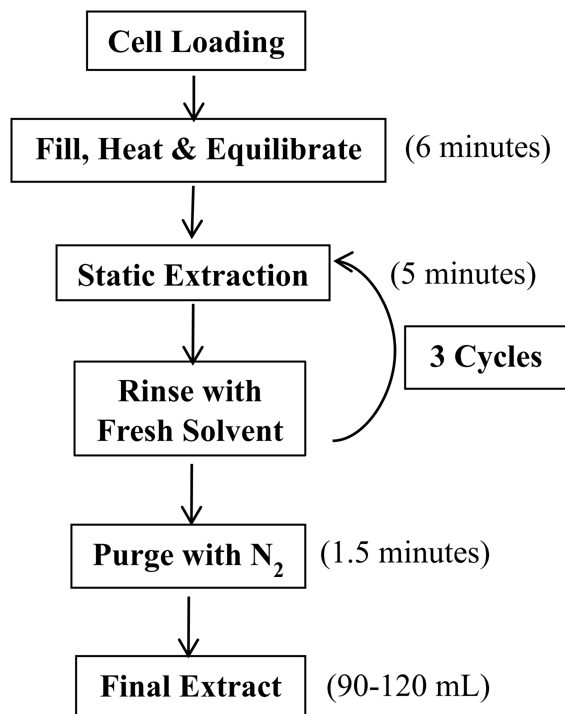

Figure 3.1 Extraction of phenolic compounds using ASE

3.4.2 Accelerated solvent extraction (ASE)

The phenolics in the canola meal samples were extracted using an accelerated solvent extractor (ASE 300, Dionex, Thermofisher Scientific, Mississauga, ON, Canada) (**Figure 3.1**). Both sieved samples were mixed with Ottawa sand in a ratio of 1:5 to optimize the extraction efficiency. Extraction was carried out using 33-mL cells. Cellulose filter paper (Fisher Scientific, Ottawa, ON, Canada) was placed at the bottom of each cell followed by filling it with samples. ASE was carried out at three different temperatures (140, 160, and 180 °C). Extraction solvents were methanol and ethanol in different concentrations (70%, 60%, 40%, and 30% v/v) to establish

the optimum conditions for the extraction of individual phenolic compounds with water as the control. The final extracts were concentrated using the rotary evaporator (BÜCHI Rotavapor® R-100, BÜCHI Labortechnik AG, Flawil, Switzerland) with samples freeze-dried in a Labconco 6 Freezone Freeze Dryer (Labconco Corp., Kansas City, MO, USA) at a temperature of -50 °C for 48 to 36 hr. All freeze-dried samples were reconstituted in methanol (≥ 99.9% v/v) and brought to the final volume of 30.0 mL (**Figure 3.2**).

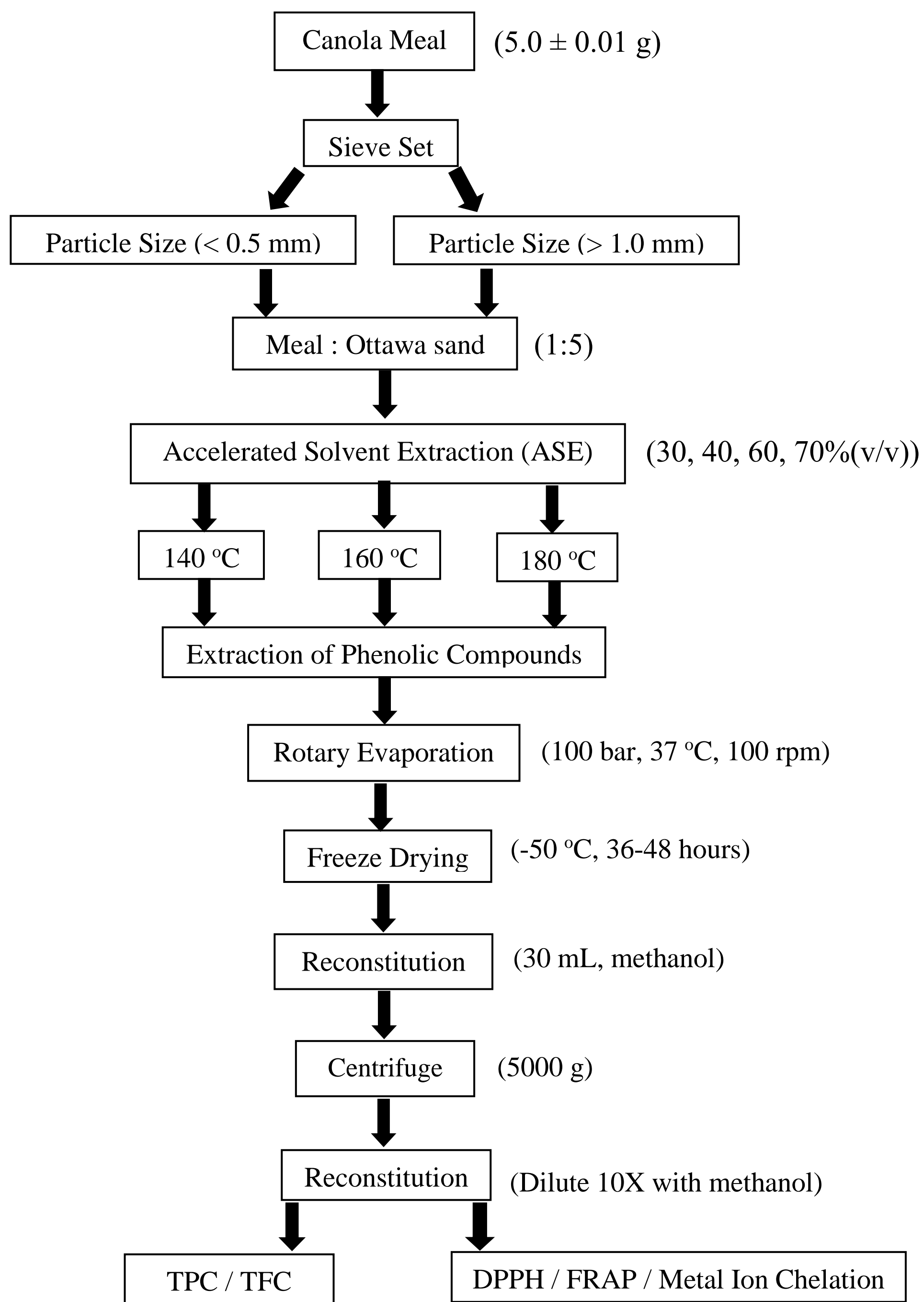

Figure 3.2 Extraction of phenolic compounds and quantification of antioxidant capacity

3.4.3 Determination of total phenolic content

The total phenolic content (TPC) of each extract was determined using the method described by Thiyam, Stöckmann, and Schwarz (2006) with slight modifications. Briefly, reconstituted canola meal extracts (0.5 mL) with Folin-Ciocalteu's reagent (0.5 mL) were added to a 10-mL conical flask. The sample mixture was vortexed, and the reaction was counterbalanced by the addition of 1.0 mL of 19% sodium carbonate solution and water (up to 10.0 mL). The reaction mixture was kept in the dark for an hour with intermittent shaking at 30-min intervals. The absorbance of the blue-colored complex was measured with the DU 800 UV/Visible Spectrophotometer (Beckman Coulter Inc., Mississauga, ON, Canada) at 750 nm. Methanol was substituted as the blank and the standard curve was prepared using sinapic acid in methanol (100%) at 1 mg/mL concentration (R^2 = 0.9856).

3.4.4 Determination of total flavonoid content

The total flavonoid content (TFC) was determined using the method described by Dave Oomah, Mazza, and Kenaschuk (1996) with slight modifications. Briefly, reconstituted and diluted (1.0 mL) extract wasmixed with 3.0mL of distilled water, followed by the addition of 100 µL of diphenylboric acid 2-aminoethyl ester solution (1% v/v). Absorbance was measured as per the TPC at 404 nm. Methanol was substituted for the extracts as the blank and the standard curve prepared using quercetin stock solution in methanol (100%) in 1 mM concentration (R^2 = 0.9994). The TFC was expressed as micromolar quercetin equivalents per gramof the meal (µM QE/g).

3.4.5 Ferric reducing/antioxidant power assay

The antioxidant capacity was determined using the ferric reducing/antioxidant power assay (FRAP), described by Benzie and Strain (1996), with slight modifications. FRAP working solution was prepared using a mixture of 200.0 mL of acetate buffer (300 mM, pH 3.6), 20.0 mL of TPTZ solution (10 mM), 20.0 mL of ferric chloride solution (20 mM), and 24.0 mL of distilled water. Briefly, 100 µL of reconstituted and diluted canola meal extract was mixed with 900 µL of water. After the vortex, the sample with 2.0 mL of FRAP reagent was added and kept at 37°C in the dark for 30 min and the absorbance of the colored complex measured as per the TPC at 593 nm. Methanol was substituted as the blank and the standard curve was prepared using Trolox stock solution in methanol (100%) in 5, 10, 20, 40, 60, 80, and 100 µM concentrations ($R^2 = 0.9924$). The results were expressed as micromolar Trolox equivalents per gram of the meal (µM TE/g).

3.4.6 DPPH free radical-scavenging assay

Antioxidant capacity based on free radical–scavenging activity was determined using the DPPH assay according to the method described by Thiyam et al. (2006) with some modifications. In brief, 50 uL of reconstituted and diluted canola meal extract was mixed with 2.95 mL of 0.1 mM DPPH solution in methanol. The absorbance of the colored complex was measured as per the TPC at 516 nm after 10 min.

Radical-scavenging activity was calculated using the following equation:

$$\text{Scavenging Effect (\%)} = \left[\frac{\text{Control Å}_{(516\,nm)} - \text{Sample Å}_{(516\,nm)}}{\text{Control Å}_{(516\,nm)}}\right] \times 100$$

3.4.7 Ferrous ion chelating activity assay

The ferrous ion chelation ability of different was measured using the method depicted by Dinis, Maderia, and Almeida (1994) with slight modifications. In summary, 0.4 mL of reconstituted and diluted extract was mixed with 0.05 mL of ferrous chloride solution (2 mM). The reaction was initiated by the addition of 0.2 mL of ferrozine solution and 3.35-mL water. The final solution was mixed thoroughly and kept at room temperature for 10 min. The absorbance of the colored complex was measured as per the TPC at 562 nm. Metal-chelating activity of the extract was calculated using the following equation:

$$\text{Scavenging Effect (\%)} = \left[\frac{\text{Control Å}_{(562\ nm)} - \text{Sample Å}_{(562\ nm)}}{\text{Control Å}_{(562\ nm)}}\right] \times 100$$

3.4.8 High performance liquid chromatography - diode array detection (HPLC-DAD) analysis of sinapic acid derivatives

The changes in phenolic composition, from extraction at high pressure and temperature, were evaluated using the High-Performance Liquid Chromatography (HPLC) method described by Harbaum-Piayda et al. (2010) with modifications. Individual major phenolic compounds present in canola meal were analyzed by reversed-phase High Performance Liquid Chromatography-Diode Array Detection (HPLC-DAD) (Ultimate 3000, Dionex, Sunnyvale, Torrance, CA, USA). The separation was carried out on a Kinetex® Biphenyl C_{18} 100 Å RP column (2.6 mm, 150 × 4.6 mm, Phenomenex, Torrance, CA, USA), with a 0.4 mL/min flow rate and a 10 µL injection volume. The column oven was maintained at 30°C. Extracts of both 70% ethanol and 70% methanol were used in the analysis. Major phenolic compounds, including sinapine, sinapic acid, and canolol, were identified using the authenthic standards with a detection limit of 0.001 mg/mL. Calibration curves for each standard were obtained from 1.0 to 100 µg/mL (n = 11) concentration range with $R^2 = 0.9982$ for sinapic acid, $R^2 = 0.9999$ for canolol, and $R^2 = 0.9995$ for sinapine. Separation was conducted using a gradient elution with water (0.1% [v/v] formic acid) as solvent A and methanol (0.1% [v/v] formic acid) as solvent B. The gradient elution system operated as follows: 25% to 25% B (0 to 3 min), 25% to 40% B (3 to 8 min), 40% to 40% B (8 to 13 min), 40% to 60% B (13 to 25 min), 60% to 70% B (25 to 38 min), 70% to 100% (38 to 41 min), 100% to 100% (41 to 44 min), 100% to 25% (44 to 47 min), and 25% to 25% (47 to 57 min). The chromatograms were acquired at 270 nm (canolol) and 330 nm (sinapine and sinapic acid) using Chromeleon software Version 7.2 SR4 (Dionex Canada Ltd., Oakville, ON, Canada).

3.5 Statistical analysis

All the experiments were carried out in triplicate. Results were presented as the mean ± standard deviation of the triplicate analysis. The assumptions of normal distribution and constant variance were verified by examining the residuals (Pallant, 2011). Data points were checked for their normality using the normality test and necessary transformation of the raw data was done accordingly to obtain the normalized data. For the current experiment, square-root transformations were conducted according to the obtained normalized data (Pallant, 2011). A factorial design was applied for the study with four independent factors, including particle size, type of solvent, solvent concentration, and temperature. Data analysis was carried out using the general linear multiple regression model using the two-way analysis of variance and multiple mean comparisons were achieved with Tukey's test with differences at the 5% level ($P < 0.05$) considered statistically significantly different (Pallant, 2011). All the data analysis tests were assessed by SPSS statistical software version 22 (IBM, New York, NY, United States).

3.6 Results & Discussion

3.6.1 Model fit statistics for ASE extracts

According to the mean sum of squares in the model fit statistics, the concentration of the solvent, as well as the extraction temperature, was the most important factor affecting the TPC (**Table 3.1**). With the exception of particle size, all other parameters (temperature, solvent type, and concentration) contributed significantly to the TPC of the extracts. Furthermore, the interaction effects of the parameters illustrated two-way interactions including, size∗concentration, solvent∗concentration, concentration∗temperature, size∗temperature, and solvent∗temperature, which were significant ($P < 0.05$). In addition, all three-way interactions were also significantly different. The particle size, however, had no significant effect on the TPC of the canola meal extractants ($P = 0.23$). A similar trend was observed for the total flavonoid content where, except for the particle size ($P = 0.83$), all other independent variables were significant ($P < 0.05$), including both two-way and three-way interactions (**Table 3.1**). Despite the similarities, according to model fit statistics, the concentration of the solvent and the extraction temperature were the most important factors contributing to the TFC of the extracts. The antioxidant activity of the extractants also showed a similar pattern towards FRAP and DPPH with independent factors demonstrating significance towards the model fit statistics with the exception of particle size ($P = 0.23$ and $P = 0.87$) (**Table 3.2**). For FRAP antioxidant activity, solvent concentration and temperature were the factors that contributed the most towards the model fit statistics, whereas DPPH radical activity was mainly regulated by the type and the concentration of the solvents (**Table 3.2**). Thus, metal ion chelation activity demonstrated a similar trend as FRAP did, where solvent concentration and temperature contributed towards the model fit statistics. However, all four independent factors

(particle size, temperature, solvent type, and concentration) contributed to the significance of the model. Furthermore, all the results obtained for TPC, TFC, DPPH, FRAP, and ion chelation of the canola meal extracts had high (>95%) coefficients of variance: R^2 = 0.982, 0.924, 0.948, 0.958, and 0.922, respectively. Multiple mean comparisons were conducted using Tukey's post-hoc analysis for TPC, TFC, and antioxidant assays. According to the post-hoc analysis, the temperature had a positive effect on TPC, TFC, FRAP, DPPH and ion chelation for all three types of solvents (ethanol, methanol, and water). Furthermore, solvent concentration, and the type of solvent, also had a significant effect on TPC, TFC, and the antioxidant activity of the extractants.

Table 3.1 Effect of particle size, solvent, temperature and concentration on TPC & TFC

Source	Sum of Squares	DF	Mean Square	Significance
**Total Phenolic Content (TPC)*				
Size	0.002	1	0.002	0.23
Solvent (sol)	0.183	1	0.183	0.00
Concentration (con)	14.486	3	4.829	0.00
Temperature (temp)	6.775	2	3.388	0.00
Size * Con	0.632	3	0.211	0.00
Solvent * Con	1.331	3	0.444	0.00
Con * Temp	0.262	6	0.044	0.00
Size * Temp	0.060	2	0.030	0.00
Solvent * Temp	0.139	2	0.070	0.00
Size * Solvent * Con	0.354	3	0.118	0.00
Size * Con * Temp	0.565	6	0.094	0.00
Solvent * Con * Temp	0.751	6	0.125	0.00
Error	0.522	361	0.001	
Total	343.577	415		
**Total Flavonoid Content (TFC)*				
Size	6.097E-7	1	6.097E-7	0.83
Solvent (sol)	0.001	1	0.001	0.00
Concentration (con)	0.022	3	0.007	0.00
Temperature (temp)	0.002	2	0.001	0.00
Size * Con	0.001	4	0.000	0.00
Solvent * Con	0.000	3	6.820E-5	0.00
Solvent * Temp	0.001	2	0.000	0.00
Size * Solvent * Con	0.001	4	0.000	0.00
Size * Con * Temp	0.000	16	2.717E-5	0.01
Size * Solvent * Con * Temp	0.000	12	3.156E-5	0.01
Error	0.004	260	1.354E-5	

*Square root transformations were conducted to obtain the normality of data

Table 3.2 Effect of particle size, solvent, temperature and concentration on antioxidant activity

Source	Sum of Squares	DF	Mean Square	Significance
**FRAP – Ferric Reducing Antioxidant Power*				
Size	8.050E-5	1	8.050E-5	0.23
Solvent (sol)	0.004	1	0.004	0.00
Temperature (temp)	0.041	2	0.021	0.00
Concentration (con)	0.194	3	0.065	0.00
Size * Con	0.011	4	0.003	0.00
Solvent * Con	0.004	3	0.001	0.00
Temp * Con	0.008	6	0.001	0.00
Size * Temp	0.001	2	0.000	0.01
Solvent * Temp	0.001	2	0.000	0.00
Size * Solvent * Con	0.004	4	0.001	0.00
Size * Temp * Con	0.007	8	0.001	0.00
Solvent * Temp * Con	0.003	6	0.000	0.00
Size * Solvent * Temp * Con	0.002	6	0.000	0.00
Error	0.020	364	5.485E-5	
Total	11.620	416		
**DPPH – Free Radical Scavenging Effect*				
Size	0.001	1	0.001	0.87
Solvent (sol)	94.815	1	94.815	0.00
Concentration (con)	200.255	3	66.752	0.00
Temperature (temp)	3.326	2	1.663	0.00
Size * Con	3.871	4	0.968	0.00
Solvent * Con	37.085	3	12.362	0.00
Con * Temp	2.381	8	0.298	0.00
Size * Temp	0.833	2	0.416	0.00
Size * Solvent * Con	1.748	4	0.437	0.00
Size * Con * Temp	6.727	8	0.841	0.00
Solvent * Con * Temp	1.265	8	0.158	0.00
Size * Solvent * Con * Temp	2.110	8	0.264	0.00
Error	19.077	374	0.051	
Total	20582.024	428		
**Metal Ion Chelation Effect*				
Size	3.292	1	3.292	0.00
Solvent (sol)	0.989	1	0.989	0.00
Concentration (con)	16.739	3	5.580	0.00
Temperature (temp)	24.434	2	12.217	0.00
Size * Con	3.150	3	1.050	0.00
Solvent * Con	7.985	3	2.662	0.00
Con * Temp	1.748	6	0.291	0.00
Size * Solvent	1.646	1	1.646	0.00
Solvent * Temp	0.872	2	0.436	0.00
Size * Solvent * Con	17.894	2	8.947	0.00
Size * Con * Temp	2.482	10	0.248	0.00
Solvent * Con * Temp	0.667	6	0.111	0.00
Size * Solvent * Con * Temp	1.452	6	0.242	0.00
Error	8.195	340	0.024	
Total	14958.189	391		

3.6.2 Structure-related antioxidant activity

Due to their diversity in structure, phenolic compounds provide different functions, including sensory (color, bitterness, astringency, taste, and so on), nutritional quality, radical scavengers, antimutagens, and so on. (Kraljić et al., 2015). Therefore, it is important to understand the structure-function relationship by phenolic compounds that change during processing. Khattab, Eskin, and Thiyam-Hollander (2014) reported sinapine was converted into sinapic acid at a high temperature under microwave conditions and further converted into canolol via decarboxylation. Khattab et al. (2014) reported that the optimum conditions for the conversion of sinapic acid to canolol were 300 W of microwave power for 13 min, which yielded 4.2 mg/g of canolol (58.4% conversion rate). They also observed that around 96.1% of TPC in canola meal was converted into sinapic acid, of which 58.3% was decarboxylated to canolol. Moreover, Gaspar et al. (2008) found that the antioxidant activity of the cinnamic acid derivatives were not significantly ($P < 0.05$) affected by its halogenation reactions. They explained that the presence of a methoxy group in position-5 in sinapic acid increased the stabilization of the peroxyl radical, which improved its antioxidant activity. Thus, the application of ASE provides a better understanding of the impact that high pressure and temperature have on the structure-related antioxidant activity of TPC and TFC in the extracts. In addition to the method of extraction, the type and concentration of solvents, as well as the temperature all have a greater impact on the structure-related phenolic antioxidant activity. The impact of each factor on the structure-related antioxidant activity of TPC and TFC will be discussed in detail in the following sections.

3.6.3 Accelerated solvent extraction (ASE)

The high pressure and temperature associated with ASE produce better extraction results than conventional methods do. Application of high pressure and temperature aid the concurrent extraction and phenolic transformation during the ASE process, which improve the antioxidant activity of the extract by converting sinapic acid to canolol and its derivatives (Li & Guo, 2016a). This was confirmed by the higher antioxidant activity and TPC observed with the increase in temperature. These researchers also observed the TPC content at higher extraction temperatures (>180°C) was significantly greater ($P < 0.05$) than that at lower temperatures (<160°C). Furthermore, extractants obtained using concurrent extraction at temperatures above 180°C improved the antioxidant activity by increasing the levels of sinapic acid and canolol (Li & Guo, 2016a). The current study was consistent with the above findings as antioxidant activity was also temperature dependent, with the highest activity recorded at 180°C ($P > 0.05$) and the lowest at 140°C (**Table 3.3**). In addition, application of high-pressure further aids in the disruption of plant tissues with the release of the intracellular phenolic compounds into the extraction medium. This would also enhance the antioxidant activity as well TPC by improving the solubilities of phenolic compounds. Our results indicate that the TPC of extracts was over 10% higher (**Table 3.3**) compared to that obtained through the more conventional extraction methods described by Siger and Józefiak (2016) and Wroniak, Rękas, Siger, and Janowicz (2016).

The above differences are often attributed to higher solute diffusion rates, mass transfer rate, and higher solubilities of the targeted compounds connected with ASE (Li & Guo, 2016a). In general, methanol and ethanol have relatively lower boiling points, so using ASE could increase the extraction temperatures to up to 200°C without any combustion. At elevated temperatures

(about 200°C), concurrent extraction resulted in the transformation of phenolic compounds and increased the TPC value, which improved the antioxidant activity (Li & Guo, 2016a). This was confirmed by the antioxidant assays, and the higher content of total phenolics and flavonoids (**Table 3.3**). The increase in TPC and TFC values at higher temperatures showed that ASE facilitated a one step extraction and transformation process, making it an ideal method of extraction for studying the structure-function relationship of phenolic compounds. Li and Guo (2016a) also found that high-pressure extractions improved the antioxidant activity of the extract by converting sinapine and sinapic acid to canolol and its derivatives. In comparison to conventional extraction methods, most of the steps linked with ASE are automated, thereby providing a labor-free environment for analysis (Luthria et al., 2004). The individual parameters associated with ASE, including temperature, and the nature and concentration of solvents, will be further discussed.

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Table 3.3 Effect of particle size, temperature, solvent type, and concentration of solvent on antioxidant activity

Particle Size	Temperature	Type of Solvent	Concentration of Solvent	DPPH Radical Activity (%)	Metal Ion Chelation Activity (%)	FRAP (μ mol TE / g DM)[3]	TFC[4] (μ mol QE/ g DM)[5]	TPC[6] (mg SAE / g DM)[7]
0.5 mm	140	Water	100% Water	42.64 ± 0.39	35.03 ± 0.65	226.31 ±0.01	1.00 ± 0.00	3.29 ± 0.25
		Ethanol	30% EtOH[1]	26.26 ± 0.24	28.77 ± 1.18	113.51 ± 0.04	2.60 ± 0.22	3.14 ± 0.41
			40% EtOH	49.56 ± 0.92	32.16 ± 2.39	196.13± 0.01	2.65 ± 0.05	4.33 ± 0.44
			60% EtOH	43.47 ± 0.25	31.52 ± 0.34	622.81 ± 0.02	3.12 ± 0.46	6.21 ± 0.10
			70% EtOH	69.08 ± 9.74	42.54 ± 2.18	666.80 ± 0.02	5.65 ± 0.69	10.59 ± 0.53
		Methanol	30% MeOH[2]	33.67 ± 1.03	33.72 ± 0.45	109.18 ± 0.04	1.85 ± 0.27	3.40 ± 0.78
			40% MeOH	31.11 ± 4.99	31.95 ± 0.32	333.34 ± 0.01	1.65 ± 0.05	2.04 ± 0.20
			60% MeOH	48.84 ± 0.74	31.82 ± 0.13	435.17 ± 0.02	1.75 ± 0.16	7.13 ± 0.04
			70% MeOH	66.18 ± 4.36	28.77 ± 1.18	459.27 ± 0.02	3.70 ± 0.11	11.06 ± 0.80
1.0 mm	140	Water	100% Water	46.09 ± 0.62	35.94 ± 0.55	473.05 ± 0.05	1.30 ± 0.00	3.37 ± 0.34
		Ethanol	30% EtOH	41.70 ± 0.80	32.16 ± 2.39	780.31 ± 0.01	2.20 ± 0.22	1.94 ± 0.12
			40% EtOH	47.26 ± 0.92	34.71 ± 0.41	890.74 ± 0.06	2.60 ± 0.22	4.67 ± 0.18
			60% EtOH	53.96 ± 1.49	34.16 ± 1.52	1224.88 ± 0.05	3.50 ± 0.07	5.16 ± 0.31
			70% EtOH	60.76 ± 7.27	36.62 ± 0.40	1747.33 ± 0.02	5.00 ± 0.99	10.13 ± 0.55
		Methanol	30% MeOH	33.14 ± 0.26	32.94 ± 1.34	605.25 ± 0.04	1.60 ± 0.11	2.91 ± 0.28
			40% MeOH	26.28 ± 0.48	35.33 ± 2.02	1105.62 ± 0.06	1.65 ± 0.05	2.04 ± 0.28
			60% MeOH	47.26 ± 0.92	39.95 ± 4.58	1585.48 ± 0.07	3.10 ± 0.11	7.45 ± 0.41
			70% MeOH	65.75 ± 5.52	34.48 ± 3.34	2441.26 ± 0.02	3.80 ± 0.11	10.42 ± 0.75
0.5 mm	160	Water	100% Water	43.06 ± 0.31	36.50 ± 0.77	103.58 ± 0.01	1.00 ± 0.00	10.43 ± 0.23
		Ethanol	30% EtOH	35.40 ± 1.26	32.57 ± 2.36	1167.70 ± 0.03	2.60 ± 0.33	4.16 ± 0.28
			40% EtOH	52.00 ± 1.27	35.73 ± 0.68	1123.81 ± 0.01	2.75 ± 0.60	5.22 ± 0.34
			60% EtOH	56.64 ± 1.41	33.06 ± 1.00	1687.73 ± 0.09	3.43 ± 0.37	7.39 ± 0.71
			70% EtOH	67.79 ± 7.10	46.67 ± 0.92	2905.69 ± 0.06	5.00 ± 0.99	15.56 ± 4.07
		Methanol	30% MeOH	38.67 ± 1.18	37.88 ± 0.60	659.76 ± 0.05	1.65 ± 0.16	6.84 ± 0.86
			40% MeOH	29.00 ± 3.59	33.71 ± 0.78	1555.99 ± 0.08	2.45 ± 0.05	7.58 ± 0.24
			60% MeOH	52. 00 ± 1.27	40.73 ± 2.16	2040.99 ± 0.02	2.95 ± 0.05	7.98 ± 0.43
			70% MeOH	66.39 ± 2.55	32.62 ± 2.58	2968.75 ± 0.01	4.15 ± 0.05	12.97 ± 0.98

Table 3.3 (cont`d)

Particle Size	Temperature	Type of Solvent	Concentration of Solvent	DPPH Radical Activity (%)	Metal Ion Chelation Activity (%)	FRAP (μ mol TE / g DM)[3]	TFC[4] (μ mol QE/ g DM)[5]	TPC[6] (mg SAE / g DM)[7]
1.0 mm	160	Water	100% Water	46.57 ± 1.47	37.35 ± 0.12	486.66 ± 0.02	1.45 ± 0.05	10.92 ± 0.45
		Ethanol	30% EtOH[1]	34.64 ± 0.85	35.73 ± 0.68	744.20 ± 0.02	2.45 ± 0.49	2.55 ± 0.14
			40% EtOH	46.67 ± 1.12	36.09 ± 0.99	1406.21 ± 0.02	2.95 ± 0.60	4.45 ± 0.17
			60% EtOH	52.06 ± 1.10	37.23 ± 1.80	2992.99 ± 0.03	3.23 ± 0.59	6.49 ± 0.42
			70% EtOH	67.42 ± 2.43	42.06 ± 2.37	989.19 ± 0.01	5.15 ± 0.05	14.75 ± 0.89
		Methanol	30% MeOH[2]	34.87 ± 1.17	37.38 ± 0.42	1112.89 ± 0.07	1.60 ± 0.22	3.95 ± 0.52
			40% MeOH	29.67 ± 1.13	32.71 ± 0.61	1388.81 ± 0.09	1.95 ± 0.16	3.63 ± 0.54
			60% MeOH	46.67 ± 1.12	41.53 ± 0.31	1762.44 ± 0.06	3.35 ± 0.16	8.45 ± 0.44
			70% MeOH	67. 16 ± 9.40	48.66 ± 5.65	3086.53 ± 0.05	4.55 ± 0.60	14.59 ± 2.82
0.5 mm	180	Water	100% Water	45.73 ± 0.24	39.67 ± 0.80	486.64 ± 0.03	1.60 ± 0.11	13.08 ± 0.08
		Ethanol	30% EtOH	35.09 ± 0.61	34.87 ± 1.77	1328.24 ± 0.02	2.80 ± 0.22	5.52 ± 0.52
			40% EtOH	49.72 ± 1.09	38.24 ± 2.86	1394.68 ± 0.01	2.65 ± 0.05	6.26 ± 0.66
			60% EtOH	57.91 ± 0.71	36.89 ± 2.26	1943.02 ± 0.10	3.70 ± 0.09	9.65 ± 0.70
			70% EtOH	68.74 ± 10.10	53.53 ± 3.55	3710.03 ± 0.06	6.30 ± 0.55	24.71 ± 2.77
		Methanol	30% MeOH	42.98 ± 0.76	46.36 ± 4.70	976.20 ± 0.02	2.25 ± 0.05	4.23 ± 0.39
			40% MeOH	30.62 ± 2.73	40.72 ± 0.53	1885.96 ± 0.04	3.35 ± 0.27	8.89 ± 0.60
			60% MeOH	49.72 ± 1.09	48.17 ± 2.34	2762.54 ± 0.10	3.58 ± 0.13	9.84 ± 0.50
			70% MeOH	62.53 ± 1.64	34.82 ± 1.50	3436.70 ± 0.09	5.00 ± 0.33	15.75 ± 1.18
1.0 mm	180	Water	100% Water	45.54 ± 0.19	39.89 ± 1.43	759.95 ± 0.08	1.80 ± 0.00	15.90 ± 1.28
		Ethanol	30% EtOH	36.44 ± 0.69	38.24 ± 2.86	990.50 ± 0.10	2.30 ± 0.00	4.19 ± 0.53
			40% EtOH	52.84 ± 1.29	39.47 ± 1.18	1460.10 ± 0.09	2.90 ± 0.22	5.84 ± 0.78
			60% EtOH	58.16 ± 6.97	41.91 ± 2.34	2227.11 ± 0.06	3.75 ± 1.15	7.38 ± 0.37
			70% EtOH	61.35 ± 3.44	50.90 ± 1.99	3264.41 ± 0.01	5.10 ± 0.88	16.40 ± 0.47
		Methanol	30% MeOH	36.88 ± 0.53	38.44 ± 2.93	1196.08 ± 0.02	2.20 ± 0.11	5.63 ± 0.68
			40% MeOH	31.29 ± 1.57	45.00 ± 0.27	1226.56 ± 0.02	2.40 ± 0.11	5.07 ± 0.56
			60% MeOH	52.84 ± 1.29	62.66 ± 2.38	2453.55 ± 0.02	4.40 ± 0.22	11.79 ± 0.87
			70% MeOH	67.14 ± 7.11	42.01 ± 9.29	3718.46 ± 0.02	5.45 ± 0.27	20.72 ± 1.47

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Values are mean ± standard deviations in triplicates (n = 3). [1]EtOH - aqueous ethanol (70% v/v), [2]MeOH - aqueous methanol (70% v/v), [3]µ mol TE / g DM - results expressed as micromoles of trolox equivalents per gram of dry matter, [4]TFC – total flavonoid content, [5]µ mol QE/ g DM - results expressed as micromoles of quercetin equivalents per gram of dry matter, [6]TPC - total phenolic content, [7]mg SAE / g DM - results expressed as milligrams of sinapic acid equivalents per gram of dry matter
Note: Values are mean ± standard deviations in triplicates (n = 3).

Table 3.4 Effect of particle size, solvent type, and concentration of solvent on antioxidant activity at room temperature (25ºC)

Particle Size	Temperature	Type of Solvent	Concentration of Solvent	DPPH Radical Activity (%)	Metal Ion Chelation Activity (%)	FRAP (µ mol TE / g DM)[3]	TFC[4] (µ mol QE/ g DM)[5]	TPC[6] (mg SAE / g DM)[7]
0.5 mm	RT*	Ethanol	70% EtOH[1]	46.45 ± 1.96	34.22 ± 1.47[a]	405.21 ± 0.08[a]	1.22 ± 0.17[a]	9.64 ± 1.29
		Methanol	70% MeOH[2]	47.43 ± 1.55	28.97 ± 3.53[b]	102.99 ± 0.04[b]	1.95 ± 0.27[b]	10.26 ± 1.81
1.0 mm	RT	Ethanol	70% EtOH	46.22 ± 0.43	31.88 ± 0.48	382.50 ± 0.01[a]	1.47 ± 0.33	12.86 ± 0.91[a]
		Methanol	70% MeOH	45.13 ± 0.57	30.76 ± 3.61	925.44 ± 0.06[b]	1.85 ± 0.49	19.02 ± 0.17[b]

*RT - Room Temperature (25ºC), Values are mean ± standard deviations in triplicates (n = 3). [1]EtOH - aqueous ethanol (70% v/v), [2]MeOH - aqueous methanol (70% v/v), [3]µ mol TE / g DM - results expressed as micromoles of trolox equivalents per gram of dry matter, [4]TFC – total flavonoid content, [5]µ mol QE/ g DM - results expressed as micromoles of quercetin equivalents per gram of dry matter, [6]TPC - total phenolic content, [7]mg SAE / g DM - results expressed as milligrams of sinapic acid equivalents per gram of dry matter, a,b – statistically significantly different

3.6.4 Impact of temperature

The effect of the heating temperature on the extraction of phenolic compounds is often associated with different type of bonds present in plant species (Teh & Birch, 2014). These researchers found that the high temperatures during the extraction process broke the phenolic–matrix bonds inside the cellular matrix and altered its chemical structures. This phenomenon would impact the inter-cellular pressure, with the resulting solutes, including phenolic compounds, passing through the cellular membranes (Teh & Birch, 2014). It has often been reported that ASE might not be very effective in extracting total phenolic compounds; however, it could be applied to extract some targeted phenolic compounds (Bonoli, Marconi, & Caboni, 2004) and free phenolic compounds (Okuda, Yamashita, Tanaka, Matsukawa, & Tanabe, 2009). Therefore, application of Accelerated Solvent Extraction (ASE)/Pressurized Solvent Extraction (PSE) would be a better choice for extracting specific phenolic compounds, including canolol and its derivatives. This was confirmed in a study by Sorensen et al. (2013), who found that canolol and other phenolic compounds could be extracted via defatted meal at higher temperatures, that is, 185°C. This was consistent with the results of Hossain, Barry-Ryan, Martin-Diana, and Brunton (2011), who optimized the extraction of antioxidant compounds from rosemary, oregano, and marjoram using ASE at 130°C, although the results with the solvent concentration varied between different samples. Gaspar et al. (2008) also stated that the antioxidant activity of cinnamic acid derivatives could be thermodynamically favored. They suggested that a higher number of hydroxyl substituents attached to the structure, or the presence of catechol moieties are often associated with a lower electrochemical and/or redox potential due to o-quinone formation. In the current study, there was an increase in the antioxidant activity at the higher temperatures. The highest amount of antioxidant activity was found at 180°C ($P > 0.05$), while the

lowest was found at 140°C (**Table 3.3**). Compared to our control, it was evident that temperature was a factor affecting the composition and extraction efficiency of phenolic compounds.

The TPC of the extracts varied from 9.64 to 12.86 mg SAE/g DM for 70% ethanol extracts at room temperature. This was comparatively lower than the TPC of the 70% ethanol extracts obtained at a higher temperature, which ranged from 10.13 to 24.71 mg SAE/g DM (**Table 3.3** and **Table 3.4**). This was further confirmed via the antioxidant activity (FRAP) of the two extracts, which ranged from 0.383 to 0.405 mmol TE/g DM at room temperature, which was considerably lower than the extracts obtained at a high temperature, which ranged from 0.667 to 3.710 mmol TE/g DM (**Table 3.3** and **Table 3.4**). Our results were parallel to the results obtained by Li and Guo (2016a), who observed a relatively higher TPC with an increase in temperature. Using a similar method of extraction at 75°C for 20 min, both Cai and Arntfield (2001) and Zago et al. (2015) reported TPC values of 19.90 and 17.40 mg/g DW, respectively. These values were comparable with those obtained using our ASE extracts at 25°C (room temperature) (12.86 mg SAE/g), confirming the extraction efficiency of ASE/PSE.

The highest radical activity and TPC were both obtained at 180°C for both 70% ethanol and methanol extractants (**Table 3.3** and **Table 3.4**). The greater extraction efficiency at high temperature and pressure could account for the high antioxidant activity and TPC. This was consistent with the results reported by Zago et al. (2015), who observed higher TPC and antioxidant activity with superheated steam at 160°C with a hydration step. They reported that 2 hr of hydration of the meal prior to microwave treatment increased the TPC value by 12% (22 mg SAE g/DM) compared to the non-hydrated meal. They suggested that partial breakdown of the plant cell walls during the heat treatment would enhance the release of bound phenolic compounds. Furthermore, a lower thermal

degradation of the phenolic compounds during the microwave treatment would also enhance the TPC values and antioxidant activity of the extracts. Therefore, a combination of ASE with microwave radiation could be a potential novel technique for the green extraction of phenolics (Li & Guo, 2016a; Siger & Józefiak, 2016; Yang et al., 2014; Zago et al., 2015).

These results agreed with those in the work by Spielmeyer, Wagner, and Jahreis (2009), who reported that heating rapeseed increased the phenolic content, particularly canolol, 120-fold compared to the unheated seeds. Harbaum-Piayda et al. (2010) also reported that the high reactivity of canolol at relatively higher temperatures could potentially produce new phenolic compounds, including dimer and trimer derivatives of canolol, which would have higher antioxidant properties than either sinapine or sinapic acid. These researchers also confirmed that these oligomeric compounds, including dimers, had twofold higher antioxidant activity than canolol did, which was comparable to that of quercetin (Harbaum-Piayda et al., 2010).

3.6.5 Impact of extraction solvents

The solvent extractants and method of extraction both had a significant ($P < 0.05$) effect on the antioxidant capacity of the canola meal. Both ethanol and methanol, at the highest concentration of 70%, enhanced the antioxidant activity of canola meal (**Table 3.3**). Water as an extractant, however, demonstrated relatively higher antioxidant activity at higher temperatures (>160°C), but it was much lower than that demonstrated by either ethanol and methanol. The higher antioxidant activity resulted from the improved extraction of phenolic compounds by ASE at high pressure and high temperature. Water also proved to be a better extractant for TPC when the temperature was raised from 140 to 180°C, with the TPC increasing from 3.29 to 15.90 mg SAE/g DM, which was accompanied by an increase in FRAP activity from 0.226 to 0.760 mmol TE/g DM (**Table 3.3**). This

increase was still markedly lower than that obtained using either ethanol or methanol extractants and confirms the efficacy of ACE as an efficient green extraction method for phenolic compounds.

In addition, the different degrees of polarity associated with the different solvent concentrations could also affect the extraction of the phenolic compounds and their different antioxidant properties (Teh & Birch, 2013). In fact, even pure water at higher temperatures and pressures demonstrated lower polarity, which was associated with higher antioxidant activity. Li and Guo (2016a) observed that water at higher temperature and pressure could be an effective green extractant. They showed it was a good extraction medium for sinapine and sinapic acid due to its relatively high H-bonding donor and accepting ability (Li & Guo, 2016a). These authors also suggested that ASE was capable of concurrent extraction of phenolic compounds and their transformations. Li and Guo (2016a) also reported that different solvents yielded different phenolic compositions of sinapine, sinapic acid, and canolol. The current study suggests that high pressure and temperature facilitates the conversion of sinapic acid to canolol, which is further improved using methanol and ethanol extractants. The use of high pressure and temperature is often linked with the elimination of a number of the hydroxyl substituents attached to the structure, improving its redox potential and increasing the antioxidant activity (Gaspar et al., 2008). This was further demonstrated by introducing a halogen substituent in the ortho position of the phenolic group in cinnamic acid, which increased its lipophilicity and rendered it a novel lipophilic antioxidant. Our study established 70% ethanol and 70% methanol as the optimum extractants for obtaining phenolic compounds with higher antioxidant activity compared to the corresponding 60%, 40%, and 30% ethanol and methanol extractants (**Table 3.3**). These results are in agreement with those in the work by Li and Guo (2016a) and Thiyam, Kuhlmann, Stöckmann, and Schwarz (2004), both of whom demonstrated the effectiveness of 70% ethanol and 70% methanol extractants at a high temperature (180°C). Compared

to methanol, however, 70% ethanol would be a better eco-friendly choice for extracting phenolic compounds. In the current study, adjustment of solvent concentration enhanced the extraction efficiency of the TPC and antioxidant activity consistent with that reported by Alothman, Bhat, and Karim (2009). These researchers reported that the recovery of antioxidative phenolics was primarily affected by the solubility of the phenolic compounds, which is a major limitation in developing an overall standard extraction procedure. However, the use of high pressure and temperature may overcome the differences in solubility between the phenolic compounds and facilitate more complete extraction with higher yields of TPC and greater antioxidant activity (Alothman et al., 2009). The type and extractability of the phenolic compounds also depend on the raw material type (variety), as well as storage temperature, moisture level, amount of impurities, and maturity stage of the seeds (Siger & Józefiak, 2016).

3.6.6 Determination of TPC

TPC and TFC are the major determinants of antioxidant activity and include a wide range of phenolic compounds, both phenolic acids and polymers (Teh, Bekhit, & Birch, 2014). The TPC of the soluble and insoluble fractions of canola meal extracts was determined using the FC assay. In the current method, under alkaline conditions, phenolic groups are degraded to phenolate ions, which reduce the phosphotungstic-phosphomolybdic complex in the FC reagent to form a blue-colored complex (Chandrasekara & Shahidi, 2010). The TPC obtained using FC assays includes both free and soluble conjugates, which are responsible for the invitro antioxidant capacity of the extracts (Chandrasekara & Shahidi, 2010). In this study, the TPC of the canola meal extracts was expressed as mg SA per g of the defatted meal (**Table 3.3**). In general, for both particle sizes (0.5 and 1.0 mm), the TPC increased with the concentration of the solvent, with 70% ethanol yielding 24.71 and 16.40

mg SAE/g DM, respectively. A similar but reverse effect was observed for the two particles sizes extracted with 70% methanol, which yielded 15.75 and 20.72 mg SAE/g DM, respectively. A higher TPC was also observed when the temperature was increased to 180 °C with the highest value reported for all three (ethanol 24.71, methanol 20.72, and water 15.90 mg SAE/g DM) extractants. Overall, however, particle size had no significant effect ($P > 0.05$) on TPC.

These results were consistent with those in the work by Siger, Czubinski, Dwiecki, Kachlicki, and Nogala-Kalucka (2013), who reported 80% methanol extracts of canola meal had the highest phenolic content, ranging from 15.77 to 18.07 mg/g DM. Furthermore, Terpinc, Čeh, Ulrih, and Abramovič (2012) also reported that the amount of extractable phenolic compounds increased with a decrease in the polarity index of the solvent (water-1.000, methanol-0.762, and ethanol-0.654), which was consistent with our results showing higher TPC levels with lower polar solvent combinations including ethanol (24.71 mg SAE/g DM) and methanol (20.72 mg SAE/g DM). We recorded slightly higher TPC values compared to those reported by Siger et al. (2013), who reported TPC values of around 15.77 to 18.07 mg/g DM. This discrepancy in TPC values can be attributed to the variety of rapeseeds, pH, type of extraction, and the nature of phenolic compounds extracted (Chandrasekara & Shahidi, 2010). Furthermore, it was reported that the solvent extractant could also affect the TPC, as well as the individual phenolic compounds (Terpinc et al., 2012). These researchers reported that phenolic extraction was much more efficient with 70% methanol than with 96% ethanol, consistent with the results obtained by Alothman et al. (2009) and Matthäus (2002), as well as with our current findings. This may be attributed to an increase in the rate of diffusion of the solutes from the solid phase to the solvent at higher temperatures (Chandrasekara & Shahidi, 2010). However, during the ASE process, the presence of simple amino acids, carbohydrates, and tannins in the crude extract could interfere with the extraction of phenolic compounds and affect the TPC.

3.6.7 Antioxidant activity of canola meal

Canola meal is rich in several phenolic compound groups, including phenolic acids, flavones, and flavanols. However, based on the antioxidant activity, sinapine, sinapic acid, and canolol have been identified as the most active antioxidant components in canola meal extracts. These major phenolic compounds have demonstrated structure-related activity related to the high-pressure and high-temperature extraction of ASE. This was evident through the changes in phenolic composition with different solvents and at different temperatures. Furthermore, with the increase in polarity, the extraction efficiency of the phenolic compounds increased with 70% ethanol and/or 70% methanol with the highest antioxidant activity recorded.

3.6.7.1 Ferric reducing/antioxidant power assay (FRAP)

FRAP has been used extensively to identify the reducing power of phenolic compounds, which is a common measure of antioxidant activity. In general, reducing power is used as an indicator of electron donating ability, which is important for assessing the antioxidant activity of phenolic compounds. Reducing activity is measured based on the transformation of ferric ions (Fe^{3+}) to ferrous (Fe^{2+}) in the presence of TPTZ. The principle of the FRAP assay is based on the antioxidant strength required for reducing ferric-tripyridyltriazine complex (light brown) to its ferrous form(blue). Antioxidant compounds could serve as reductants in the presence of Fe^{3+}, so the intensity of the blue-colored complex is proportional to the ferrous ion concentration, providing a measure of its antioxidant capacity. In this study, canola meal extracts demonstrated a relatively higher reducing power depending on the type of solvent and the temperature (**Table 3.3**). Particle size, however, had very little effect on the reducing power of the compounds. The reducing power of canola meal extract ranged from 0.109 to 3.718 mmol TE/g DM of the defatted meal (**Table 3.3**). These results agree with

data reported by Siger et al. (2013), who found that various solvent extracts from canola meal produced significant differences in reducing power. Moreover, the levels of reducing power of the canola meal extracts at higher temperature treatments, ranged in decreasing order of magnitude with the solvent extractants, were as follows: 70% > 60% > 40% > 30%. For example, at 180 °C, the FRAP values for the 0.5-mm particle size canola meal extracts were 3.710 > 1.943 > 1.395 > 1.328 mmol TE/g DM for 70% > 60% > 40% > 30% ethanol extractants, respectively. The corresponding changes in FRAP were 3.437 > 2.763 > 1.886 > 0.976 mmol TE/g DM for the corresponding methanol extracts (**Table 3.3**). These significant differences ($P < 0.05$) among canola meal extracts suggest that it was not only the solvent type but also the solvent concentration and temperature that affected the reducing power and antioxidant activity (Teh & Birch, 2014). Furthermore, Gaspar et al. (2008) stated that oxidation of both free and bound forms of cinnamic acid derivatives could take place via electron transfer mechanisms, with the higher number of hydroxyl groups being responsible for the lower electrochemical potential.

Teh et al. (2014) also found that the recovery of phenolics using different extraction techniques increased at higher temperatures and correlated with higher antioxidant activity as measured by FRAP and DPPH. Moreover, the results of TPC and TFC correlated with the reducing power of the canola meal extracts, in agreement with previous findings. Our results further suggest that the phenolic compounds present in canola meal extracts could be possible electron donors.

3.6.7.2 DPPH free radical-scavenging assay

DPPH radical scavenging activity is a widely used spectroscopic method for rapidly evaluating the antioxidant activity of phenolic compounds. This assay is based on the ability of antioxidant compounds to scavenge free radicals via an electron donating mechanism. A higher

antioxidant capacity is demonstrated by a decrease in its absorbance. Generally, the methanolic extracts demonstrated a relatively higher radical-scavenging activity than the corresponding ethanol extracts from canola meal did, ranging from 26.26% to 69.08% for the 30% to 70% methanol extractants. These results are in agreement with those in the work by Terpinc et al. (2012) and Li and Guo (2016a), who found that 70% methanol and 70% ethanol extracts both exhibited relatively higher electron donating ability compared to other solvent extracts. These results were consistent with our findings, where we observed higher radical scavenging activity for both 70% ethanol and 70% methanol extracts, with the average radical-scavenging activity of the extracts being around 50% to 60% for all the solvent types and concentrations. Terpinc et al. (2012) and Li and Guo (2016a) also reported relatively higher antioxidant activity for their extracts. This was attributed to the possible synergistic effect between phospholipids and the other antioxidant compounds present, including canolol (Li & Guo, 2016b). This further confirms that the higher polarities of the solvents were more effective in extracting phenolic compounds with greater radical-scavenging activity (Li & Guo, 2016a). Gaspar et al. (2008) proposed a mechanism in which cinnamic acid moiety was modified by the presence of a catechol moiety that generated o-quinone formation, decreasing the redox potential of cinnamic acid derivatives. Moreover, Li and Guo (2016a) also found that the radical-scavenging activity and the TPC of the canola meal extracts obtained using ASE for 5 min (21.92 and 28.42 SAE/g) had the same efficacy ($P > 0.05$) as that obtained by Soxhlet extraction at 100°C for 12 hr. They also found that aqueous solvent extractants were more effective than the corresponding pure solvents, with 70% to 80% solvent extractants producing the highest antioxidant activity (Li & Guo, 2016b).

Table 3.5 Effect of particle size, temperature, solvent type on major phenolic composition

Particle Size	Concentration of Solvent	Temperature (°C)	Sinapine (µg/g DW)	Sinapic Acid (µg/g DW)	Canolol (µg/g DW)
0.5 mm	100% Water*	140	483.72 ± 16.20[a]	8.25 ± 0.53[b]	0.26 ± 0.09[b]
		160	471.17 ± 14.74[a]	7.97 ± 1.42[b]	0.73 ± 0.73[b]
		180	1470.00 ± 17.10[b]	57.01 ± 2.36[a]	4.51 ± 1.58[a]
	70% EtOH	140	10110.02 ± 853.58[a]	432.05 ± 1.81[c]	203.29 ± 9.73[a]
		160	12082.81 ± 1094.54[a]	550.96 ± 3.49[b]	297.98 ± 51.25[a]
		180	5716.92 ± 375.24[b]	734.95 ± 3.49[a]	212.10 ± 18.76[a]
	70% MeOH	140	8596.55 ± 224.31[b]	592.55 ± 25.46[c]	179.61 ± 11.63[c]
		160	9064.80 ± 88.40[a]	626.03 ± 14.15[b]	251.72 ± 16.80[a]
		180	7048.20 ± 508.47[c]	806.56 ± 21.57[a]	216.18 ± 19.44[b]
1.0 mm	100% Water	140	1590.92 ± 204.24[a]	74.64 ± 10.23[a]	7.27 ± 0.84[a]
		160	1466.86 ± 160.44[b]	80.17 ± 21.51[a]	9.36 ± 1.12[a]
		180	1416.98 ± 215.40[c]	89.15 ± 6.82[a]	10.71 ± 2.20[a]
	70% EtOH	140	6672.78 ± 857.02[a]	398.69 ± 99.68[b]	59.37 ± 1.41[c]
		160	6944.35 ± 168.07[a]	473.37 ± 6.56[b]	145.06 ± 32.84[b]
		180	6184.98 ± 134.47[a]	627.02 ± 50.99[a]	342.48 ± 37.83[a]
	70% MeOH	140	9495.33 ± 864.30[a]	691.01 ± 66.75[b]	187.12 ± 51.43[c]
		160	8165.73 ± 158.53[a]	501.64 ± 140.63[b]	281.33 ± 35.46[b]
		180	7468.67 ± 193.10[b]	859.02 ± 12.09[a]	491.29 ± 42.58[a]

MeOH – methanol, EtOH – ethanol, µg – microgram, DW – dry weight, a,b,c – statistically significantly different, *statistical significance for each solvent type at different temperature levels for each particle size

3.6.8 Determination of major phenolic compounds (HPLC-DAD)

Canola meal consists of many phenolic compounds, including vanillic acid, protocatechuic acid, syringic acid, *p*-coumaric acid, ferulic acid, caffeic acid, and chlorogenic acid (Kozlowska, Naczk, Shahidi, & Zadernowski, 1990). However, the most abundant compound is sinapine, which accounts for over 80% of the total phenols. Also, the hydrolyzed product of sinapine, which is sinapic acid, is the most common free phenolic acid present in canola meal (65% to 85%) (Li & Guo, 2016b; Quinn et al., 2017). Moreover, as mentioned earlier, the application of high temperature and pressure converts sinapic acid by decarboxylation into canolol (decarboxylated product), a compound of great interest due to its antioxidant potential. These phenolic compounds sinapine, sinapic acid, and canolol were readily identified by HPLC analysis in extracts obtained by 70% ethanol, 70% methanol, and water extractants. The separation of these phenolic compounds by HPLC is based on their degree of polarity, which ranges in increasing order from canolol < sinapic acid < sinapine. Our study demonstrated that the most polar solvent, water, yielded the lowest amount of canolol, compared to the less polar 70% ethanol and 70% methanol extractants (**Table 3.5**). The lower polarity of canolol accounts for its higher extraction by the low polar organic solvents. Szydłowska-Czerniak, Amarowicz, and Szłyk (2010) also reported that the polarity of extracting solvents determined the antioxidant activity as well as the TPC of their canola meal extracts, which was enhanced with an increase in temperature. The results of the present study showed water was an effective green extractant for sinapine at a high temperature (180°C). However, the yields obtained were much lower compared to those obtained with either 70% methanol or 70% ethanol extractants (**Table 3.5**). The latter extractants were more efficient in extracting both the less polar canolol and more polar sinapine. These results are in agreement with

those obtained by Li and Guo (2016a) and Cai and Arntfield (2001), who both found 70% ethanol and 70% methanol were the ideal extractants for phenolic compounds. Besides TPC, a similar effect was observed for antioxidant activity, in which temperature was the main factor affecting the changes in the major phenolic compounds of the extractants as well. Such changes in the major phenolic compounds, associated with an increase in temperature, were attributed to the degree of decarboxylation of sinapic acid. Statistical analysis demonstrated that temperature and solvent concentration were the two main factors significantly ($P < 0.05$) affecting the antioxidant activity (**Table 3.1** and **3.2**). Khattab et al. (2010) previously reported that the conversion of sinapic acid into canolol at higher temperatures involved decarboxylation. These results were consistent with those in the work by Siger et al. (2013), in which greater extraction of phenolic compounds, including canolol, was observed at higher temperatures. Our results confirm that under conditions of high pressure and temperature, solvents can effectively extract phenolic compounds from canola meal extracts (**Table 3.5**).

3.7 Conclusions

The recovery of phenolic and flavonoid compounds from canola meal was shown to be dependent on the type and polarity of the solvent extractants, the method of extraction, and temperature. Compared to the pure solvents, both 70% aqueous ethanol and methanol extractants were more effective in extracting phenolic compounds using the ASE method. Of the two extractants examined, 70% ethanol was not only more effective, but was the environmentally preferred extractant. Our results confirmed a positive correlation between the phenolic content of the meal and its antioxidant activity. Furthermore, the data also showed that water could be used for extraction of minimum phenolic compounds, particularly sinapic acid and canolol. The application of ASE provides a novel effective method for the green extraction of antioxidative phenolics. Furthermore, more information is needed to better understand the structure-based antioxidant activity of phenolic compounds, as affected by the high pressure and temperature of ASE. This information should lead to better utilization of canola by-products, such as canola meal. In summary, such antioxidants could be incorporated into food products to enhance shelf life by suppressing lipid oxidation. Further, fractionation and identification of individual phenolic compounds in canola meal, however, is needed to provide a new source of nutraceuticals for improving human health.

3.8 Acknowledgments

This project was funded by NSERC Discovery Grant RGPIN- 2015-03809 (grant holder, Usha Thiyam-Hollander), which is kindly acknowledged. Univ. of Manitoba's GETS scholarship for Ruchira Nandasiri is kindly acknowledged. Dr. Rasheda Rabbani, Faculty Biostatistician, Department of George & Fay Yee Centre for Healthcare Innovation (CHI), Community Health Sciences (CHS), University of Manitoba is kindly acknowledged for her statistical advice. Postdoctoral research fellow Dr. Erika Zago is gratefully acknowledged.

3.9 Supporting information

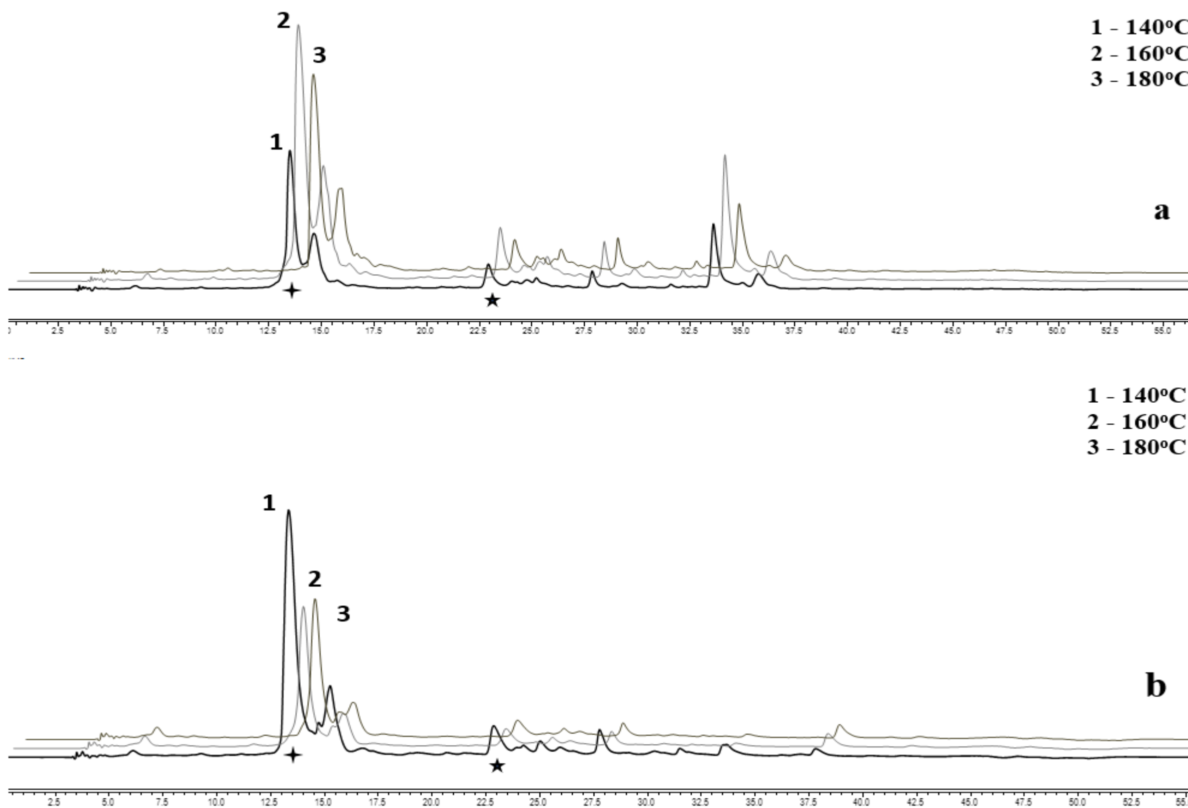

Figure 3S1 HPLC-DAD for Canola Meal Extracts with the particle size of 1.0 mm for Different Temperatures (1-140ºC, 2-160ºC, 3-180ºC) at the Wavelength of 320 nm (a - 70% methanol, b - 70% ethanol) ✦- Sinapine, ★- Sinapic acid

Table 3S1 Post-Hoc analysis for multiple comparisons of the mean effects studied in the general linear regression of the factorial design for total phenolic content and total flavonoid content

Multiple Comparisons		Mean Difference (I-J)	Standard Error (SE)	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Total Phenolic Content						
Solvent (I)	Solvent (J)					
Ethanol	Methanol	-.0487*	.00397	.000	-.0580	-.0393
	Water	-.1301*	.00616	.000	-.1446	-.1156
Methanol	Ethanol	.0487*	.00397	.000	.0393	.0580
	Water	-.0814*	.00616	.000	-.0959	-.0669
Water	Ethanol	.1301*	.00616	.000	.1156	.1446
	Methanol	.0814*	.00616	.000	.0669	.0959
Concentration (I)	Concentration (J)					
30	40	.0502*	.00549	.000	.0352	.0653
	60	-.1433*	.00570	.000	-.1589	-.1276
	70	-.4670*	.00555	.000	-.4822	-.4518
	100	-.2420*	.00672	.000	-.2604	-.2236
40	30	-.0502*	.00549	.000	-.0653	-.0352
	60	-.1935*	.00570	.000	-.2091	-.1779
	70	-.5172*	.00555	.000	-.5324	-.5020
	100	-.2922*	.00672	.000	-.3107	-.2738
60	30	.1433*	.00570	.000	.1276	.1589
	40	.1935*	.00570	.000	.1779	.2091
	70	-.3237*	.00575	.000	-.3395	-.3080
	100	-.0988*	.00689	.000	-.1177	-.0799
70	30	.4670*	.00555	.000	.4518	.4822
	40	.5172*	.00555	.000	.5020	.5324
	60	.3237*	.00575	.000	.3080	.3395
	100	.2250*	.00677	.000	.2064	.2435
100	30	.2420*	.00672	.000	.2236	.2604
	40	.2922*	.00672	.000	.2738	.3107
	60	.0988*	.00689	.000	.0799	.1177
	70	-.2250*	.00677	.000	-.2435	-.2064
Temperature (I)	Temperature (J)					
140	160	-.2042*	.00455	.000	-.2149	-.1935
	180	-.3203*	.00462	.000	-.3312	-.3094
160	140	.2042*	.00455	.000	.1935	.2149
	180	-.1161*	.00454	.000	-.1268	-.1054
180	140	.3203*	.00462	.000	.3094	.3312

Table 3S1 (cont'd) Multiple Comparisons		Mean Difference (I-J)	Standard Error (SE)	Sig.	95% Confidence Interval Lower Bound	Upper Bound
	160	.1161*	.00454	.000	.1054	.1268
Total Flavonoid Content						
Solvent (I)	Solvent (J)					
Ethanol	Methanol	.0057*	.00044	.000	.0046	.0067
	Water	.0221*	.00069	.000	.0205	.0238
Methanol	Ethanol	-.0057*	.00044	.000	-.0067	-.0046
	Water	.0164*	.00069	.000	.0148	.0181
Water	Ethanol	-.0221*	.00069	.000	-.0238	-.0205
	Methanol	-.0164*	.00069	.000	-.0181	-.0148
Concentration (I)	Concentration (J)					
30	40	.0021*	.00064	.011	.0003	.0039
	60	-.0081*	.00061	.000	-.0098	-.0064
	70	-.0210*	.00061	.000	-.0227	-.0194
	100	.0120*	.00075	.000	.0100	.0141
40	30	-.0021*	.00064	.011	-.0039	-.0003
	60	-.0102*	.00064	.000	-.0120	-.0084
	70	-.0231*	.00064	.000	-.0249	-.0214
	100	.0099*	.00078	.000	.0078	.0120
60	30	.0081*	.00061	.000	.0064	.0098
	40	.0102*	.00064	.000	.0084	.0120
	70	-.0129*	.00061	.000	-.0146	-.0113
	100	.0201*	.00075	.000	.0181	.0222
70	30	.0210*	.00061	.000	.0194	.0227
	40	.0231*	.00064	.000	.0214	.0249
	60	.0129*	.00061	.000	.0113	.0146
	100	.0331*	.00075	.000	.0310	.0351
100	30	-.0120*	.00075	.000	-.0141	-.0100
	40	-.0099*	.00078	.000	-.0120	-.0078
	60	-.0201*	.00075	.000	-.0222	-.0181
	70	-.0331*	.00075	.000	-.0351	-.0310
Temperature (I)	Temperature (J)					
140	160	-.0024*	.00052	.000	-.0036	-.0012
	180	-.0070*	.00051	.000	-.0082	-.0058
160	140	.0024*	.00052	.000	.0012	.0036
	180	-.0046*	.00051	.000	-.0058	-.0034
180	140	.0070*	.00051	.000	.0058	.0082
	160	.0046*	.00051	.000	.0034	.0058

*The mean difference is significant at the 0.05 level, Sig.-level of significance

Table 3S2 Post-Hoc analysis for multiple comparisons of the mean effects studied in the general linear regression of the factorial design for antioxidant activity

Multiple Comparisons		Mean Difference (I-J)	Standard Error (SE)	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
DPPH Radical Activity (%)						
Solvent (I)	Solvent (J)					
Ethanol	Methanol	.9775*	.02317	.000	.9230	1.0321
	Water	.6751*	.03645	.000	.5893	.7608
Methanol	Ethanol	-.9775*	.02317	.000	-1.0321	-.9230
	Water	-.3025*	.03652	.000	-.3884	-.2165
Water	Ethanol	-.6751*	.03645	.000	-.7608	-.5893
	Methanol	.3025*	.03652	.000	.2165	.3884
Concentration (I)	Concentration (J)					
30	40	.3066*	.03260	.000	.2172	.3959
	60	-.1223*	.03295	.002	-.2126	-.0319
	70	-1.5562*	.03260	.000	-1.6455	-1.4668
	100	-.1539*	.03993	.001	-.2633	-.0444
40	30	-.3066*	.03260	.000	-.3959	-.2172
	60	-.4288*	.03295	.000	-.5191	-.3385
	70	-1.8627*	.03260	.000	-1.9521	-1.7734
	100	-.4604*	.03993	.000	-.5698	-.3510
60	30	.1223*	.03295	.002	.0319	.2126
	40	.4288*	.03295	.000	.3385	.5191
	70	-1.4339*	.03295	.000	-1.5242	-1.3436
	100	-.0316	.04021	.935	-.1418	.0786
70	30	1.5562*	.03260	.000	1.4668	1.6455
	40	1.8627*	.03260	.000	1.7734	1.9521
	60	1.4339*	.03295	.000	1.3436	1.5242
	100	1.4023*	.03993	.000	1.2929	1.5118
100	30	.1539*	.03993	.001	.0444	.2633
	40	.4604*	.03993	.000	.3510	.5698
	60	.0316	.04021	.935	-.0786	.1418
	70	-1.4023*	.03993	.000	-1.5118	-1.2929
Temperature (I)	Temperature (J)					
140	160	-.0942*	.02681	.001	-.1573	-.0311
	180	-.1791*	.02681	.000	-.2422	-.1161
160	140	.0942*	.02681	.001	.0311	.1573
	180	-.0849*	.02662	.004	-.1476	-.0223
180	140	.1791*	.02681	.000	.1161	.2422

Table 3S2 (cont'd) Multiple Comparisons		Mean Difference (I-J)	Standard Error (SE)	Sig.	95% Confidence Interval Lower Bound	Upper Bound
	160	.0849*	.02662	.004	.0223	.1476
FRAP Antioxidant Activity						
Solvent (I)	Solvent (J)					
Ethanol	Methanol	-.0531*	.00922	.000	-.0748	-.0314
	Water	.6441*	.01438	.000	.6103	.6780
Methanol	Ethanol	.0531*	.00922	.000	.0314	.0748
	Water	.6972*	.01425	.000	.6637	.7308
Water	Ethanol	-.6441*	.01438	.000	-.6780	-.6103
	Methanol	-.6972*	.01425	.000	-.7308	-.6637
Concentration (I)	Concentration (J)					
30	40	.1299*	.01337	.000	.0932	.1665
	60	-.1469*	.01275	.000	-.1818	-.1119
	70	-.6040*	.01275	.000	-.6390	-.5691
	100	.5042*	.01561	.000	.4614	.5470
40	30	-.1299*	.01337	.000	-.1665	-.0932
	60	-.2768*	.01337	.000	-.3134	-.2401
	70	-.7339*	.01337	.000	-.7706	-.6973
	100	.3743*	.01613	.000	.3301	.4185
60	30	.1469*	.01275	.000	.1119	.1818
	40	.2768*	.01337	.000	.2401	.3134
	70	-.4572*	.01275	.000	-.4921	-.4222
	100	.6511*	.01561	.000	.6083	.6939
70	30	.6040*	.01275	.000	.5691	.6390
	40	.7339*	.01337	.000	.6973	.7706
	60	.4572*	.01275	.000	.4222	.4921
	100	1.1082*	.01561	.000	1.0654	1.1510
100	30	-.5042*	.01561	.000	-.5470	-.4614
	40	-.3743*	.01613	.000	-.4185	-.3301
	60	-.6511*	.01561	.000	-.6939	-.6083
	70	-1.1082*	.01561	.000	-1.1510	-1.0654
Temperature (I)	Temperature (J)					
140	160	-.1803*	.01071	.000	-.2055	-.1551
	180	-.3349*	.01056	.000	-.3598	-.3101
160	140	.1803*	.01071	.000	.1551	.2055
	180	-.1547*	.01056	.000	-.1795	-.1298
180	140	.3349*	.01056	.000	.3101	.3598
	160	.1547*	.01056	.000	.1298	.1795

Table 3S2 (cont'd) Multiple Comparisons		Mean Difference (I-J)	Standard Error (SE)	Sig.	95% Confidence Interval Lower Bound	Upper Bound
Metal Ion Chelation Activity (%)						
Solvent (I)	Solvent (J)					
Ethanol	Methanol	-.1066*	.01676	.000	-.1460	-.0671
	Water	.0286	.02595	.513	-.0325	.0897
Methanol	Ethanol	.1066*	.01676	.000	.0671	.1460
	Water	.1352*	.02652	.000	.0728	.1976
Water	Ethanol	-.0286	.02595	.513	-.0897	.0325
	Methanol	-.1352*	.02652	.000	-.1976	-.0728
Concentration (I)	Concentration (J)					
30	40	-.0433	.02493	.413	-.1116	.0251
	60	-.2416*	.02310	.000	-.3050	-.1783
	70	-.6131*	.02298	.000	-.6761	-.5501
	100	-.1676*	.02872	.000	-.2463	-.0888
40	30	.0433	.02493	.413	-.0251	.1116
	60	-.1984*	.02451	.000	-.2656	-.1311
	70	-.5698*	.02440	.000	-.6367	-.5029
	100	-.1243*	.02987	.000	-.2062	-.0424
60	30	.2416*	.02310	.000	.1783	.3050
	40	.1984*	.02451	.000	.1311	.2656
	70	-.3715*	.02253	.000	-.4332	-.3097
	100	.0741	.02836	.070	-.0037	.1518
70	30	.6131*	.02298	.000	.5501	.6761
	40	.5698*	.02440	.000	.5029	.6367
	60	.3715*	.02253	.000	.3097	.4332
	100	.4455*	.02826	.000	.3680	.5230
100	30	.1676*	.02872	.000	.0888	.2463
	40	.1243*	.02987	.000	.0424	.2062
	60	-.0741	.02836	.070	-.1518	.0037
	70	-.4455*	.02826	.000	-.5230	-.3680
Temperature (I)	Temperature (J)					
140	160	-.3240*	.01904	.000	-.3688	-.2792
	180	-.7054*	.01938	.000	-.7510	-.6598
160	140	.3240*	.01904	.000	.2792	.3688
	180	-.3813*	.01930	.000	-.4268	-.3359
180	140	.7054*	.01938	.000	.6598	.7510
	160	.3813*	.01930	.000	.3359	.4268

*The mean difference is significant at the 0.05 level, Sig.-level of significance

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BRIDGE TO CHAPTER 4

In chapter 3, the optimization of the extraction parameters with the emphasis on its changes on the antioxidant activity was determined using different *in-vitro* assays including FRAP, DPPH and metal ion chelation activity. Different extraction conditions yield different amount of phenolic compounds. It is often important to determine the optimized extraction condition for individual phenolic compounds to improve its extractability. The removal of phenolic compounds from the canola meal might have positive impact towards the palatability. Differences in particle sizes, extraction temperatures, extraction solvents, and solvent concentrations impact the final yield of the phenolic composition. Chapter 4 comprises with identification of quantification of major sinapates of canola meal using HPLC coupled with mass spectrometry.

AUTHOR CONTRIBUTIONS FOR CHAPTER 4

Attenuation of sinapic acid and sinapine-derived flavor-active compounds using a factorial-based pressurized high-temperature processing. **Nandasiri R.**, Zago, E., Eskin N. A. M., Thiyam-Holländer U. Journal of American Oil Chemists Society, 2021, 98 (7), 779-794. Ruchira Nandasiri designed the study, performed the experiments, developed the HPLC method, conducted the statistical analysis, and wrote the first draft of the manuscript. Dr. Erika Zago synthesized the canolol and developed of the HPLC method and helped with review comments. Both Dr. Michael Eskin and Dr. Usha Thiyam-Holländer provided critical feedback on the manuscript with proof reading and assisted with the review comments. Dr. Usha Thiyam-Holländer obtained the funding.

CHAPTER 4

MANUSCRIPT II

ATTENUATION OF SINAPIC ACID AND SINAPINE-DERIVED FLAVOR-ACTIVE COMPOUNDS USING A FACTORIAL-BASED PRESSURIZED HIGH-TEMPERATURE PROCESSING

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Published in Journal of American Oil Chemists Society, 2021, 98 (7), 779-794.

4.1 Abstract

De-oiled canola meals are sources of protein-containing flavor-active phenolic compounds. Conventional canola oil processing utilizes an excess amount of solvents and is associated with the release of high-intensity bitter flavor-active phenolic compounds, limiting the use of the canola meal. Recent advances in the extraction and isolation of the bitter favor-active phenolic compounds from canola by-products produce protein isolates, however, would benefit the industry by producing a side-stream ingredient rich in phenolics. High temperature and pressure-aided processing, namely the accelerated solvent extraction (ASE) was investigated to extract the flavor-active bitter molecules from the canola meal. The extractability of flavor-active phenolic compounds including the major sinapates, kaempferol derivatives, and other thermo-generative compounds including thomasidioic acid (TA) was evaluated. The effects of temperature, solvent extractant and concentration, and the particle size of the meal, were examined on the extraction efficiency of these phenolic compounds. Extraction temperature (180°C) was the primary determinant ($p<0.05$) for the attenuation of major sinapates including sinapine and sinapic acid. Both ethanol and methanol extractants at a concentration of 70% (v/v) significantly ($p<0.05$) extracted the flavor-active phenolic compounds. The pressurized high temperature through optimized ASE conditions attenuated the bitter undesirable flavor-active phenolic molecules from canola meal thereby facilitating a potential value-added phenolic-rich by-product.

Keywords – accelerated solvent extraction (ASE), high temperature, de-oiled canola, bitter compounds, processing, sinapine

4.2 Introduction

Currently, up to 60% of the world's dietary protein is provided by plant-based sources (Gorissen & Witard, 2018). With the current emphasis on sustainable ingredients, plant-based protein has garnered interest by the food and feed protein industry to meet consumer demands for new and alternative sources. Both canola and pea protein blends are reported to exceed the protein quality of meat and dairy and would play a key aspect in fulfilling the future protein demand for humans (Gläser et al., 2020; Hald et al. 2019). However, the presence of undesirable bitter complexes initiated by compounds such as glucosinolates, phytates, tannins, phenolics, and its high fibre content limits the use of canola meal in food sources (Khattab et al., 2010; Naczk et al., 1998). Moreover, the associations between the proteins and the tannins further contributes to the bitter taste in the protein products (Naczk et al. 1998). Recent advancements in canola industry have led to produce valuable protein isolates and other protein ingredients from canola meal. Hence, the residual meal after isolation of the protein fractions may impart as a value-added by product to produce bitter flavor-active phenolic compounds to introduce in the nutraceutical industry.

The phenolic compounds in canola can be categorized as free, esterified, and insoluble bound (with benzoic and/or cinnamic acid) (Alu'datt et al., 2017; Li & Guo, 2016b; Quinn et al., 2017). Kozlowska *et al*., (1983) reported the content of insoluble and bound phenolic compounds in canola meal ranged from 32-50 mg/kg. The predominant free phenolic compounds in rapeseed meal were sinapic acid, vanillic acid, protocatechuic acid, syringic acid, p-coumaric acid, ferulic acid, caffeic acid, and chlorogenic acid (Kozlowska et al., 1990). The predominant phenolic compounds in canola by-products are esterified, with sinapine accounting for over 80%, and

sinapic acid occurring as the major free form (Li & Guo, 2016b; Quinn et al., 2017). The traditional processing methods require large amount of extraction solvents (for example 1 g meal requires 70 mL ethanol). This is considered environmentally undesirable even though up to 85% of the phenolics can be removed (Li & Guo, 2016b; Quinn et al., 2017). The abundance of sinapates and kaempferol derivatives present in the meal before and after solvent extraction warrants further investigation. Moreover, these bitter-flavoring phenolic compounds conjugate with other food ingredients including proteins, peptides, and lipids (Alu'datt et al., 2017). Consequently, the amount, bonding, and structure can have a profound effect on the extraction of these complex phenolic compounds; for example, their initial concentration determines the tannin-protein, protein-phenolic and lipid-phenolic-protein complexes (Alu'datt et al., 2017; Mišan et al., 2010).

The targeted removal and co-extraction of these bitter flavor-contributing compounds, especially sinapine, and kaempferol derivatives will contribute to further innovative processing of canola by-products. Furthermore, these value-added by products could be introduced as a source of nutraceuticals with high antioxidant activity (Alu'datt et al., 2017; Li & Guo, 2016b). Apart from sinapine, both sinapic acid and canolol are both reported as strong antioxidative, anti-radical and anti-mutagenic molecules (Cao et al., 2015; Chen, 2016; Morley et al., 2013). The formation of canolol is closely associated with high temperature processing as temperature-dependent parameters are necessary to improve the functional properties of canolol (Li & Guo, 2016a; Nandasiri et al., 2019). Hence, the isolation and purification of these flavor-active phenolic compounds and other antioxidative compounds would be an asset to the industry. Thus, a targeted efficient extraction method capable of releasing or separating the bitter-flavor active phenolic compounds from proteinaceous matter would be advantageous to the industry.

Both pressurized solvent extraction (PSE) and accelerated solvent extraction (ASE) have recently been applied by the natural product industry to extract phenolic compounds at a relatively high temperature (~200°C), and pressure (~2000 psi) (Li & Guo, 2016a; Nandasiri et al., 2019). The higher phenolic extraction efficiency associated with these methods facilitate attenuation of the bitter-flavoring compounds in the meal, by impacting the extraction of the major sinapic acid derivatives, primarily sinapine and kaempferol derivatives (Li & Guo, 2016a, 2016b; Nandasiri et al., 2019). Thermal processing and the high pressure associated with ASE have many advantages including reduction in the surface tension and viscosity of the extracting solvents, which improves the solubility and mass transfer of targeted phenolics (Li & Guo, 2016a). ASE is also equipped with a closed chamber so that an inert supply with N_2 ensures the stability of the crude extracts with a higher yield of phenolic compounds (Nandasiri et al., 2019).

Previous research reported that structural alterations of phenolics resulted from the application of high pressure, and high temperature (Nandasiri et al., 2019), which generated canolol and flavor-active novel dimers and trimers (Harbaum-Piayda et al., 2010; Kraljić et al., 2015). These previous works discussed extraction yields and instability of these flavor-active phenolic compounds, however on a lab-scale, and further investigation is yet to be considered. A potential major drawback in converting them at both bench-top and industrial scale is absent so far. Consequently, targeted extraction of bitter flavor-active phenolic co-stream ingredients from canola meal should substantially increase its value as a source of nutraceuticals. The present study investigated the pressurized temperature processing (ASE) as method of extraction of flavor-active phenolic compounds. Two different particle sizes (0.5 mm and 1.0 mm) and two extractants (methanol and ethanol) at different concentrations (30%, 40%, 60%, and 70% v/v) under high pressure (1500 psi) at three different temperatures (140, 160, and 180°C) were examined in the

current study. The present study investigated important parameters for extracting the bitter compounds, sinapine, sinapic acid, thomasidioc acid (TA), and major flavor-active kaempferol derivatives. Furthermore, the application of pressurized temperature processing via ASE with the targeted extraction of canolol was investigated. The targeted extraction has implications in co-processing of the canola meal to produce value-added phenolic compounds.

4.3 Materials

Mechanical crushed (double expeller pressed) canola meal containing an oil content of 4-6% (*Brassica napus* L.) was used in this study. All the raw materials were obtained from the Viterra group, St. Agathe, Manitoba. Sinapic acid (purity > 98%) were purchased from Fisher scientific Canada Ltd (Ottawa, ON, Canada). Sinapine (purity > 97%) was purchased from ChemFaces Biochemical Co., Ltd (Wuhan, Hubei, China) Canolol was synthesized in the lab (purity > 97%) and its purity confirmed via HPLC. Cellulose filter papers were purchased from Thermo Scientific Canada Ltd (Mississauga, ON, Canada). All the extraction solvents were purchased from Fisher scientific Canada Ltd (Ottawa, ON, Canada).

4.4 Methods

4.4.1 Sample preparation

Canola meal was sieved (Mesh sieve size of 0.5 and 1.0 mm, Ro-Tap Testing Sieve Shaker Model B, WS Tyler, Mentor, Ohio, USA) to obtain two different particle sizes. A Mastersizer 2000 (Malvern Instruments Ltd, Malvern, United Kingdom) was used to confirm the particle size. Samples were defatted using the Soxtec 2050 (Foss-Tecator, Foss North America, Minneapolis, MN, USA) and stored at -20oC until further analyzed (Khattab, et al., 2010).

4.4.2 Synthesis and purification of canolol

The synthesis of canolol was carried out as described by Simpson *et al*. (2005) and Zago *et al*. (2015) by Knoevenagel condensation. In a 200 mL flask, syringaldehyde (3,5-dimethoxy-4-hydroxybenzaldehyde) (8.23 µmol, 1.5 mg), malonic acid (12.35 µmol, 1.3 mg) and piperidine (41.17 µmol, 4.07 mL) was dissolved in toluene (21.0 mL). The reaction mixture was heated to

reflux (115ºC) with continuous magnetic stirring (200 rpm). Traces of piperidine were eliminated by adding 20 mL of toluene to the precipitate under vacuum evaporation (Zago et al., 2015). The remaining precipitate was purified using a glass column filled with silica gel 60 Å as the stationery and n-hexane/ethyl acetate (70/30, v/v) as the mobile phase. Fraction separation was followed by applying drops of each collector tube to TLC (EMD Millipore Silica Gel 60 F254) plates which were developed with n-hexane/ethyl acetate/formic acid (70/30/1, v/v/v), dried and directly analyzed using a UV lamp (Zago et al., 2015).

4.4.3 Extraction of bitter compounds using accelerated solvent extraction (ASE)

Extraction of bitter compounds was performed using aqueous methanol and ethanol at different concentrations (30%, 40%, 60%, and 70% v/v) under high pressure (1500 psi) and at three different temperatures (140, 160, and 180ºC) using ASE (ASE 300, Dionex, New York, NY, USA). Sieved samples were mixed with Ottawa sand in a ratio of 1:5 to optimize the yield of the compounds (**Figure 4.1**). Extracts were concentrated using the rotary evaporator (BÜCHI Rotavapor® R-100, BÜCHI Labortechnik AG, Flawil, Switzerland) and freeze-dried in a freeze dryer (6 Freezone, Labconco Corporation, Kansas City, MO, USA) at -50ºC for 36 to 48 hours. All freeze-dried samples were reconstituted with 100% methanol to a final volume of 30.0 mL and diluted up to 10- times prior to HPLC analysis (**Figure 4.1**).

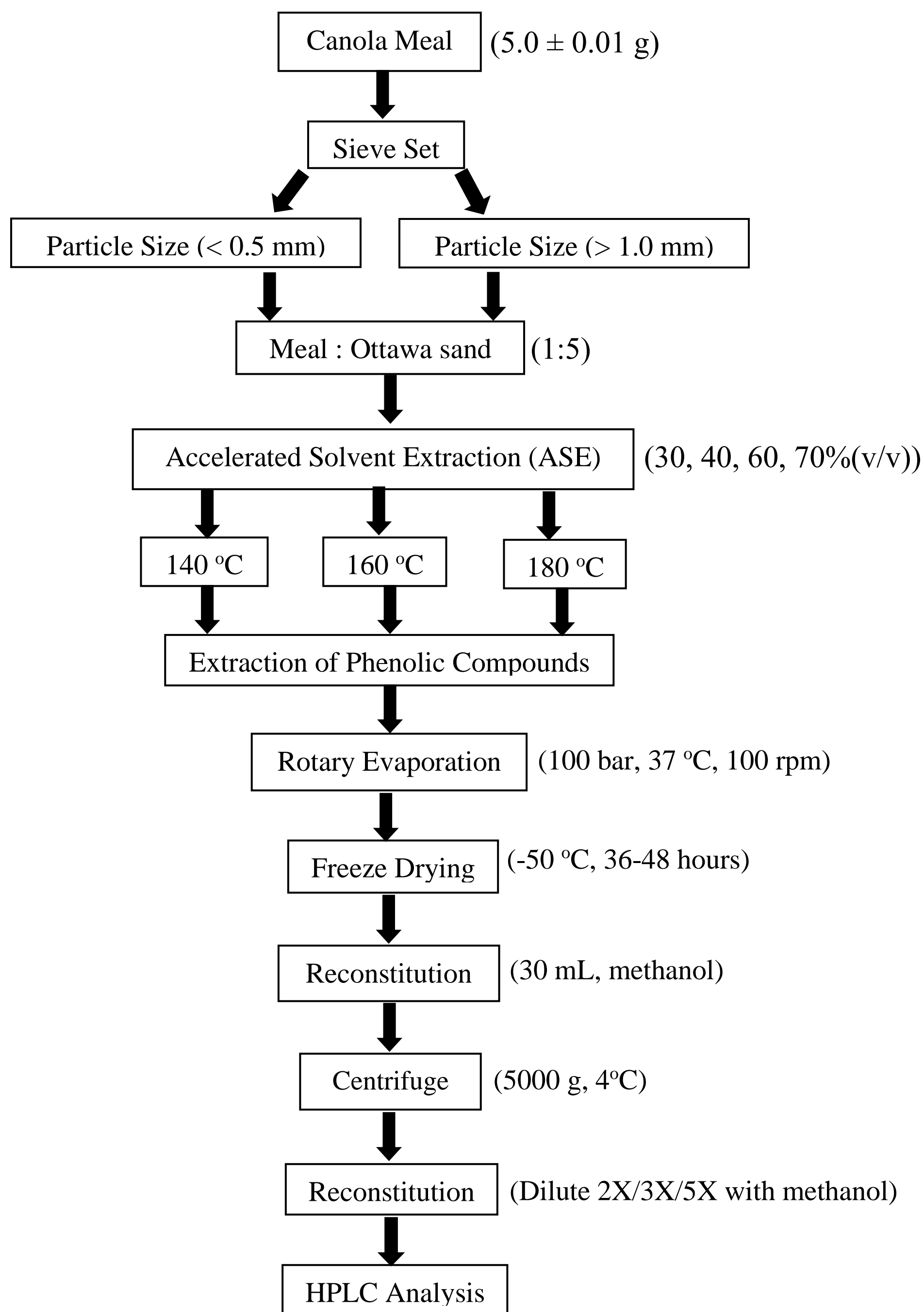

Figure 4.1 Extraction of phenolic compounds using Accelerated Solvent Extractor (ASE)

4.4.4 Effect of acidification on bitter flavor compounds

Canola meal matrix was acidified with 1.5% *O*-phosphoric acid solution and extracted at 160°C with three different extractants (100% (v/v) water, 70% (v/v) methanol, and 70% (v/v) ethanol) as described in **4.4.3** and subjected to HPLC as described in **4.4.5**.

4.4.5 Identification of flavor-active bitter phenolics by HPLC-MS/MS

HPLC analysis was adapted and carried out on a Kinetex® Biphenyl C_{18} 100 Å RP column (2.6 mm, 150 x 4.6 mm, Phenomenex, Canada) maintained at 30°C with 0.4 mL/min flow rate, and 10 μL injection volume as Harbaum-Piayda *et al*. (2010) as described in Nandasiri *et al*. (2019). The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). Chromatograms were acquired at 270 and 330 nm in triplicate by Chromeleon software Version 7.2 SR4 (Dionex Canada Ltd, Oakville, ON Canada). Calibration curves of sinapine, sinapic acid, and canolol were obtained from a series of standard solutions in methanol from 1.0 to 100 μg/mL (n = 11) with $R^2 = 0.998$ for sinapic acid, $R^2 = 0.999$ for canolol, and $R^2 = 0.999$ for sinapine with detection limit of each compound at 0.001 mg/mL.

Structural elucidation of kaempferol-3-*O*-(2‴-*O*-sinapoyl-*β*-sophoroside), kaempferol-3-*O*-sophoroside, thomasidioic acid (TA) were tentatively identified by liquid chromatography with mass spectrometry and tandem mass spectrometry (LC-MS) using the HPLC method described above. Fractions were collected at one-minute intervals and were dried (N_2) and analyzed by ESI-MS-MS/MS. Positive ion mode (ESI^+) was used, and spectra recorded on a Bruker Compact high resolution quadrupole time of flight mass spectrometer (Q-TOF-MS) (Bruker Daltonics, Billerica, Massachusetts, USA). MS mode was applied during the formula generation and the mass range

was from 50 m/z to 2500 m/z was used. The elute pump was operated at a maximum pressure of 10150 psi, with a capillary voltage of 3500V at a dry gas flow rate of 4.0 L/min with a drying temperature of 200^oC. MS/MS tuning was carried out with 5.0 eV (ion energy) and 10.0 eV (collision energy). The obtained fragments were compared with the literature values in confirming the phenolic structures (Cai et al. 1999; Hald et al. 2019; Rubino et al. 1996).

4.5 Statistical analysis

All the experiments were carried out in triplicates. Results were presented as mean ± standard deviation of triplicate analysis. Data points were checked for their normality, and required transformations were carried out to obtain normalized data (Pallant, 2011). For the current experiment, logarithmic and square root transformations were conducted accordingly to obtain normalized data (Pallant, 2011). A factorial design consists with four independent factors including particle size (0.5 and 1.0 mm), type of extraction solvent (ethanol and methanol), concentration of the solvent (30%, 40%, 60%, and 70%, v/v) and extraction temperature (140, 160 and 180^oC). Data analysis was carried out using the general linear multiple regression model using the two-way analysis of variance (ANOVA). Multiple mean comparison was performed using Tukey's test at the level of significant of 0.05 ($p < 0.05$) (Pallant, 2011). To identify the correlation between each phenolic compound partial correlation analysis and a regression analysis was conducted for the major phenolic compounds to elucidate the structure-function relationship. All the data analysis tests were assessed by SPSS statistical software version 22 (IBM, New York, NY, USA).

4.6 Results & Discussion

4.6.1 Extraction efficiency of major sinapates

The hydrolysis of sinapine to sinapic acid is considered the major structural-alteration pathway contributing to the flavor-active properties present in canola meal (Li & Guo, 2016a; Nandasiri et al., 2019; Siger et al., 2013). Apart from sinapine, other sinapate derivatives including sinapic acid and canolol also contributes to the flavor properties of the canola meal (Morley et al., 2013; Thiyam et al., 2009; Thiyam et al., 2006). Furthermore, the decarboxylation of sinapic acid to canolol takes place at higher processing temperatures (Zago et al., 2015). Hence, the higher processing temperatures (>100°C) are associated with the improved extractability of the bitter flavor-active phenolic compounds (Nandasiri et al. 2020). Thus, our findings demonstrated that both extraction temperature and extractant concentration appears to be the most important parameters for attenuating the major sinapates from the canola meal. Statistical analysis further illustrated that the extraction efficiency of these sinapates including sinapine, sinapic acid and canolol, were influenced by concentration of the extractant, type of solvent, and extraction temperature (**Table S1 a-f**). It was previously reported that solvent concentration is an important factor affecting the rate and the degree of decarboxylation of sinapic acid (Li & Guo, 2016a; Nandasiri et al., 2019; Siger et al., 2013). Current study further confirmed that both the extractant concentration and the extraction temperature are the dominant factors attenuating the major sinapates. However, the particle size of the meal was the least important factor in extracting the flavor-active bitter molecules including the sinapates.

The extractability of sinapine, the major flavor-active phenolic compound present in canola meal (Thiyam et al., 2009) was primarily dependent on the extractant concentration and the

extraction temperature. According to the model fit statistics both particle size ($p = 0.12$) and type of solvent ($p = 0.15$), had no significant effect on the extractability of sinapine (**Table S1a**). This further confirms that the removal of sinapine was much less affected by the particle size of the dried canola meal compared to the type of solvent extractant (methanol, ethanol). A similar trend was observed for the extractability of sinapic acid, another flavor-active phenolic acid present in canola meal by-products. Except for particle size ($p = 0.81$), type of solvent ($p = 0.30$), and size*concentration interaction ($p = 0.24$), all other independent variables were significant ($p < 0.05$) (**Table S1b**) for extracting sinapic acid using the pressurized temperature processing. However, the extractability of canolol was mainly dependent on both the extractant concentration and type of extractant including the extraction temperature (**Table S1c**). The size of canola meal particles ($p = 0.11$) had a negligible effect on extractability of the canolol. The statistical analysis of the model accuracy was further conformed with the higher co-efficiencies of variances for all the major sinapates (sinapine - $R^2 = 0.998$, sinapic acid - $R^2 = 0.990$, and canolol - $R^2 = 0.982$).

The polarity of the extractant solvent could affect the extractability of phenolic compounds and its antioxidant properties (Teh & Birch, 2013). Furthermore, Li & Guo (2016a) reported that different polarities of the extractant solvents yield different distributions of major sinapates. The application of pressurized heat via ASE further facilitates the concurrent extraction of phenolic compounds and their transformations (Li & Guo, 2016a; Nandasiri et al., 2019). It was reported that the application of pressurized heat improves the H-bonding donor and accepting ability (Li & Guo, 2016a). Furthermore, the pressurized heat would further eliminate the number of hydroxyl groups and other attachments attached to the phenolic structure thereby improving the extractability of the phenolic compounds (Gaspar et al., 2008). The current study validated 70% (v/v) of both ethanol (**Figure S3**) and methanol (**Figure S2**) aqueous extractants as the optimum

concentration for extracting the major sinapates compared to their corresponding concentrations. Hence, the extractability of phenolic compounds increases with a decrease in the polarity index of the type of extractant (Terpinc et al., 2012). Considering the polarity index of both methanol (0.762) and ethanol (0.654) with having similar polarities confirms the current research findings. These results agree with previous reports where major sinapates including canolol was extracted at higher temperatures and when the optimum aqueous solvent concentration was 70% (v/v) (Li & Guo, 2016b, 2016a; Nandasiri et al., 2019; Thiyam et al., 2004; Zago et al., 2015).

The above results confirmed that the extractability of these three flavor-active sinapates were minimally affected by particle size ($p > 0.05$). Generally, the higher extraction efficiency of hydroxycinnamic acids is solely attributed to thermal degradation. For example, the generation of aroma compounds such as 4-vinylguaiacol (the product of the decarboxylation of ferulic acid), guaiacol and vanillin from ferulic acid and the bitter series *O*-caffeoyl-, *O*-feruloyl-, *O*-dicaffeoyl- and quinide derivatives derived from chlorogenic and quinic acids (Rahman et al., 2020).

4.6.2 Extraction efficiency of other flavor-active minor compounds

Apart from the major flavor-active sinapates, other classes of phenolics also serve as active bitter flavoring compounds such as kaempferol 3-*O*-*β*-sophoroside (KS) and, kaempferol 3-*O*-(2‴-*O*-sinapoyl-*β*-sophoroside) (KSS) (Hald et al., 2019; Yang et al., 2015). A sensory study conducted by Hald *et al*. (2019) demonstrated that protein isolates of rapeseed (canola) containing kaempferol 3-*O*-*β*-sophoroside (KS) exhibited a bitter taste above the low threshold concentration of 3.4 μmol/L confirming the as the key flavor-active molecule of the protein isolates. Kaempferol 3-*O*-*β*-sophoroside was also reported as a flavor-active phenolic compound found to be present in *Brassica* family (Yang et al., 2015). Extraction, identification, and quantification of these unique minor compounds would advance the avenues for biorefinery approach as well as feed formulations targeting the removal of off flavors. Liquid chromatography (LC) coupled with mass spectrometry and tandem mass spectrometry (MS/MS-MS) identified this unique flavor-active molecule to be present in our extracts. The quantification of this molecule was done based on sinapic acid equivalents (SAE) to understand the impact of extraction parameters including concentration of the extractant, type of solvent, extraction temperature and the particle size.

The statistical analysis indicated that extractability of KSS was impacted by all the extraction parameters including concentration of the extractant, type of solvent, extraction temperature and the particle size, indicating the stability of this unique flavor active molecule (**Table S1e**). Nevertheless, post-hoc analysis using Tukey's test indicated that both 60% (v/v) and 70% (v/v) for both methanol and ethanol extractants had a minimal impact on the extractability of KSS (**Table 4.1a**). This further confirms that lower solvent polarities enable the extraction of this unique flavor-active molecule, thereby attenuating the bitter off flavors from the meal. The application of less organic solvents and other harmful chemicals are often rewarded by the

industries and the government, and often provide many economic benefits (Chen et al., 2014). However, the other kaempferol derivative, KS showed a different extractability compared to KSS. The extractability of KS was mainly depended on both solvent concentration and the particle size. Interestingly, both solvent type ($p = 0.26$) and extraction temperature ($p = 0.50$) had a minimal impact on its extractability (**Table S1d**). The results further indicated that this minor compound showed relatively higher thermal stability than the other flavor-active compounds. Further, post-hoc analysis indicated that each concentration level had a significant impact on the extractability of KS (**Table 4.1a**). Hence, the use of smaller particle size meal with higher polarity aided a relatively higher concentration of KS. The above results confirmed that the extractability of these two unique flavor active minor compounds (KSS and KS) differed considerably. Thus, the results further confirmed the structural alterations in the phenolic compounds would affect the extractability parameters and may impact its flavor profile.

Thomasidioic acid (TA) is another flavor-active molecule but the structural alteration due to processing and extraction has not received much attention in recent years. Both Rubino *et al*. (1996) and Cai *et al*. (1999) reported that TA was not a natural phenolic compound but formed during the high temperature processing in the presence of oxygen at both acidic and alkaline pH. The formation of TA takes place in the acidic medium with the precursor sinapic acid with dehydrosinapic acid lactone as its intermediary product (Rubino, Arntfield, & Charlton, 1995). TA is categorized under the phenolic group of lignans. These lignans were reported to convert into hormone like compounds by the gut microflora inside the body, which protects the body against hormone dependent cancers (Ward, 1993). The quantification of this thermo-generative compound was conducted to understand the impact of each extraction parameter.

The statistical analysis indicated that extractability of TA was primarily depended on both extraction temperature and the concentration of the extractant (**Table S1f**). Both the size of the canola meal particles ($p = 0.48$) and the type of solvent ($p = 0.14$) had a minimum impact on the extractability of TA agreeing with the previous reports. On the contrary, at higher extraction temperatures, these lignan compounds further converts to other complex phenolic compounds including its dimers, trimers, and oligomers (Harbaum-Piayda et al., 2010; Morley et al., 2013; Oehlke et al., 2017; Siger et al., 2013). Consequently, the concentration of free TA would decrease with the formation of these phenolic derivatives. This was further confirmed via the statistical analysis showing that both 140 and 180°C processing temperatures had no significant differences on the extractability of TA (**Table 4.1c**).

Table 4.1 Post-Hoc analysis for multiple comparisons of the mean effects studied in the general linear regression of the factorial design for main flavor-active phenolic compounds

Multiple Comparisons		Mean Difference (I-J)	Standard Error (SE)	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
(a) Kaempferol 3-*O*-(2‴-*O*-sinapoyl-*β*-sophoroside) (KSS)						
Concentration (I)	Concentration (J)					
30	40	-117.96*	13.50	0.00	-153.07	-82.86
	60	-286.40*	13.50	0.00	-321.51	-251.30
	70	-274.20*	13.50	0.00	-309.30	-239.10
40	30	117.96*	13.50	0.00	82.86	153.07
	60	-168.44*	13.50	0.00	-203.54	-133.34
	70	-156.23*	13.50	0.00	-191.34	-121.13
60	30	286.40*	13.50	0.00	251.30	321.51
	40	168.44*	13.50	0.00	133.34	203.54
	70	12.21	13.50	0.80	-22.90	47.31
70	30	274.20*	13.50	0.00	239.10	309.30
	40	156.23*	13.50	0.00	121.13	191.34
	60	-12.21	13.50	0.80	-47.31	22.90
Temperature (I)	Temperature (J)					
140	160	-58.91*	11.70	0.00	-86.61	-31.21
	180	146.45*	11.70	0.00	118.75	174.15
160	140	58.91*	11.70	0.00	31.21	86.61
	180	205.36*	11.70	0.00	177.67	233.06
180	140	-146.45*	11.70	0.00	-174.15	-118.75
	160	-205.36*	11.70	0.00	-233.06	-177.67
(b) Kaempferol 3-*O*-*β*-sophoroside (KS)						
Concentration (I)	Concentration (J)					
30	40	-2.09*	0.63	0.01	-3.71	-0.46
	60	-12.93*	0.63	0.00	-14.56	-11.31
	70	-27.15*	0.63	0.00	-28.78	-25.53
40	30	2.09*	0.63	0.01	0.46	3.71
	60	-10.84*	0.63	0.00	-12.47	-9.22
	70	-25.06*	0.63	0.00	-26.69	-23.44
60	30	12.93*	0.63	0.00	11.31	14.56
	40	10.84*	0.63	0.00	9.22	12.47
	70	-14.22*	0.63	0.00	-15.84	-12.59
70	30	27.15*	0.63	0.00	25.53	28.78
	40	25.06*	0.63	0.00	23.44	26.69
	60	14.22*	0.63	0.00	12.59	15.84
(c) Thomasidioic acid (TA)						
Concentration (I)	Concentration (J)					
30	40	-3.01*	0.43	0.00	-4.20	-1.83
	60	-8.99*	0.43	0.00	-10.19	-7.79
	70	-9.85*	0.43	0.00	-11.04	-8.66
40	30	3.01*	0.43	0.00	1.82	4.20
	60	-5.98*	0.43	0.00	-7.17	-4.78
	70	-6.84*	0.43	0.00	-8.02	-5.66

(Table 4.1 Cont'd) Multiple Comparisons		Mean Difference (I-J)	Standard Error (SE)	Sig	95% Confidence Interval	
					Lower Bound	Upper Bound
60	30	8.99*	0.43	0.00	7.79	10.19
	40	5.98*	0.43	0.00	4.78	7.17
	70	-0.86	0.43	0.27	-2.06	0.33
70	30	9.85*	0.43	0.00	8.66	11.04
	40	6.84*	0.43	0.00	5.66	8.02
	60	0.86	0.43	0.27	-0.33	2.06
Temperature (I)	Temperature (J)					
140	160	-2.34	0.35	0.00	-3.17	-1.51
	180	-0.19	0.35	0.84	-1.02	0.63
160	140	2.34	0.35	0.00	1.51	3.17
	180	2.15	0.35	0.00	1.31	2.98
180	140	0.19	0.35	0.84	-0.63	1.02
	160	-2.15	0.35	0.00	-2.98	-1.31
(d) Sinapine						
Concentration (I)	Concentration (J)					
30	40	-0.10*	0.00	0.00	-0.11	-0.09
	60	-0.49*	0.00	0.00	-0.50	-0.48
	70	-0.83*	0.00	0.00	-0.84	-0.82
40	30	0.10*	0.00	0.00	0.09	0.11
	60	-0.39*	0.00	0.00	-0.40	-0.38
	70	-0.73*	0.00	0.00	-0.74	-0.72
60	30	0.49*	0.00	0.00	0.48	0.50
	40	0.39*	0.00	0.00	0.38	0.40
	70	-0.34*	0.00	0.00	-0.35	-0.33
70	30	0.83*	0.00	0.00	0.82	0.84
	40	0.73*	0.00	0.00	0.72	0.74
	60	0.34*	0.00	0.00	0.33	0.35
Temperature (I)	Temperature (J)					
140	160	-0.10*	0.00	0.00	-0.11	-0.09
	180	-0.01*	0.00	0.01	-0.02	-0.00
160	140	0.10*	0.00	0.00	0.09	0.11
	180	0.09*	0.00	0.00	0.08	0.09
180	140	0.01*	0.00	0.01	0.00	0.02
	160	-0.08*	0.00	0.00	-0.09	-0.08
(e) Sinapic acid						
Concentration (I)	Concentration (J)					
30	40	-0.08*	0.01	0.00	-0.11	-0.05
	60	-0.37*	0.01	0.00	-0.39	-0.34
	70	-0.87*	0.01	0.00	-0.90	-0.85
40	30	0.08*	0.01	0.00	0.05	0.11
	60	-0.29*	0.01	0.00	-0.32	-0.26
	70	-0.79*	0.01	0.00	-0.82	-0.77
60	30	0.37*	0.01	0.00	0.34	0.39
	40	0.29*	0.01	0.00	0.26	0.32
	70	-0.51*	0.01	0.00	-0.54	-0.48

(Table 4.1 Cont'd) Multiple Comparisons		Mean Difference (I-J)	Standard Error (SE)	Sig	95% Confidence Interval	
					Lower Bound	Upper Bound
70	30	0.87*	0.01	0.00	0.85	0.90
	40	0.79*	0.01	0.00	0.77	0.82
	60	0.51*	0.01	0.00	0.48	0.54
Temperature (I)	Temperature (J)					
140	160	-0.18*	0.01	0.00	-0.21	-0.16
	180	-0.08*	0.01	0.00	-0.10	-0.05
160	140	0.18*	0.01	0.00	0.16	0.21
	180	0.10*	0.01	0.00	0.08	0.13
180	140	0.08*	0.01	0.00	0.05	0.10
	160	-0.10*	0.01	0.00	-0.13	-0.08
(f) Canolol						
Concentration (I)	Concentration (J)					
30	40	-0.01	0.02	0.95	-0.05	0.03
	60	-0.60*	0.02	0.00	-0.64	-0.55
	70	-0.90*	0.02	0.00	-0.94	-0.86
40	30	0.01	0.02	0.95	-0.03	0.05
	60	-0.59*	0.02	0.00	-0.63	-0.54
	70	-0.89*	0.02	0.00	-0.93	-0.85
60	30	0.60*	0.02	0.00	0.55	0.64
	40	0.59*	0.02	0.00	0.54	0.63
	70	-0.30*	0.02	0.00	-0.35	-0.26
70	30	0.90*	0.02	0.00	0.86	0.94
	40	0.89*	0.02	0.00	0.85	0.93
	60	0.30*	0.02	0.00	0.26	0.35
Temperature (I)	Temperature (J)					
140	160	-0.36*	0.01	0.00	-0.39	-0.32
	180	-0.57*	0.01	0.00	-0.61	-0.54
160	140	0.36*	0.01	0.00	0.32	0.39
	180	-0.21*	0.01	0.00	-0.25	-0.18
180	140	0.57*	0.01	0.00	0.54	0.61
	160	0.21*	0.01	0.00	0.18	0.25

Sig: level of significance ($P < 0.05$); *: mean difference is significantly different ($P < 0.05$)

4.6.3 Impact of pressurized heat on flavor-active phenolic compounds

The literature generally supported that thermal processing affected sinapates. The high temperature (up to 200ºC) and pressure (~1500 psi) of ASE facilitates the removal of the aglycone moieties attached to phenolic compounds by hydrolysis with minimal interference on its original composition (Yang et al., 2015). The application of ASE yielded comparatively higher amounts of phenolic compounds compared to conventional methods as well as ultrasound extraction (Li & Guo, 2016a; Nandasiri et al., 2019). This was attributed to the high pressure of ASE which increased the solubility of the targeted compounds and the diffusion rates as well as the mass transfer rates of the solutes (Li & Guo, 2016a). The concurrent extraction of ASE also facilitated the structural transformations of sinapine to sinapic acid and canolol, at elevated temperatures (Li & Guo, 2016a).

These transformations would enable the attenuation of bitter flavor-active phenolic compounds while improving its co-processing. For example, the decreasing content of sinapine, largely impacted by the increase in temperature (**Table 4.2**) is attributed to the decomposition or hydrolysis pathway (Khattab, et al., 2014; Oehlke et al., 2017). Results indicated that the concentration of sinapine decreased significantly ($p > 0.05$) from 9.75 mg/g DW to 5.12 mg/g DW with the increase in temperature from 140ºC to 180ºC with 70% (v/v) ethanol whereas, the concentration of sinapine further decreased from 12.1 mg/g DW to 5.12 mg/g DW with increase in temperature from 160ºC to 180ºC (**Table 4.2**). This confirms the transformation of sinapine at higher temperatures, either from the bound and free forms (Chen, et al., 2014; Khattab, et al., 2010). The thermal decomposition order of the phenolic compounds showed the following decreasing pattern; sinapine > sinapic acid > canolol (Khattab, et al., 2010).

High temperature pre-conditioning and thermal processing treatments can also significantly ($p < 0.05$) influence the structure of phenolic compounds besides sinapine, as well as sinapic acid and canolol (Siger et al., 2013; Siger et al., 2015; Thiyam et al., 2009; Wroniak et al., 2016). Temperatures namely, 160°C and 180°C with a high pressure induced the hydrolysis of sinapine into sinapic acid which is consequently produces canolol by decarboxylation (Li & Guo, 2016a; Morley et al., 2013; Zago et al., 2015). In this study, the higher concentrations of sinapic acid and canolol produced by ASE confirm the conversion of sinapine to sinapic acid and canolol at the higher temperatures (**Table 4.2**). Thus, the combined treatment of ASE with microwave improved the quantity of phenolic compounds at relatively higher processing temperatures ranging from 160 to 180°C (Li & Guo, 2016a; Siger & Józefiak, 2016; Wroniak et al., 2016). The visually apparent darker brown/black colored extracts obtained at higher processing temperatures (180°C) by ASE was indicative of the presence of higher amounts of Maillard reaction products apart from the phenolics (Chen et al., 2014; Rubino et al., 1996).

The highest concentration of sinapic acid was attained at 160°C for both organic extractants (70% (v/v) methanol - 0.55 mg/g DW and 70% (v/v) ethanol - 0.63 mg/g DW) compared to 180°C (**Table 4.2**). A reduction in total sinapic acid and canolol content observed at temperatures above 160°C may be due to the loss of the *cis*-isomer of sinapic acid at temperatures higher than 140°C (Siger et al., 2015). Above 140°C, the *cis*-sinapic acid content decreased rapidly, and was undetectable at temperatures of 160°C and 180°C. Furthermore, both Harbaum-Piayda *et al*. (2010) and Kraljić *et al*. (2015) reported that canolol at high temperatures (>180°C) is converted into its other forms including dimers, trimers, and oligomers. Therefore, a reduction in both sinapic acid and canolol is observed under higher processing temperatures. Spielmeyer et al. (2009) noted that the optimal temperature for extracting canolol was 160°C. Moreover, Morley et al. (2013) also

reported that optimum roasting temperature for the formation of canolol is at the extraction temperature of 160°C. These findings are in agreement with our results, which also found that the highest level of canolol formation was at 160ºC. In addition, Zago et al. (2015) reported 2-hour hydration of the meal before the treatment of super-heated steam (160ºC) increased both the antioxidant activity and its total phenolic content (TPC) by 12% (22 mg SAEg/DM) compared to the non-hydrated meal further in agreement with our current findings. The authors suggest that the increase in its TPC may be due to the release of the bound phenolic compounds via the partial breakdown of the plant cell walls during the super-heated steam. The extraction conditions of ASE would facilitate similar properties yielding higher phenolic composition.

Table 4.2 Effect of particle size, temperature, type, and concentration of solvent on changes in flavor active bitter compounds

Particle Size (mm)	Temperature (°C)	Concentration of Solvent (v/v%)	[ϕ]KSS* (µg [¥]SAE/g [ψ]DW)	[δ]KS* (µg SAE/g DW)	Thomasidioic acid* (µg SAE/g DW)	Sinapine (µg/g DW)	Sinapic Acid (µg/g DW)	Canolol (µg/g DW)
0.5 mm	140°C	30% [σ]EtOH	423.53 ± 12.76	569.03 ± 16.01	228.71 ± 21.42	2350.00 ± 359.30	79.10 ± 3.90	9.91 ± 3.14
		40% EtOH	925.42 ± 32.35	1056.14 ± 38.64	541.50 ± 16.91	4869.12 ± 127.27	147.55 ± 9.35	8.10 ± 2.57
		60% EtOH	763.45 ± 75.17	1388.19 ± 48.34	822.56 ± 46.93	6671.58 ± 62.43	198.16 ± 8.43	47.74 ± 3.81
		70% EtOH	952.80 ± 33.58	2725.38 ± 30.93	1795.71 ± 180.35	9757.71 ± 152.22	432.05 ± 1.81	203.29 ± 9.73
		30% [τ]MeOH	773.54 ± 48.11	1041.12 ± 38.27	564.29 ± 13.98	4153.79 ± 116.74	150.81 ± 2.24	11.94 ± 2.32
		40% MeOH	1025.87 ± 112.39	1203.77 ± 83.95	529.73 ± 29.61	4464.86 ± 119.55	72.33 ± 11.66	20.83 ± 11.87
		60% MeOH	220.67 ± 26.68	1021.74 ± 47.10	270.38 ± 53.36	4494.62 ± 389.31	71.82 ± 26.70	15.67 ± 2.39
		70% MeOH	500.08 ± 70.22	1855.12 ± 23.67	1410.60 ± 207.05	6160.44 ± 128.36	592.55 ± 25.46	179.61 ± 11.63
1.0 mm	140°C	30% EtOH	212.36 ± 18.42	538.31 ± 29.34	305.35 ± 17.40	2630.46 ± 80.35	81.09 ± 7.56	13.90 ± 0.91
		40% EtOH	179.33 ± 4.70	293.78 ± 16.97	103.60 ± 5.94	1078.76 ± 6.27	260.67 ± 4.86	26.76 ± 3.28
		60% EtOH	663.18 ± 32.70	1321.10 ± 7.11	758.73 ± 55.50	5896.32 ± 118.79	260.01 ± 5.48	87.37 ± 3.76
		70% EtOH	655.94 ± 91.98	1892.20 ± 10.08	1339.62 ± 101.51	5797.95 ± 52.55	398.69 ± 99.68	238.63 ± 1.41
		30% MeOH	557.33 ± 37.61	671.63 ± 37.48	331.69 ± 34.29	2478.50 ± 139.97	47.14 ± 7.70	21.20 ± 2.85
		40% MeOH	422.34 ± 27.27	748.28 ± 48.60	422.34 ± 27.27	2842.48 ± 110.01	31.14 ± 5.65	26.24 ± 1.80
		60% MeOH	470.89 ± 32.56	677.43 ± 1.51	382.29 ± 0.46	3060.95 ± 15.05	293.01 ± 1.62	43.33 ± 12.73
		70% MeOH	950.34 ± 56.04	2347.79 ± 44.25	1364.79 ± 16.98	8206.31 ± 41.74	675.26 ± 9.02	187.12 ± 51.43
0.5 mm	160°C	30% EtOH	132.04 ± 4.70	324.81 ± 12.26	220.76 ± 30.65	1806.70 ± 48.24	88.69 ± 8.80	16.75 ± 2.90
		40% EtOH	713.21 ± 30.99	1423.91 ± 53.32	881.39 ± 48.29	6721.77 ± 126.14	205.79 ± 11.95	15.11 ± 3.20
		60% EtOH	703.43 ± 56.04	1658.42 ± 41.94	888.99 ± 69.62	6913.73 ± 201.36	246.82 ± 19.85	249.58 ± 6.50
		70% EtOH	653.75 ± 11.37	3307.61 ± 56.60	2185.33 ± 120.68	12082.81 ± 1094.54	550.96 ± 3.49	297.98 ± 51.25
		30% MeOH	1032.76 ± 95.57	1769.61 ± 74.13	874.26 ± 77.21	6100.58 ± 305.36	281.17 ± 24.91	10.62 ± 7.05
		40% MeOH	945.42 ± 13.78	942.79 ± 66.71	754.34 ± 182.43	3656.16 ± 89.12	143.51 ± 16.10	25.22 ± 8.51
		60% MeOH	788.04 ± 92.81	1687.69 ± 169.78	1041.97 ± 105.25	7366.38 ± 962.85	243.78 ± 62.75	173.27 ± 9.35
		70% MeOH	536.46 ± 84.48	1971.82 ± 14.88	1425.79 ± 113.94	5533.98 ± 41.71	626.03 ± 14.15	251.72 ± 16.80
1.0 mm	160°C	30% EtOH	563.51 ± 9.85	651.23 ± 6.79	326.62 ± 9.61	2849.35 ± 30.21	89.99 ± 7.96	23.84 ± 7.96
		40% EtOH	240.35 ± 2.47	377.58 ± 32.05	426.46 ± 14.46	4329.83 ± 58.55	191.75 ± 14.80	64.67 ± 6.34
		60% EtOH	582.44 ± 7.12	1701.65 ± 27.95	961.67 ± 43.71	7792.17 ± 276.81	165.47 ± 10.25	72.95 ± 21.53
		70% EtOH	506.57 ± 38.60	2509.03 ± 29.50	1693.58 ± 65.86	6944.35 ± 168.07	473.37 ± 6.56	125.36 ± 39.88

Table 4.2 (cont'd)

		30% MeOH	431.01 ± 19.05	768.74 ± 33.12	359.51 ± 30.99	2852.97 ± 148.04	163.64 ± 16.25	42.58 ± 2.38
		40% MeOH	447.51 ± 40.02	680.26 ± 45.54	383.71 ± 61.37	3184.11 ± 149.76	43.57 ± 10.31	32.13 ± 3.89
		60% MeOH	776.68 ± 83.43	960.46 ± 138.32	960.46 ± 138.32	6706.03 ± 535.48	237.91 ± 41.59	192.06 ± 18.37
		70% MeOH	676.04 ± 77.82	1665.96 ± 124.64	1482.51 ± 272.82	8165.73 ± 158.53	501.64 ± 140.63	281.33 ± 35.46
0.5 mm	180°C	30% EtOH	51.06 ± 40.92	1585.41 ± 49.94	65.60 ± 8.72	769.38 ± 24.57	84.28 ± 1.24	52.62 ± 6.36
		40% EtOH	133.95 ± 42.32	395.01 ± 37.90	239.64 ± 15.61	2228.62 ± 77.84	181.89 ± 5.31	77.24 ± 12.59
		60% EtOH	417.21 ± 40.22	1058.33 ± 38.95	653.53 ± 44.18	4240.96 ± 104.62	215.43 ± 2.91	217.58 ± 7.34
		70% EtOH	718.54 ± 36.52	3358.75 ± 90.12	1967.23 ± 158.57	5716.92 ± 375.24	675.62 ± 65.04	212.10 ± 18.76
		30% MeOH	204.08 ± 14.59	609.28 ± 25.28	597.53 ± 21.22	3072.81 ± 136.20	86.40 ± 23.80	101.49 ± 23.61
		40% MeOH	583.45 ± 76.96	634.79 ± 75.82	581.09 ± 95.73	2308.56 ± 118.78	116.01 ± 12.02	84.53 ± 7.63
		60% MeOH	663.16 ± 83.59	1681.52 ± 72.77	890.77 ± 37.04	5265.03 ± 285.80	277.83 ± 17.82	153.64 ± 20.33
		70% MeOH	714.49 ± 47.80	3687.18 ± 95.28	2416.61 ± 90.00	6162.62 ± 360.52	806.56 ± 21.57	216.18 ± 19.44
1.0	180°C	30% EtOH	237.15 ± 20.37	621.43 ± 30.48	352.20 ± 19.12	2879.21 ± 60.17	150.76 ± 3.92	59.02 ± 3.15
		40% EtOH	98.55 ± 7.51	283.02 ± 9.33	186.63 ± 10.88	1846.17 ± 28.03	170.46 ± 8.09	20.90 ± 6.03
		60% EtOH	903.42 ± 64.20	2181.98 ± 136.64	1312.03 ± 21.67	6617.90 ± 260.27	397.36 ± 2.11	308.55 ± 4.72
		70% EtOH	659.67 ± 14.50	2742.36 ± 13.98	1682.31 ± 86.85	6184.98 ± 134.47	595.39 ± 62.95	342.48 ± 37.83
		30% MeOH	115.03 ± 13.81	375.86 ± 9.68	290.80 ± 10.17	1534.56 ± 40.30	132.49 ± 2.14	49.06 ± 8.27
		40% MeOH	560.68 ± 26.02	1212.32 ± 30.48	598.51 ± 43.39	4854.27 ± 289.56	312.28 ± 36.12	182.61 ± 28.62
		60% MeOH	679.42 ± 77.55	1473.98 ± 94.02	603.08 ± 4.19	3814.86 ± 17.49	185.60 ± 2.24	214.76 ± 14.68
		70% MeOH	612.62 ± 42.10	2691.18 ± 17.56	1971.61 ± 90.88	7468.67 ± 193.10	859.02 ± 12.09	467.05 ± 49.98

Results are expressed as mean values ± standard deviations. [ϕ]KSS: kaempferol 3-O-(2‴-O-sinapoyl-β-sophoroside); [δ]KS: kaempferol 3-O-β-sophoroside; nd: not detected; [τ]MeOH: methanol; [σ]EtOH: ethanol; [¥]SAE: sinapic acid equivalents; µg: microgram; g: gram; mm: millimeter; [ψ]DW: dry weight; °C: centigrade

*Data of these phenolic compounds were obtained from the original manuscript published in *LWT. Food Sci. Technol.* 138. .doi.org/10.1016/j.lwt.2020.110397.

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Khattab *et al*. (2014) reported that over 95% of sinapine was converted to sinapic acid using 70% (v/v) methanol:water by microwave extraction from canola meal. However, approximately 55% of sinapic acid was then decarboxylated to canolol with a yield of 4.2 g/kg. Thus, the relatively lower conversion rate of sinapic acid to canolol can be explained with the formation of other intermediaries of sinapic acid at higher extraction temperatures. The formation of TA at high temperature at acidic pH conditions with the precursor sinapic acid is a good example for the lower conversion rate of sinapic acid to canolol (Rubino et al., 1995).

In another note, Cai *et al*. (1999) reported that autoclaving of sinapic acid at 121°C for 15 minutes at 0.1 MPa pressure would also produce TA, which further confirms the processing conditions applied in ASE (200°C and 1500 psi) is ideal for the formation of TA at relatively higher temperature and pressure levels. The formation of these lignan derivatives and the Maillard reaction products at higher temperatures could directly influence the antioxidant activity as well as the total phenolic and flavonoid content of the extracts (Chen et al., 2014; Rubino et al., 1996). These lignans directly impact the flavor-profile at the higher processing temperatures and pressure conditions although the existing literature have not discussed this aspect. The use of high pressure and temperature on the other hand is ideal for a short-time treatment to obtain these flavor-active compounds. Thus, shorter extraction time (~10-20 minutes) associated with the ASE provides the ideal environment for extracting these flavor-active minor compounds.

A recent sensory analysis conducted by Hald *et al*. (2019) further confirmed that the bitter flavor of canola meal by-products was due to the presence of kaempferol 3-*O*-(2‴-*O*-sinapoyl-*β*-sophoroside). They further reported that of these esterified products, KSS and KS were the most

influential bitter compounds affecting the flavor profile (Hald et al., 2019; Yang et al., 2015). Further work by Siger *et al*. (2013) reported that other kaempferol derivatives are present in canola extracts including kaempferol 3-dihexoside-7-sinapoyl-hexoside (30 mg/100 g DW). They further reported that the concentration of these kaempferol derivatives increased with acid hydrolysis (Siger et al., 2013), which is relevant to the production and precipitation of protein concentrates.

On the contrary, most flavonoids are easily oxidized under aerated conditions, so the presence of an inert gas (N_2) is important to attenuate the oxidation (Nandasiri et al., 2019). Thus, an oxygen-free environment is essential for the extraction and the co-processing of the flavonoid-based flavor-active phenolic compounds. Apart from wet heat and high pressure, ASE's closed system equipped with inert gas (N_2) could facilitate the preservation of phenolic compounds and their antioxidant properties, which otherwise will be detrimental at such high and pressured conditions. Furthermore, this technique can readily recover highly reactive phenolic compounds and prevent their auto-oxidation. Frolov *et al*. (2013) reported that a closed system equipped with inert gas during ASE extraction minimized the rate of oxidative degradation by the complete evacuation of air from the extractants. Moreover, Li & Guo (2016a) stated that the formation and stability of canolol may be affected by shorter extraction times and the method of cooling after each extraction. The centrifugation of the extractants at 4°C immediately after each extraction step, in our method facilitated the higher recovery of phenolic compounds including canolol. Thus, an efficient cooling procedure is recommended soon after the thermal extraction to produce higher yields of flavor-active phenolic compounds including canolol, after ASE extraction. These extraction conditions correspondingly disfavor the Wessely-Moser regrouping thereby improving the extraction efficiency of flavor-active bitter-phenolics (Wang, 2010).

4.6.4 Relationship between the major phenolic compounds

Understanding the relationship between major sinapates and other flavor-active bitter phenolic compounds would help to clarify questions regarding the extraction of these compounds. A partial correlation analysis was conducted to evaluate the degree and direction of these major flavor-active phenolic compounds (**Table 4.3**). The results confirmed a moderate positive correlation between sinapine (r = 0.50) and sinapic acid (r = 0.59) with canolol (**Table 4.3**). Similarly, sinapic acid had a moderate to strong correlation of 0.57 with sinapine (**Table 4.3**). All the correlation values were significant for sinapine, sinapic acid and canolol.

In terms of other flavor-active phenolic compounds, a poor correlation was observed between KSS and all the other phenolic compounds except KS (r = 0.59). On the contrary, KS demonstrated a moderate correlation among all the other phenolic compounds with having the highest correlation with sinapic acid (r = 0.66) (**Table 4.3**). Although, the statistical correlation between sinapic acid and TA was insignificant, the negative correlation proved that sinapic acid is a likely precursor to produce TA (Rubino et al., 1996).

Table 4.3 Partial correlation analysis of main bitter flavor-active phenolic compounds

Control Variables			$^{\alpha}$CL	$^{\beta}$SA	$^{\gamma}$SP	$^{\phi}$KSS	$^{\delta}$KS	$^{\eta}$TA
Size	CL	corr		0.59	0.50	0.06	0.40	0.07
Solvent		sig		0.00	0.00	0.42	0.00	0.36
Concentration	SA	corr	0.59		0.57	0.09	0.66	-0.04
Temperature		sig	0.00		0.00	0.27	0.00	0.62
	SP	corr	0.50	0.57		0.22	0.59	0.32
		sig	0.00	0.00		0.00	0.00	0.00
	KSS	corr	0.06	0.09	0.22		0.59	0.32
		sig	0.42	0.27	0.00		0.00	0.00
	KS	corr	0.40	0.66	0.59	0.47		0.34
		sig	0.00	0.00	0.00	0.00		0.00
	TA	corr	0.07	-0.04	0.32	0.57	0.34	
		sig	0.36	0.62	0.00	0.00	0.00	

corr: correlation; sig: level of significance; $^{\alpha}$CL: canolol; $^{\beta}$SA: sinapic acid; $^{\gamma}$SP: sinapine; $^{\phi}$KSS: kaempferol 3-O-(2‴-O-sinapoyl-β-sophoroside); $^{\delta}$KS: kaempferol 3-O-β-sophoroside; $^{\eta}$TA: thomasidioic acid

Table 4.4 Linear Relationship between major phenolic compounds

Regression Analysis	Sum of Squares	DF	Mean Square	Std Error of Estimates	R^2	Adjusted R^2	Significance
$^{\gamma}$SP Vs $^{\beta}$SA (transformed data)							
Regression	28.91	1	28.91	0.22	0.77	0.77	0.00
Residual	8.45	176	0.05				
Total	37.35	177					
SP Vs $^{\alpha}$CL (transformed data)							
Regression	23.66	1	23.66	0.35	0.51	0.51	0.00
Residual	22.65	182	0.12				
Total	46.31	183					
SA Vs CL (transformed data)							
Regression	18.52	1	18.52	0.38	0.43	0.42	0.00
Residual	24.96	170	0.15				
Total	43.48	171					
SA Vs $^{\eta}$TA (transformed data)							
Regression	3183.99	1	3183.99	356.56	0.35	0.35	0.00
Residual	5829.94	173	33.70				
Total	9013.92	174					
SP Vs TA (transformed data)							
Regression	5607.53	1	5607.53	5.38	0.51	0.51	0.00
Residual	5350.65	185	28.92				
Total	10958.18	186					
CL Vs TA (transformed data)							
Regression	2473.14	1	2473.14	6.64	0.24	0.23	0.00
Residual	7893.77	179	44.10				
Total	10366.91	180					
CL Vs $^{\delta}$KS (transformed data)							
Regression	14348.58	1	14348.58	9.69	0.46	0.45	0.00
Residual	17082.53	182	93.86				
Total	31431.11	183					

DF: degrees of freedom; R^2: coefficient of correlation; Adj R^2 - adjusted coefficient of correlation; $^{\alpha}$CL: canolol; $^{\beta}$SA: sinapic acid; $^{\gamma}$SP: sinapine; $^{\delta}$KS: kaempferol 3-O-β-sophoroside; $^{\eta}$TA: thomasidioic acid

Apart from the partial correlation analysis, linear regression model was used to evaluate the relationship between major flavor-active phenolic compounds. Our results indicated a linear relationship for the conversion of sinapine to sinapic acid (R^2 = 0.77) (**Table 4.4**). A linear response further demonstrated that at higher temperatures alteration of the sinapine structure could occur with sinapic acid formed by elimination of the choline-ester (Khattab et al., 2010; Thiyam et al., 2009). The higher extraction temperatures would enable the dissociation of the choline ester from the sinapine, which increases the yield of sinapic acid at higher extraction temperatures (Khattab et al., 2010; Thiyam et al., 2009). Li & Guo (2016b) further suggested that the higher level of sinapic acid associated with increase in temperature, could be due to either its greater extraction from the meal at higher temperatures or the concurrent conversion of sinapine to sinapic acid. Thus, high pressure further promotes the cleavage of hydrogen bonds in the water molecules thereby increasing the concentration of protons (H^+) in the medium making it more acidic with lower pH. This phenomenon would also facilitate the conversion of sinapine to sinapic acid during the ASE extraction (Nandasiri et al., 2019).

The lower linearity between sinapic acid and canolol (R^2 = 0.42) suggests that the formation of canolol could be associated with other factors besides temperature (**Table 4.4**). The lipophilic nature of canolol and its attachment to the cell and other biological membranes, was thought to make it unavailable for other types of reactions (Khattab et al., 2014). Furthermore, the lower polarity and higher reactivity of canolol may further lowers the availability of canolol thereby reducing the linearity between sinapic acid and canolol (Chen et al., 2014; Khattab et al., 2010). Likewise, the linearity between the sinapic acid and TA was low (R^2 = 0.35) (**Table 4.4**). Both lower linearity relationships between the above phenolic compounds directs the structure-based activity of sinapic acid between TA and canolol. Furthermore, both Spielmeyer *et al*. (2009)

and Morley *et al*. (2013) reported that the optimal temperature for the extraction of canolol was 160°C. At processing temperatures above 180°C, canolol forms dimers and trimers which will affect its concentration (Harbaum-Piayda et al., 2010; Kraljić et al. 2019; Kraljić et al., 2015). Thus, improving the extractability of these flavor-active minor compounds via pressurized temperature processing would aid the production of value-added by-products including phenolic antioxidants. These by-products may hold promising results in the nutraceutical industry.

The effect of acidification was found to have a minimal effect on the phenolic composition (**Table 4.5**). The amounts of sinapine, sinapic acid and canolol did not significantly increase with acidification of the medium. Harbaum-Piayda *et al*. (2010) pretreated canola oil distillate with phosphoric acid followed by methanol extraction and reported that that acidification/protonation together with high temperature, produced higher yields of phenolics including canolol and its derivatives. The extraction of flavor compounds from the corresponding canola meal in this study, however, did not to show any significant differences among phenolic compounds at 160°C using either 70% (v/v) ethanol/water or 70% (v/v) methanol/water as extractants (**Table 4.5**).

Table 4.5 Changes in major sinapic acid derivatives on acidification vs non-acidification

Solvent	Temperature (°C)	Acidification (1.5% $^{\theta}$PA)	Sinapine (mg/g $^{\psi}$DW)	Sinapic Acid (mg/g DW)	Canolol (µg/g DW)
Water	160	NA	1.14 ± 0.01[a]	0.22 ± 0.00[a]	67.84 ± 6.01[a]
Water	160	A	7.06 ± 2.52[b]	2.23 ± 1.59[b]	386.80 ± 6.50[b]
$^{\tau}$MeOH (70%)	160	NA	4.80 ± 0.06	0.50 ± 0.00	304.13 ± 26.01
MeOH (70%)	160	A	4.60 ± 0.62	0.55 ± 0.24	333.74 ± 47.21
$^{\sigma}$EtOH (70%)	160	NA	5.94 ± 0.02	0.56 ± 0.01	118.31 ± 7.10[a]
EtOH (70%)	160	A	6.11 ± 2.57	0.65 ± 0.08	402.79 ± 82.14[b]

$^{\theta}$PA – Phosphoric acid, NA – Not Applied, A- Applied, $^{\tau}$MeOH – methanol, $^{\sigma}$EtOH – ethanol, mg – milligram, µg – microgram, $^{\psi}$DW – dry weight, a,b – statistically significantly different

4.7 Conclusions

The occurrence of major sinapates, namely sinapine, sinapic acid, and canolol and other active molecules including TA and kaempferol derivatives imparts flavor to canola meal. The targeted extraction and co-processing using ASE proved to be an efficient method for extracting these flavor-active molecules while attenuating the bitter molecules from the canola meal. The use of shorter extraction times (20 minutes) and lower solvent usage improved concurrent and targeted extractability of flavor-active phenolic molecules. Therefore, the use of ASE could enable the creation of co-streams of phenolic rich antioxidants. These phenolic rich antioxidative compounds from the meal characterize an additional potential source for use in the food and nutraceutical industries. These new co-streams can be piloted with canola protein industries to benefit the ongoing strong demand for alternative plant-based natural preservatives and shelf-life improving agents.

4.8 Acknowledgments

This project was funded by NSERC Discovery Grant RGPIN-2015-03809. The grant holder, Dr. Usha Thiyam-Holländer, sadly passed away on December 24th, 2020, and this publication is one several manuscripts submitted after her death. Dr. Michael Eskin was a close collaborator of Dr. Thiyam-Holländer. University of Manitoba's GETS scholarship for Ruchira Nandasiri is kindly acknowledged. Ms. Emy Komatsu, Technician, Department of Chemistry, University of Manitoba is acknowledged for her technical support on mass spectrometry. In addition, we would like to acknowledge Dr. Rasheda Rabbani, Faculty Biostatistician, Department of George & Fay Yee Centre for Healthcare Innovation (CHI), Community Health Sciences

(CHS), University of Manitoba is kindly acknowledged for her statistical advice. There is no conflict of interest between the authors to declare.

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Supplementary Materials

Table S1a Effect of particle size, solvent type, solvent concentration, and temperature on sinapine concentration

HPLC Analysis – Sinapine (LOG Transformation)	Sum of Squares	DF	Mean Square	Significance
Con	20.48	3	6.83	0.00
Temp	0.39	2	0.19	0.00
Size * Con	0.17	3	0.06	0.00
Solvent * Con	0.58	3	0.20	0.00
Con * Temp	1.71	6	0.29	0.00
Size * Temp	0.53	2	0.27	0.00
Solvent * Temp	0.12	2	0.06	0.00
Size * Solvent * Con	1.65	3	0.55	0.00
Size * Con * Temp	0.31	6	0.05	0.00
Solvent * Con * Temp	0.57	6	0.09	0.00
Size * Solvent * Temp	0.20	2	0.10	0.00
Size * Solvent * Con * Temp	0.18	6	0.03	0.00
Error	0.06	142	0.00	
Total	2203.18	190	6.83	
R^2 - 0.998				
Adj R^2 - 0.997				

DF: degrees of freedom; LOG: logarithmic; Con: concentration; Temp: Temperature; HPLC: high performance liquid chromatography; R^2: coefficient of correlation; Adj R^2 - adjusted coefficient of correlation

Table S1b Effect of particle size, solvent type, solvent concentration, and temperature on sinapic acid concentration

HPLC Analysis – Sinapic acid (LOG Transformation)	Sum of Squares	DF	Mean Square	Significance
Con	21.36	3	7.12	0.00
Temp	1.40	2	0.70	0.00
Solvent * Con	1.69	3	0.56	0.00
Con * Temp	2.84	6	0.47	0.00
Size * Solvent	0.80	1	0.80	0.00
Size * Temp	1.58	2	0.79	0.00
Solvent * Temp	1.87	2	0.93	0.00
Size * Solvent * Con	1.53	3	0.51	0.00
Size * Con * Temp	1.24	6	0.21	0.00
Solvent * Con * Temp	2.30	6	0.38	0.00
Size * Solvent * Temp	0.24	2	0.12	0.00
Size * Solvent * Con * Temp	2.05	5	0.41	0.00
Error	0.37	131	0.00	
Total	926.81	178		
R^2 - 0.990				
Adj R^2 - 0.987				

DF: degrees of freedom; LOG: logarithmic; Con: concentration; Temp: Temperature; HPLC: high performance liquid chromatography; R^2: coefficient of correlation; Adj R^2 - adjusted coefficient of correlation

Table S1c Effect of particle size, solvent type, solvent concentration, and temperature on canolol concentration

HPLC Analysis – Canolol (LOG Transformation)	Sum of Squares	DF	Mean Square	Significance
Solvent	0.38	1	0.38	0.00
Con	25.94	3	8.65	0.00
Temp	9.38	2	4.69	0.00
Size * Con	0.34	3	0.11	0.00
Solvent * Con	1.19	3	0.40	0.00
Con * Temp	1.19	6	0.20	0.00
Size * Temp	0.46	2	0.23	0.00
Solvent * Temp	0.36	2	0.18	0.00
Size * Solvent * Con	0.31	4	0.08	0.00
Size * Con * Temp	1.32	6	0.22	0.00
Solvent * Con * Temp	1.29	6	0.21	0.00
Size * Solvent * Con * Temp	1.79	8	0.22	0.00
Error	0.84	136	0.01	
Total	688.80	184		
R^2 - 0.982				
Adj R^2 - 0.976				

DF: degrees of freedom; LOG: logarithmic; Con: concentration; Temp: Temperature; HPLC: high performance liquid chromatography; R^2: coefficient of correlation; Adj R^2 - adjusted coefficient of correlation

Table S1d Effect of particle size, solvent type, solvent concentration, and temperature on kaempferol 3-*O*-*β*-sophoroside (KS) concentration

HPLC Analysis – KS (SQRT Transformation)	Sum of Squares	DF	Mean Square	Significance
Size	563.17	1	563.17	0.00
Con	22282.23	3	7427.41	0.00
Size * Con	546.82	3	182.27	0.00
Solvent * Con	1614.98	3	538.33	0.00
Con * Temp	1500.90	6	250.15	0.00
Size * Temp	578.81	2	289.41	0.00
Size * Solvent * Con	1506.08	3	502.03	0.00
Size * Con * Temp	349.79	6	58.30	0.00
Solvent * Con * Temp	972.49	6	162.08	0.00
Size * Solvent * Temp	239.68	2	119.84	0.00
Size * Solvent * Con * Temp	337.43	6	56.24	0.00
Error	1350.60	144	9.38	
Total	271600.79	192		
R^2 - 0.958				
Adj R^2 - 0.945				

DF: degrees of freedom; SQRT: square root; KS: kaempferol 3-*O*-*β*-sophoroside; Con: concentration; Temp: Temperature; HPLC: high performance liquid chromatography; R^2: coefficient of correlation; Adj R^2 - adjusted coefficient of correlation

Table S1e Effect of particle size, solvent type, solvent concentration, and temperature on kaempferol 3-*O*-(2‴-*O*-sinapoyl-*β*-sophoroside) (KSS) concentration

HPLC Analysis – KSS	Sum of Squares	DF	Mean Square	Significance
Size	302716.48	1	302716.48	0.00
Solvent	281870.43	1	281870.43	0.00
Con	2688694.01	3	896231.34	0.00
Temp	1431323.84	2	715661.92	0.00
Size * Con	2318117.40	3	772705.80	0.00
Solvent * Con	1952004.37	3	650668.12	0.00
Con * Temp	2138094.49	6	356349.08	0.00
Size * Temp	443444.35	2	221722.17	0.00
Size * Solvent * Con	1160887.26	3	386962.42	0.00
Size * Con * Temp	575877.71	6	95979.62	0.00
Solvent * Con * Temp	725714.48	6	120952.41	0.00
Size * Solvent * Temp	849788.95	2	424894.48	0.00
Size * Solvent * Con * Temp	586533.03	6	97755.51	0.00
Error	630321.94	144	4377.24	
Total	78186801.31	192		
R^2 - 0.961				
Adj R^2 - 0.948				

DF: degrees of freedom; KSS: kaempferol 3-*O*-(2‴-*O*-sinapoyl-*β*-sophoroside); Con: concentration; Temp: Temperature; HPLC: high performance liquid chromatography; R^2: coefficient of correlation; Adj R^2 - adjusted coefficient of correlation

Table S1f Effect of particle size, solvent type, solvent concentration, and temperature on thomasidioic acid (TA) concentration

HPLC Analysis – TA (SQRT Transformation)	Sum of Squares	DF	Mean Square	Significance
Con	3156.47	3	1052.16	0.00
Temp	143.54	2	71.77	0.00
Con * Temp	839.30	6	139.88	0.00
Solvent * Temp	188.80	2	94.40	0.00
Solvent * Con	2777.30	3	925.77	0.00
Size * Con	394.18	3	131.39	0.00
Size * Temp	81.15	2	40.58	0.00
Size * Solvent * Con	814.61	3	271.54	0.00
Size * Con * Temp	249.71	6	41.62	0.00
Solvent * Con * Temp	580.45	6	96.74	0.00
Size * Solvent * Temp	131.53	2	65.76	0.00
Size * Solvent * Con * Temp	130.10	6	21.68	0.00
Error	698.99	159	4.40	
Total	122771.70	213		
R^2 - 0.956				
Adj R^2 - 0.941				

DF: degrees of freedom; SQRT: square-root; TA: thomasidioic acid; Con: concentration; Temp: Temperature; HPLC: high performance liquid chromatography; R^2: coefficient of correlation; Adj R^2 - adjusted coefficient of correlation

BRIDGE TO CHAPTER 5

The canola meal limits its use in both food and feed industry due to its bitter-flavor active phenolic compounds. The elimination of these phenolic compounds using ASE may improve the economic value of the meal as well as provide a side stream ingredient of natural antioxidants. Hence, identification and quantification of specific bitter flavor-active phenolic compounds are important. Chapter 5 was an extension of chapter 4, where the identification and quantification of major bitter flavor-active phenolic compounds from canola meal was determined. Besides sinapates other phenolic compounds including kaempferol derivatives as well as other thermo-generated phenolic compounds including thomasidioic acid was identified using the tandem mass spectrometry. HPLC column performance and recovery was further evaluated for the quantification of the sinapates.

AUTHOR CONTRIBUTIONS FOR CHAPTER 5

Attenuation of sinapic acid and sinapine-derived flavor-active compounds using a factorial-based pressurized high-temperature processing. **Nandasiri R.**, Eskin N. A. M., Komatsu, E., Perreault, H., Thiyam-Holländer U. LWT-Food Science and Technology, 2021, 81-96. Ruchira Nandasiri designed the study, performed the experiments, conducted the statistical analysis, and wrote the first draft of the manuscript. Ms Emy Komatsu conducted the mass spectrometry analysis helped in identification of phenolic compounds. Dr. Helene Perreault helped in identification of phenolic compounds and addressing the review comments. Both Dr. Michael Eskin and Dr. Usha Thiyam-Holländer provided critical feedback on the manuscript with proof reading and assisted with the review comments. Dr. Usha Thiyam-Holländer obtained the funding.

CHAPTER 5

MANUSCRIPT III

VALORIZATION OF CANOLA BY-PRODUCTS: CONCOMITANCE OF FLAVOR-ACTIVE BITTER PHENOLICS USING PRESSURIZED HEAT TREATMENTS

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Published in LWT-Food Science and Technology, 2021, 81-96

5.1 Abstract

Canola by-products have considerable potential as functional ingredients and antioxidants due to the presence of unique phenolic compounds. Thus, extraction of flavor-active phenolics present in canola by-products is important for their co-production and valorization. The quantity and quality of these unique phenolic compounds could affect the sensory and organoleptic properties of any product or ingredients derived postprocessing of the canola by-products. This study evaluated, both quantitatively and qualitatively, the formation of these flavor-active compounds under pressurized temperature processing. Particle size, extraction temperature, type of solvent extractants (70% v/v methanol or ethanol), were factors examined for extracting the major flavor-active phenolic compounds including thomasidioic acid (TA), kaempferol 3-*O*-*β*-sophoroside (KS), kaempferol 3-*O*-(2‴-*O*-sinapoyl-*β*-sophoroside) (KSS). Two other kaempferol derivatives, kaempferol 3-(2″-hydroxypropionylglucoside)-4′-glucoside (K-4-G) and kaempferol 3-[2″-glucosyl-6″-acetyl-galactoside]-7-glucoside (K-7-G) were also tentatively identified in the extract. The results confirmed that extraction temperature and solvent type had a significant impact ($p < 0.05$) on the extraction of flavor-active compounds TA and KS. Thus, all parameters had a significant impact ($p < 0.05$) on the extricability of KSS. Particle size and concentration had a significant impact on extraction of K-7-G whereas all other parameters excluding particle size had a significant impact ($p < 0.05$) on the extraction of K-4-G.

Keywords: Canola by-products, kaempferol derivatives, thomasidioic acid, hydroxycinnamic acid, flavor-active phenolics

5.2 Introduction

Canola processing by-products and wastes represent a significant source of value-added flavor-active antioxidants, phytochemicals, and novel functional ingredients. The use of these by-products, however, are currently limited to the feed industry (Frolov et al., 2013; Zago et al., 2015). The marked bitter taste associated with such animal feed is attributed to their rich content of phenolic compounds (Yang et al., 2015). In general, the flavor-active phenolic content of meal ranges between 6400 and 18400 µg/g on dry weight basis (Sánchez et al., 2018; Zago et al., 2015). However, advances in the isolation and extraction of these bitter phenolic compounds are limited. New isolation techniques, such as pressurized heat processing, have not been extensively examined (Kraljić et al., 2015; Nandasiri et al., 2019). In fact, high pressure and temperature processing of canola by-products have been reported to modify phenolic compounds with the formation of dimers and oligomers as well as other flavonoid and hydroxycinnamic acid derivatives (Harbaum-Piayda et al., 2010; Kraljić et al., 2015; Nandasiri et al., 2019). Such complex phenolic compounds formed during high pressure and temperature processing may affect the sensory and organoleptic properties depending on their level of concentration (Hald et al., 2019). Thus, characterization, identification, and quantification of these flavor-active phenolic compounds from canola-by products are needed to determine their sensory and organoleptic properties (Wolfram et al., 2010).

It has been reported that high pressure and temperature processing yields novel phenolic compounds the structures of which have yet to be confirmed (Harbaum-Piayda et al., 2010; Kraljić et al., 2015). For example, the structural transformation of sinapic acid to canolol by decarboxylation at higher temperatures and pressures has recently been reported (Harbaum-Piayda

et al., 2010; Kraljić et al., 2015; Li & Guo, 2016b). In addition to the hydroxycinnamic acid, derivatives, kaempferol derivatives can also undergo structural changes under high pressure and high temperature processing (Hald et al., 2019). A novel phenolic compound responsible for the bitter taste of canola protein isolates was identified recently (Hald et al., 2019; Wolfram et al., 2010). Kaempferol derivatives including kaempferol 3-*O*-(2‴-*O*-sinapoyl-*β*-sophoroside) (KSS) and kaempferol 3-*O*-*β*-sophoroside (KS) were identified as the major phenolic compounds responsible for the bitter taste of the canola by-products including meal (Hald et al., 2019). To-date there is limited information available on the repercussion of heat-based processing on these phenolic compounds. Furthermore, it was reported that sinapic acid was transformed into other structural forms including thomasidioic acid (TA) at different processing conditions including alkaline buffers as well as at lower pH conditions (Rubino et al., 1996). The beneficial properties of such lignin products in canola by-products, however, have yet to be fully determined. A wide variety of chromatographic methods have been published for determining phenolic compounds based on the nature and the number of phenolic compounds present in matrices (Luthria et al., 2004). The limited availability of standards and the similarity and overlapping of the UV-spectra of the different phenolic compounds (isomers), however, are serious limitations with the use of HPLC. Consequently, the use of mass spectrometry (MS), could facilitate the tentative identification of individual phenolic compounds with greater sensitivity and selectivity (Frolov et al., 2013).

This study examined the effects of pressurized heat treatment on the structural transformation of kaempferol derivatives, hydroxycinnamic acid derivatives and thomasidioic acid. These results should expand the versatility of canola by-products for human nutrition and nutraceutical industry, by producing canola proteins with high nutritive value more economically.

To our knowledge this is the first report of the simultaneous identification and quantification of phenolic monomers, and their degradation products in canola by-product extracts using HPLC and mass spectrometry as affected by pressurized wet heat treatment.

5.3 Materials and Methods

5.3.1. Materials

5.3.1.1. Samples

Canola meal (Viterra group, St. Agathe, Manitoba) obtained by mechanical crushing (double expeller pressed) and containing an oil content of 4-6% (*Brassica napus* L.), was used in this study.

5.3.1.2. Standards and reagents

Sinapine (>97%) was purchased from ChemFaces Biochemical Co., Ltd (Wuhan, Hubei, China). Canolol was synthesized in the lab (>97%) and its purity was confirmed via HPLC (Zago et al., 2015). All other standards including Sinapic acid (>98%), 4-(-hydroxy-3methoxyphenyl)-2-butanone (97%), ethyl 4-hydroxy-3-methoxycinnamate (99%), and trans-cinnamaldehyde (>98%) were purchased from Fisher scientific Canada Ltd (Ottawa, ON, Canada). All the extraction solvents including methanol (70% v/v) and ethanol (70% v/v) were also purchased from Fisher scientific Canada Ltd (Ottawa, ON, Canada).

5.3.2. Methods

5.3.2.1. Sample preparation and extraction of phenolic compounds

Canola meal at two particle sizes (0.5 and 1.0 mm) were obtained using the sieve set (Ro-Tap Testing Sieve Shaker Model B, WS Tyler, Mentor, Ohio, USA). The size of the canola meal was confirmed via the Mastersizer 2000 (Malvern Instruments Ltd, Malvern, United Kingdom).

Flavor-active phenolic compounds present in canola meal by-products were extracted using an accelerated solvent extractor (ASE 300, Dionex) as described by Nandasiri et al. (2019). The extraction was carried out at a pressure of 1500 psi using two different solvents (methanol and ethanol) at 30, 40, 60, and 70% (v/v) concentration. Three different temperatures (140, 160, and 180°C) were studied to examine the structural transformations of flavor-active phenolics. Extracts obtained from ASE were concentrated (BÜCHI Rotavapor® R-100, BÜCHI Labortechnik AG, Flawil, Switzerland) and then freeze-dried (Labconco 6 freezone freeze dryer, Labconco Corporation, Kansas City, MO, USA) for 36-48 h at the temperature of -50°C. Freeze-dried samples were brought to a final volume of 30 mL using methanol (≥99.9% v/v).

5.3.2.2. HPLC-DAD analysis of sinapic acid derivatives

Changes in flavor-active phenolic composition, as affected by high pressure and temperature, were evaluated using the HPLC method described by Nandasiri *et al*. (2019). Changes in individual phenolic compounds were analyzed by high performance liquid chromatography (HPLC) (Ultimate 3000; Dionex, Sunnyvale, CA, USA). Separation was carried out on a Kinetex® Biphenyl C_{18} 100 Å RP column (2.6 mm, 150 × 4.6 mm, Phenomenex, Canada) maintained at 30°C with 0.4 mL/min flow rate and 10 µL injection volumes. The mobile phase was consisted of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). The gradient

elution system operated as following: 25-25% B (0-3 min), 25-40% (3-8 min), 40-40% B (8-13 min), 40-60% B (13-25 min), 60-70% B (25-38 min), 70-100% (38-41 min), 100-100% (41-44 min), 100-25% (44-47 min), 25-25% (47-57 min). Chromatograms were acquired at 270 and 330 nm with data analyzed using the Chromeleon software Version 7.2 SR4 (Dionex Canada Ltd, Oakville, ON Canada). Major phenolic compounds present in canola by-products were identified using both external standards including sinapine (97%), sinapic acid (98%) and canolol (97%) by comparing their relative retention times & UV absorbance wavelength.

5.3.2.3. HPLC column performance: % recovery

To determine the recovery percentage of the compounds, a spiking experiment was conducted using HPLC. Sinapic acid (>98%), was used to calculate the recovery percentage at three different processing temperatures (140, 160 and 180ºC). The methanolic extract (70%) of canola meal was spiked with sinapic acid (0.05 mg/mL) to determine the recovery percentage of each extractant (**Table 5.1a**). In addition, two other flavor-active phenolic compounds, ethyl 4-hydroxy-3-methoxycinnamate (98%), and trans-cinnamaldehyde (>98%) were used as internal standards to validate the HPLC method (**Table 5.1b**). These hydroxycinnamic derivatives do not occur naturally in canola by-products extracts, thus confirmed related to the flavor-active phenolics. The recovery percentage for each compound was determined using the following equation with reference to un-spiked sample.

$$\text{Recovery (\%)} = \frac{(\text{spiked sample concentration} - \text{un}-\text{spiked sample concentration}) * 100}{\text{Known spike added concentration}}$$

Table 5.1a Recovery percentages of phenolic compounds (spiked with sinapic acid)

Sample Type	Spiked sample concentration (µg/g DW)	Un-spiked samples concentration (µg/g DW)	Recovery Percentage
70 Methanol 140ºC	2457.18 ± 57.12	225.50 ± 11.81	90.82%
70 Methanol 160ºC	2364.51 ± 43.76	228.46 ± 1.84	90.34%
70 Methanol 180ºC	2498.69 ± 46.54	402.30 ± 26.51	83.90%

Results are expressed as mean values ± standard deviations. DW: Dry Weight; µg: microgram; g: gram

Table 5.1b Recovery percentages of phenolic compounds (spiked with internal standards)

Sample Type	Spiked sample concentration (µg SAE†/g DW)	Un-spiked samples concentration (µg SAE/g DW)	Recovery Percentage
trans-cinnamaldehyde	3866.00 ± 25.44	nd*	96.77%
ethyl 4-hydroxy-3-methoxycinnamate	2104.99 ± 73.45	nd	92.93%

Results are expressed as mean values ± standard deviations. DW: Dry Weight; SAE: Sinapic acid equivalents; µg: microgram; g: gram; nd: not detected
(*un-spiked samples did not contain both trans-cinnamaldehyde and ethyl 4-hydroxy-3-methoxycinnamate, as these flavor compounds are not naturally occurring in canola meal extracts)

5.3.2.4. LC-MS and MS/MS analysis of canola by-product extracts

The HPLC method described by Nandasiri *et al*. (2019) was applied to liquid chromatography with mass spectrometry and tandem mass spectrometry (LC-MS) to minimize the uncertainties in identification of major phenolic compounds. Both methanol and ethanol (70% v/v) extracts were used for the tentative identification of phenolic compounds. In a first series of experiments, on-line LC-MS was performed, allowing a first round of identifications (**Table 5S.2**). In a second round, the extracts were subjected to LC fractionation and ESI-MS-MS/MS was used on each fraction to ascertain the results from the first round. Fractionation was conducted at 1-min intervals and collected fractions were dried (N_2) and analyzed by ESI-MS-MS/MS. Positive ion mode was used, and spectra were recorded on a Bruker Compact high resolution quadrupole time of flight mass spectrometer (Q-TOF-MS) (Bruker Daltonics, Billerica, Massachusetts, USA). The instrument was operated in data-dependent mode for MS/MS. The simple MS mode allowed for empirical formula generation. Operation conditions for the HPLC were similar to those described by Nandasiri et al. (2019) with a total run time of 55 min at a flow rate of 0.4 mL/min with an injection volume of 20 µL. The Elute pump was operated at a maximum pressure of 10150 psi. The column oven was maintained at 32°C for the optimum separation of the peaks. For the formula generation the scan mode was set to MS mode and the mass range was set to 50 m/z to 2500 m/z. Electrospray ionization (ESI +) was used at capillary voltage of 3500V at a dry gas flow rate of 4.0 L/min at drying temperature of 200°C. For MS/MS, the tuning conditions included the ion energy of 5.0 eV and collision energy of 10.0 eV. MS/MS fragmentation patterns obtained were compared with the literature values (**Table 5.2**).

5.3.2.5. Statistical analysis

All the experiments were carried out in triplicate. Results were presented as the mean ± standard deviation of the triplicate analysis. The assumptions of normal distribution and constant variance were verified by examining the residuals (Pallant, 2011). Data points were checked for their normality using the normality test. For the current experiment, square-root (TA and KS) and log (K-4-G and K-7-G) transformations were done to obtain the normalized data (Pallant, 2011). No data transformation was done for KSS. A factorial design was applied for the study with four independent factors, including particle size, type of solvent, solvent proportions, and temperature. Data analysis was carried out using the general linear multiple regression model using the two-way analysis of variance (ANOVA) and multiple mean comparisons were achieved with Tukey's test with differences at the 5% level ($P < 0.05$) considered statistically significantly different (Pallant, 2011). All the data analysis tests were assessed by SPSS statistical software version 22 (IBM, New York, USA).

Table 5.2 Literature values for common phenolic compounds present in canola by-products

Method	MW	m/z	Compound
Harbaum-Piayda *et al*. (2010)	223.0	209, 208, 179, 164 149	Sinapic acid
		193, 165, 164, 163, 149	
	181.0	150, 149, 121, 93	Canolol
		121, 103, 93	
Hald *et al*. (2019)	815.3	815, 623, 609, 591, 429, 284, 254	Kaempferol 3-*O*-(2”-*O*-Sinapoyl-ß-sophoroside)
Wolfram *et al*. (2010)	611	611, 449, 287	Kaempferol 3-*O*- β-sophoroside
	815.2	815, 623, 609, 591, 429, 284	Kaempferol 3-*O*-(2’”-*O*-sinapoyl- β -sophoroside)
Frolov *et al*. (2013)	153.0	91, 108, 109, 153	Protocatechuic acid
	197.1	63, 78, 89, 91, 95, 106, 121, 123, 138, 153, 167, 182, 197	Syringic acid
	163.0	91, 93, 117, 119, 163	p-coumaric acid
	181.1	67, 95, 123, 137, 151, 165, 166, 181	Syringaldehyde
	209.1	77, 93, 95, 105, 121, 133, 151, 161, 176, 179, 191, 194, 209	Sinapyl alcohol
	223.1	65, 89, 93, 104, 117, 121, 132, 135, 148, 149, 163, 164, 165, 179, 193, 208, 223	Sinapic acid
	445	203, 297, 242, 355, 357, 401, 445	Sinapic acid dimer
Engels *et al*. (2012)	309	294, 279, 264, 223, 208	Sinapine
	977	815, 609, 447, 285	Kaempferol-sinapoyl-trihexoside
	223	208, 193, 179, 164, 149	Sinapic acid
Kraljić *et al*. (2015)	203.1	181.1, 149, 121	Canolol

	383.2	207.2, 175.2, 147.1, 143.1, 132.1, 129.1, 119.1, 115.1, 107.2, 91.2	Canolol dimer

Table 5.2 (cont'd)

Obied *et al*. (2013)		663, 422, 354, 294	Trans-sinapine 1
		663, 422, 354, 294	Trans-sinapine 2
		977, 385	Kaempferol sinapoyl triglucoside isomer
		1045, 977	Kaempferol sinapoyl triglucoside isomer
		977, 487	Kaempferol sinapoyl triglucoside isomer
		753	Disinapoyl dihexoside
		959	Trisinapoyl dihexoside
		591	Disinapoyl hexoside
		1183, 591	Tetrasinapoyl dihexoside
		591	Disinapoyl hexoside
		305, 237	Methyl sinapates

MW: molecular weight; m/z: mass to charge ratio

5.4 Results and discussion

5.4.1. Model fit statistics of the flavor-active phenolic compounds

Statistical analysis indicated that the solvent, as well as the extraction temperature, were the most important factors affecting the presence of flavor-active phenolic compounds (**Tables 5S.1a, b, c, d, and e**). Both temperature and solvent type had a significant impact ($p < 0.05$) on the concentration of KS (**Table 5S.1a**). The model fit statistics demonstrated that all parameters were major contributing factors ($p < 0.05$) affecting the concentration of KSS (**Table 5S.1b**). Moreover, the concentration of K-4-G was impacted by solvent type, concentration and temperature ($p < 0.05$) (**Table 5S.1c**). Except solvent*concentration all other parameters had a significant impact on the concentration of K-4-G. On the contrary, size and the concertation were the major contributing factors for the concentration of K-7-G (**Table 5S.1d**). Besides the kaempferol derivatives, TA, another flavor-active hydroxy cinnamic acid derived lignan compound, exhibited a similar pattern to K-4-G. Analysis showed that both concentration and the extraction temperature were significant ($p < 0.05$) dynamic factors affecting the flavor-active phenolic compounds (**Table 5S.1e**). Furthermore, all the results obtained for the flavor-active phenolic extractants including KSS, KS, K-4-G, K-7-G, and TA had high (>90%) coefficients of variance: R^2 = 0.961, 0.958, 0.950, 0.933 & 0.956 respectively. Multiple mean comparisons were conducted using the Tukey's post-hoc analysis for all three flavor-active phenolic compounds (**Table 5.3**). According to the post-hoc analysis, temperature had a positive effect on KSS, K-4-G and TA for both type of solvents. However, the post-hoc analysis of KS and K-7-G using Tukey's post-hoc analysis indicated that only solvent concentration was impacted (**Table 5.3**).

Table 5.3 Post-Hoc analysis for multiple comparisons of the mean effects studied in the general linear regression of the factorial design for main phenolic compounds

Multiple Comparisons		Mean Difference (I-J)	Standard Error (SE)	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
(a) Kaempferol 3-*O*-*β*-sophoroside (KS)						
Concentration (I)	Concentration (J)					
30	40	-2.09*	0.63	0.01	-3.71	-0.46
	60	-12.93*	0.63	0.00	-14.56	-11.31
	70	-27.15*	0.63	0.00	-28.78	-25.53
40	30	2.09*	0.63	0.01	0.46	3.71
	60	-10.84*	0.63	0.00	-12.47	-9.22
	70	-25.06*	0.63	0.00	-26.69	-23.44
60	30	12.93*	0.63	0.00	11.31	14.56
	40	10.84*	0.63	0.00	9.22	12.47
	70	-14.22*	0.63	0.00	-15.84	-12.59
70	30	27.15*	0.63	0.00	25.53	28.78
	40	25.06*	0.63	0.00	23.44	26.69
	60	14.22*	0.63	0.00	12.59	15.84
(b) Kaempferol 3-*O*-(2‴-*O*-sinapoyl-*β*-sophoroside) (KSS)						
Concentration (I)	Concentration (J)					
30	40	-117.96*	13.50	0.00	-153.07	-82.86
	60	-286.40*	13.50	0.00	-321.51	-251.30
	70	-274.20*	13.50	0.00	-309.30	-239.10
40	30	117.96*	13.50	0.00	82.86	153.07
	60	-168.44*	13.50	0.00	-203.54	-133.34
	70	-156.23*	13.50	0.00	-191.34	-121.13
60	30	286.40*	13.50	0.00	251.30	321.51
	40	168.44*	13.50	0.00	133.34	203.54
	70	12.21	13.50	0.80	-22.90	47.31
70	30	274.20*	13.50	0.00	239.10	309.30
	40	156.23*	13.50	0.00	121.13	191.34
	60	-12.21	13.50	0.80	-47.31	22.90
Temperature (I)	Temperature (J)					
140	160	-58.91*	11.70	0.00	-86.61	-31.21
	180	146.45*	11.70	0.00	118.75	174.15
160	140	58.91*	11.70	0.00	31.21	86.61
	180	205.36*	11.70	0.00	177.67	233.06
180	140	-146.45*	11.70	0.00	-174.15	-118.75
	160	-205.36*	11.70	0.00	-233.06	-177.67

Table 5.3 (cont'd) Multiple Comparisons		Mean Difference (I-J)	Standard Error (SE)	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
(c) Kaempferol 3-(2''-hydroxypropionylglucoside)-4'-glucoside (K4G)						
Concentration (I)	Concentration (J)					
30	40	-0.10*	0.04	0.04	-0.20	-0.00
	60	-0.25*	0.04	0.00	-0.35	-0.16
	70	-0.86*	0.04	0.00	-0.96	-0.76
40	30	0.10*	0.04	0.04	0.00	0.20
	60	-0.15*	0.04	0.00	-0.25	-0.06
	70	-0.76*	0.04	0.00	-0.86	-0.67
60	30	0.25*	0.04	0.00	0.16	0.35
	40	0.15*	0.04	0.00	0.06	0.25
	70	-0.61*	0.04	0.00	-0.71	-0.51
70	30	0.86*	0.04	0.00	0.76	0.96
	40	0.76*	0.04	0.00	0.67	0.86
	60	0.61*	0.04	0.00	0.51	0.71
Temperature (I)	Temperature (J)					
140	160	-0.12*	0.03	0.00	-0.19	-0.04
	180	0.19*	0.03	0.00	0.11	0.27
160	140	0.12*	0.03	0.00	0.04	0.20
	180	0.31*	0.03	0.00	0.23	0.39
180	140	-0.19*	0.03	0.00	-0.27	-0.11
	160	-0.31*	0.03	0.00	-0.39	-0.23
(d) Kaempferol 3-[2''-glucosyl-6''-acetyl-galactoside]-7-glucoside (K7G)						
Concentration (I)	Concentration (J)					
30	40	-0.13*	0.04	0.02	-0.24	-0.02
	60	-0.61*	0.04	0.00	-0.72	-0.50
	70	-0.90*	0.04	0.00	-1.01	-0.79
40	30	0.13*	0.04	0.02	0.02	0.24
	60	-0.48*	0.04	0.00	-0.58	-0.38
	70	-0.77*	0.04	0.00	-0.87	-0.67
60	30	0.61*	0.04	0.00	0.50	0.72
	40	0.48*	0.04	0.00	0.38	0.58
	70	-0.29*	0.04	0.00	-0.39	-0.19
70	30	0.90*	0.04	0.00	0.79	1.01
	40	0.77*	0.04	0.00	0.67	0.87
	60	0.29*	0.04	0.00	0.19	0.39

Table 5.3 (cont'd) Multiple Comparisons		Mean Difference (I-J)	Standard Error (SE)	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
(e) Thomasidioic acid (TA)						
Concentration (I)	Concentration (J)					
30	40	-3.01*	0.43	0.00	-4.20	-1.83
	60	-8.99*	0.43	0.00	-10.19	-7.79
	70	-9.85*	0.43	0.00	-11.04	-8.66
40	30	3.01*	0.43	0.00	1.82	4.20
	60	-5.98*	0.43	0.00	-7.17	-4.78
	70	-6.84*	0.43	0.00	-8.02	-5.66
60	30	8.99*	0.43	0.00	7.79	10.19
	40	5.98*	0.43	0.00	4.78	7.17
	70	-0.86	0.43	0.27	-2.06	0.33
70	30	9.85*	0.43	0.00	8.66	11.04
	40	6.84*	0.43	0.00	5.66	8.02
	60	0.86	0.43	0.27	-0.33	2.06
Temperature (I)	Temperature (J)					
140	160	-2.34	0.35	0.00	-3.17	-1.51
	180	-0.19	0.35	0.84	-1.02	0.63
160	140	2.34	0.35	0.00	1.51	3.17
	180	2.15	0.35	0.00	1.31	2.98
180	140	0.19	0.35	0.84	-0.63	1.02
	160	-2.15	0.35	0.00	-2.98	-1.31

Sig: level of significance ($P < 0.05$)

5.4.2. Characterization of phenolic compounds using HPLC

Our initial run with the HPLC was only able to identify 3 major phenolic compounds including sinapine, sinapic acid and canolol with the aid of standards (Nandasiri et al., 2019). The availability of only a few standards and lower sensitivity of HPLC compared to mass spectrometry limited its ability to identify other flavor-active phenolic compounds present in canola by-products. The similarity in UV-spectra and retention times of different phenolic compounds and their conjugates in the crude extracts further restricted identification by the HPLC system (Engels et al., 2012; Shao et al., 2014). In addition, canola by-products contain considerable amounts of sinapic acid derivatives including sinapine, sinapine glucopyranosyl, sinapic acid methyl ester, 3-dihexoside-7-sinapoyl-hexoside kaempferol, 3-hexoside-7-sinapoyl hexoside kaempferol, kaempferol 3-*O*-(2‴-*O*-sinapoyl-*β*-sophoroside), and kaempferol 3-*O*-*β*-sophoroside which could interfere with each other due to their similar retention times (Siger et al., 2015). Thus, analysis of crude extracts by ESI-MS-MS/MS together with the literature values provided detailed information on the structure of these novel phenolic compounds (**Table 5S.2**). Taking this into consideration, all interpreted signals provided by HPLC were labeled as unknowns and subjected to MS analysis.

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5.4.3. Identification and quantification of flavor-active phenolic compounds by mass spectrometry and tandem mass spectrometry

To identify the major flavor active phenolic compounds LC-MS-MS/ MS analysis was conducted on extracts obtained from ASE extraction. Both 70% (v/v) ethanol and 70% (v/v) methanol extracts were analyzed. Standard solutions of hydroxycinnamic acid derivatives including sinapine (SP), sinapic acid (SA) and canolol (CL) were detected in positive (+) ion mode in ESI-MS as reference materials (**Figure 5S.1**). Other flavor-active phenolic compounds were tentatively identified using the reference literature mass and retention time values (**Table 5.2**). Both sinapine and canolol showed main fragments of m/z 310.15 and 181.04 which were in agreement with the literature values (**Table 5.2**). Kraljić *et al*. (2015) observed canolol fragments at m/z $[M+Na]^+$=203.1, corresponding to the sodiated version of the m/z 180.1 fragments observed in this study. Engels *et al*. (2012) also reported sinapine at m/z 309 with major fragments at m/z 294, 279, 264, 223, 208 which is in agreement with our fragmentation patterns for both canolol and sinapine. Sinapine was the most abundant phenolic compound present in all extracts consistent with literature values (Thiyam et al., 2006). Other phenolic compounds were also identified including sinapic acid and canolol (Khattab et al., 2013). Pressurized heat treatments produce novel phenolic compounds including dimers and oligomer compounds of canolol and other hydroxycinnamic acid derivatives and their precursors (Harbaum-Piayda et al., 2010; Kraljić et al., 2015; Li & Guo, 2016a). In addition to canolol, sinapic acid can also form dimer compounds with itself and ferulic acid as a precursor (Quinn et al., 2017). Previous studies identified the formation of thomasidioic acid (TA) during heat treatment from the precursor sinapic acid (Cai et al., 1999; Cai and Arntfield, 2001; Rubino et al., 1996). In the presence of oxygen, 5% of sinapic acid is converted into TA and, 2.5% of hydrolysable sinapic acid is dimerized (Bunzel et al., 2003;

Charlton & Lee, 1997). However, identification of these flavor-active phenolic compounds is challenging due to their complex structures including glycosides, galactosides, dimers, trimers, and oligomers formed during the pressurized and high temperature processing, requiring an HPLC method that separates these phenolic compounds in a single run (Harbaum-Piayda et al., 2010; Kraljić et al., 2015; Oehlke et al., 2017; Liang et al., 2018; Mišan et al., 2010).

In addition, other flavor-active phenolic compounds such as hydroxycinnamic and kaempferol derivatives with vinyl phenols are hard to identify with HPLC due to their characteristic volatility and the lack of available standards (Hald et al., 2019; Wolfram et al., 2010). In our current analysis we were unable to identify some of the free phenolic acids, including vanillic, hydroxybenzoic, protocatechuic, *p*-coumaric, caffeic and ferulic acid. These results were consistent with the findings of Obied *et al*. (2013). However, the 70% (v/v) methanol extractant indicated the presence of other flavor-active compounds including kaempferol derivatives besides major hydroxycinnamic derivatives.

Sensory analysis conducted by Hald *et al*. (2019) confirmed that the bitter flavor of canola meal by-products was due to the presence of KSS. These flavor-active phenolic compounds hold a promising future in both food/feed and nutraceutical industry as natural antioxidants and valorization of the by-products. These sinapoyl glucoside derivatives present in canola by-products may attach to other flavonoid compounds including kaempferol would produce different array of flavor-active phenolic compounds via increasing its valorization (Obied et al., 2013). Wolfram *et al*. (2010), Shao *et al*. (2014), and Farag *et al*. (2013) found other flavor-active phenylpropanoids in rapeseed including kaempferol derivatives such as kaempferol 3-*O*-*β*-sophoroside, kaempferol 3-*O*-(2‴-*O*-sinapoyl-*β*-sophoroside), kaempferol 3-*O*-(2‴-*O*-sinapoyl-*β*-sophoroside)-7-*O*-(6⁗-

O-sinapoyl-β-glucopyranoside) and kaempferol 3-*O*-*β*-glucopyranoside-4′-*O*-(6′′′′-*O*-sinapoyl-*β*-glucopyranoside). The UV absorption data for acylated flavanols includes λ_{max} of 265 and 330 nm which are typical for flavanol glycosides (Shao et al., 2014). Our results together with the literature values tentatively identified two key metabolites of kaempferol responsible for the bitter flavor in canola meal by-products (**Figure 5S.2 d** and **e**). The LC-MS data further confirmed the presence of these flavor-active phenolic compounds at the higher temperatures of 160°C and 180°C (**Table 5.3**). Higher extractability of KSS was observed and 60% (v/v) concentration of both methanol and ethanol solvents at 1.0 mm particle size (**Table 5.4**). However, the smaller particle size had a different impact over the concentration of KSS (**Table 5.4**). The formation of these compounds is via the phenylpropanoid pathway and not closely related to sinapate biosynthesis (Wolfram et al., 2010). The authors further stated that KS was the starting metabolite that underwent glycosylation at 3-OH and 7-OH position or sinapoylation forming derivatives that affect the flavor profile of canola by-products (Shao et al., 2014). Moreover, the 4-oxo function in the C ring and the presence of 3-OH and 5-OH positions in the flavonoid structure also exert antioxidant activity, in addition to improving its flavor profile (Lema´nska et al., 2001). Our results further confirm that the concentration of KS, changes with the extraction temperature (**Table 5.4**). The highest concentration of KS was obtained at 180°C in both ethanol and methanol extracts at 70% (v/v) concentration level (**Table 5.4**). The increase in KS concentration at the higher extraction temperature was attributed to structural changes including glycosidation or sinapoylation reactions of the kaempferol derivatives (Shao et al., 2014). To our knowledge this is the first time kaempferol derivatives including K-4-G and K-7-G were reported to be present in canola meal extracts. The abundance of K-4-G in both methanolic and ethanolic extracts were comparatively higher than K-7G (**Table 5.4**), therefore we speculate that these complex compounds are also attributed to the

bitter-flavor of meal. Our results further indicated that type of solvent significantly affected the yield of both K-4-G and K-7-G. For example, at 180°C the 70% (v/v) methanol extractant yielded the highest amount of K-4-G and K-7-G for both particle sizes (**Table 5.4**).

In addition to these kaempferol derivatives TA, a lignan compound derived from sinapic acid, is another important flavor-active compound. Rubino *et al*. (1996) suggested that TA was formed under aerated conditions in buffer medium at pH of 8.5. Apart from their flavoring properties these lignan products have been extensively studied for health benefits including preventative properties against cancer (Rubino et al., 1996). HPLC analysis of our extractants showed TA was present after pressurized heat treatments. The presence of this compound in our extractants was confirmed via the UV absorption spectra (**Figure 5S.2 a**) and MS data. Some reports state that this TA is not a natural compound but produced during the extraction process (Zoia et al., 2008). The optical inactivity of the most common trans-form of TA further confirms that it is not a naturally occurring compound (Charlton & Lee, 1997; Zoia et al., 2008).

Furthermore, it was stated that the enantioselective oxidative phenol coupling of sinapic acid derivatives with amide bonds (sinapamide) can act as an intermediate in the formation of the TA (Obied et al., 2013; Zoia et al., 2008). The pressurized heat treatments in an inert environment are ideal for the formation of flavor-active sinapamide compounds such as TA. Both HPLC and LC-MS-MS/MS analyses confirmed that pressurized heat treatments yielded TA at relatively the higher temperatures of 160°C and 180°C (**Table 5.4**). The results further confirmed that the concentration of TA was affected by the extraction temperature for both 70% (v/v) ethanolic and methanolic extractants at both particle sizes (**Table 5.4**). Both extractants produced the largest yields of TA indicating it could be formed at the higher temperatures by condensation (**Table 5.4**).

Since TA is a lignan compound, we could explain its presence at relatively higher extraction temperatures (Zoia et al., 2008). Published data showed that at a higher processing temperature, the levels of complex flavor-active phenolic compounds including dimers and oligomers of hydroxycinnamic acid derivatives increase (Harbaum-Piayda et al., 2010; Kraljić et al., 2015). However, we were unable to detect or identify any dimers or oligomers of canolol in our samples. Moreover, we were also unable to detect other cyclic spermidine alkaloid compounds (Obied et al., 2013) in any of our extracts. The oxidation and the relative instability of above-mentioned compounds at higher temperatures, despite the vacuum conditions, would result in lower detection limits which might interfere with the quantification and identification (Kraljić et al., 2015; Li & Guo, 2016a).

Table 5.4 Effect of particle size, temperature, type and concentration of solvent on changes in flavor active bitter compounds

Particle Size (mm)	Temperature (°C)	Concentration of Solvent (v/v%)	kaempferol 3-*O*-(2‴-*O*-sinapoyl-*β*-sophoroside) (µg SAE/g DW)	kaempferol 3-*O*-*β*-sophoroside (µg SAE/g DW)	Kaempferol 3-(2"-hydroxypropionylglucoside)-4'-glucoside (µg SAE/g DW)	Kaempferol 3-[2"-glucosyl-6"-acetyl-galactoside] 7-glucoside (µg SAE/g DW)	Thomasidioic acid (µg SAE/g DW)
0.5 mm	140°C	30% EtOH	423.53 ± 12.76	569.03 ± 16.01	634.44 ± 56.20	nd	228.71 ± 21.42
		40% EtOH	925.42 ± 32.35	1056.14 ± 38.64	1150.77 ± 89.36	34.25 ± 2.75	541.50 ± 16.91
		60% EtOH	763.45 ± 75.17	1388.19 ± 48.34	907.28 ± 57.05	28.87 ± 8.70	822.56 ± 46.93
		70% EtOH	952.80 ± 33.58	2725.38 ± 30.93	3115.25 ± 608.74	312.55 ± 56.70	1795.71 ± 180.35
		30% MeOH	773.54 ± 48.11	1041.12 ± 38.27	2002.97 ± 98.56	57.38 ± 13.52	564.29 ± 13.98
		40% MeOH	1025.87 ± 112.39	1203.77 ± 83.95	1920.33 ± 52.45	64.09 ± 18.34	529.73 ± 29.61
		60% MeOH	220.67 ± 26.68	1021.74 ± 47.10	494.15 ± 65.57	428.97 ± 29.21	270.38 ± 53.36
		70% MeOH	500.08 ± 70.22	1855.12 ± 23.67	8404.79 ± 62.99	127.41 ± 23.39	1410.60 ± 207.05
1.0 mm	140°C	30% EtOH	212.36 ± 18.42	538.31 ± 29.34	521.01 ± 18.06	nd	305.35 ± 17.40
		40% EtOH	179.33 ± 4.70	293.78 ± 16.97	298.71 ± 7.90	15.45 ± 2.22	103.60 ± 5.94
		60% EtOH	663.18 ± 32.70	1321.10 ± 7.11	498.07 ± 54.08	51.57 ± 6.07	758.73 ± 55.50
		70% EtOH	655.94 ± 91.98	1892.20 ± 10.08	2023.68 ± 8.81	41.30 ± 23.59	1339.62 ± 101.51
		30% MeOH	557.33 ± 37.61	671.63 ± 37.48	1359.71 ± 81.85	30.42 ± 6.63	331.69 ± 34.29
		40% MeOH	422.34 ± 27.27	748.28 ± 48.60	1625.61 ± 31.76	58.48 ± 32.56	422.34 ± 27.27
		60% MeOH	470.89 ± 32.56	677.43 ± 1.51	988.29 ± 56.70	68.15 ± 13.46	382.29 ± 0.46
		70% MeOH	950.34 ± 56.04	2347.79 ± 44.25	8769.56 ± 18.62	45.82 ± 9.02	1364.79 ± 16.98
0.5 mm	160°C	30% EtOH	132.04 ± 4.70	324.81 ± 12.26	192.61 ± 37.37	nd	220.76 ± 30.65
		40% EtOH	713.21 ± 30.99	1423.91 ± 53.32	192.61 ± 37.37	83.57 ± 7.57	881.39 ± 48.29
		60% EtOH	703.43 ± 56.04	1658.42 ± 41.94	803.65 ± 24.93	72.61 ± 5.30	888.99 ± 69.62
		70% EtOH	653.75 ± 11.37	3307.61 ± 56.60	4546.18 ± 20.01	861.73 ± 90.79	2185.33 ± 120.68
		30% MeOH	1032.76 ± 95.57	1769.61 ± 74.13	2870.73 ± 134.77	80.65 ± 16.21	874.26 ± 77.21
		40% MeOH	945.42 ± 13.78	942.79 ± 66.71	2978.43 ± 178.34	39.95 ± 0.00	754.34 ± 182.43
		60% MeOH	788.04 ± 92.81	1687.69 ± 169.78	5399.95 ± 428.06	456.63 ± 101.63	1041.97 ± 105.25
		70% MeOH	536.46 ± 84.48	1971.82 ± 14.88	10356.53 ± 1252.68	68.25 ± 15.91	1425.79 ± 113.94
1.0 mm	160°C	30% EtOH	563.51 ± 9.85	651.23 ± 6.79	902.64 ± 86.01	34.54 ± 5.15	326.62 ± 9.61

Table 5.4 (cont'd)

		40% EtOH	240.35 ± 2.47	377.58 ± 32.05	287.55 ± 34.04	23.41 ± 8.98	426.46 ± 14.46
		60% EtOH	582.44 ± 7.12	1701.65 ± 27.95	646.35 ± 73.40	73.82 ± 11.53	961.67 ± 43.71
		70% EtOH	506.57 ± 38.60	2509.03 ± 29.50	5369.46 ± 64.18	126.55 ± 27.49	1693.58 ± 65.86
		30% MeOH	431.01 ± 19.05	768.74 ± 33.12	1723.51 ± 66.25	16.47 ± 1.74	359.51 ± 30.99
		40% MeOH	447.51 ± 40.02	680.26 ± 45.54	788.38 ± 72.54	37.45 ± 10.71	383.71 ± 61.37
		60% MeOH	776.68 ± 83.43	960.46 ± 138.32	5348.41 ± 562.99	210.66 ± 7.40	960.46 ± 138.32
		70% MeOH	676.04 ± 77.82	1665.96 ± 124.64	13176.68 ± 3590.69	113.05 ± 28.59	1482.51 ± 272.82
0.5 mm	180ºC	30% EtOH	51.06 ± 40.92	1585.41 ± 49.94	15.09 ± 1.94	nd	65.60 ± 8.72
		40% EtOH	133.95 ± 42.32	395.01 ± 37.90	93.87 ± 6.01	36.23 ± 3.06	239.64 ± 15.61
		60% EtOH	417.21 ± 40.22	1058.33 ± 38.95	169.24 ± 8.29	22.14 ± 4.68	653.53 ± 44.18
		70% EtOH	718.54 ± 36.52	3358.75 ± 90.12	809.51 ± 70.45	4173.66 ± 485.29	1967.23 ± 158.57
		30% MeOH	204.08 ± 14.59	609.28 ± 25.28	3459.95 ± 172.46	30.56 ± 3.42	597.53 ± 21.22
		40% MeOH	583.45 ± 76.96	634.79 ± 75.82	3780.53 ± 86.27	32.84 ± 4.74	581.09 ± 95.73
		60% MeOH	663.16 ± 83.59	1681.52 ± 72.77	4788.74 ± 204.14	259.29 ± 35.17	890.77 ± 37.04
		70% MeOH	714.49 ± 47.80	3687.18 ± 95.28	12316.82 ± 2677.22	102.79 ± 13.51	2416.61 ± 90.00
1.0	180ºC	30% EtOH	237.15 ± 20.37	621.43 ± 30.48	227.54 ± 10.69	5.87 ± 2.22	352.20 ± 19.12
		40% EtOH	98.55 ± 7.51	283.02 ± 9.33	69.81 ± 3.15	4.37 ± 1.68	186.63 ± 10.88
		60% EtOH	903.42 ± 64.20	2181.98 ± 136.64	326.91 ± 58.29	96.61 ± 11.88	1312.03 ± 21.67
		70% EtOH	659.67 ± 14.50	2742.36 ± 13.98	1075.99 ± 39.67	2453.77 ± 292.35	1682.31 ± 86.85
		30% MeOH	115.03 ± 13.81	375.86 ± 9.68	1861.52 ± 44.80	10.80 ± 2.85	290.80 ± 10.17
		40% MeOH	560.68 ± 26.02	1212.32 ± 30.48	3439.88 ± 259.96	36.67 ± 7.38	598.51 ± 43.39
		60% MeOH	679.42 ± 77.55	1473.98 ± 94.02	4811.04 ± 41.95	118.32 ± 17.00	603.08 ± 4.19
		70% MeOH	612.62 ± 42.10	2691.18 ± 17.56	22711.42 ± 3515.07	138.07 ± 11.47	1971.61 ± 90.88

Results are expressed as mean values ± standard deviations. nd: not detected; MeOH: methanol; EtOH: ethanol; SAE: sinapic acid equivalents; µg: microgram; g: gram; mm: millimeter; DW: dry weight; ºC: centigrade

1

5.5 Conclusion

Hydroxycinnamic acid, kaempferol and other derivatives of complex phenolic compounds in canola by-products impact its flavor profile. The bitter taste of the canola by-products was confirmed and attributed to the presence of kaempferol derivatives. A complete understanding of these flavor-active phenolic compounds is necessary to develop better quality varieties with improved flavor and nutritive value. The use of pressurized heat treatment via ASE could be of interest to the industry, as a green extraction method, for improving the phenolic content and flavor profile of canola by-products. To authors knowledge this is the first study to tentatively identify both kaempferol 3-(2″-hydroxypropionylglucoside)-4′-glucoside (K-4-G) and kaempferol 3-[2″-glucosyl-6″-acetyl-galactoside]-7-glucoside (K-7-G) to be present in canola meal extracts. Further confirmation of the structures of these novel flavor-active phenolic compounds will require nuclear magnetic resonance (NMR) measurements.

5.6 Acknowledgments

This project was funded by NSERC Discovery Grant, Canada, RGPIN- 2015-03809 (grant holder, Dr. Usha Thiyam-Hollander), which is kindly acknowledged. University of Manitoba's GETS scholarship for Ruchira Nandasiri is kindly acknowledged. Dr. Rasheda Rabbani, Faculty Biostatistician, Department of George & Fay Yee Centre for Healthcare Innovation (CHI), Community Health Sciences (CHS), University of Manitoba is kindly acknowledged for her statistical advice.

5.7 Supporting information

Table 5S1a Effect of particle size, solvent, temperature and concentration on kaempferol 3-*O*-*β*-sophoroside (KS) concentration

HPLC Analysis – KS (SQRT Transformation)	Sum of Squares	DF	Mean Square	Significance
Size	563.17	1	563.17	0.000
Con	22282.23	3	7427.41	0.000
Size * Con	546.82	3	182.27	0.000
Solvent * Con	1614.98	3	538.33	0.000
Con * Temp	1500.90	6	250.15	0.000
Size * Temp	578.81	2	289.41	0.000
Size * Solvent * Con	1506.08	3	502.03	0.000
Size * Con * Temp	349.79	6	58.30	0.000
Solvent * Con * Temp	972.49	6	162.08	0.000
Size * Solvent * Temp	239.68	2	119.84	0.000
Size * Solvent * Con * Temp	337.43	6	56.24	0.000
Error	1350.60	144	9.38	
Total	271600.79	192		
Corrected Total	32385.68	191		
R^2 - 0.958				
Adj R^2 - 0.945				

DF: degrees of freedom; SQRT: square root; KS: kaempferol 3-*O*-*β*-sophoroside; Con: concentration; Temp: Temperature; HPLC: high performance liquid chromatography; R^2: coefficient of correlation; Adj R^2 - adjusted coefficient of correlation

Table 5S1b Effect of particle size, solvent, temperature and concentration on kaempferol 3-*O*-(2‴-*O*-sinapoyl-*β*-sophoroside) (KSS) concentration

HPLC Analysis – KSS	Sum of Squares	DF	Mean Square	Significance
Size	302716.48	1	302716.48	0.00
Solvent	281870.43	1	281870.43	0.00
Con	2688694.01	3	896231.34	0.00
Temp	1431323.84	2	715661.92	0.00
Size * Con	2318117.40	3	772705.80	0.00
Solvent * Con	1952004.37	3	650668.12	0.00
Con * Temp	2138094.49	6	356349.08	0.00
Size * Temp	443444.35	2	221722.17	0.00
Size * Solvent * Con	1160887.26	3	386962.42	0.00
Size * Con * Temp	575877.71	6	95979.62	0.00
Solvent * Con * Temp	725714.48	6	120952.41	0.00
Size * Solvent * Temp	849788.95	2	424894.48	0.00
Size * Solvent * Con * Temp	586533.03	6	97755.51	0.00
Error	630321.94	144	4377.24	
Total	78186801.31	192		
Corrected Total	16226054.39	191		
R^2 - 0.961				
Adj R^2 - 0.948				

DF: degrees of freedom; KSS: kaempferol 3-*O*-(2‴-*O*-sinapoyl-*β*-sophoroside); Con: concentration; Temp: Temperature; HPLC: high performance liquid chromatography; R^2: coefficient of correlation; Adj R^2 - adjusted coefficient of correlation

Table 5S1c Effect of particle size, solvent, temperature and concentration on kaempferol 3-(2"-hydroxypropionylglucoside)-4'-glucoside (K-4-G) concentration

HPLC Analysis – K-4-G (Log Transformation)	Sum of Squares	DF	Mean Square	Significance
Solvent	34.54	1	34.54	0.00
Con	18.88	3	6.29	0.00
Temp	2.74	2	1.37	0.00
Size * Con	2.63	3	0.88	0.00
Con * Temp	2.55	6	0.43	0.00
Size * Solvent	0.48	1	0.48	0.00
Size * Temp	1.07	2	0.53	0.00
Solvent * Temp	10.60	2	5.30	0.00
Size * Solvent * Con	2.89	3	0.96	0.00
Size * Con * Temp	1.60	6	0.27	0.00
Solvent * Con * Temp	1.79	6	0.30	0.00
Size * Solvent * Temp	1.40	2	0.70	0.00
Size * Solvent * Con * Temp	1.24	6	0.21	0.00
Error	4.52	137	0.03	
Total	1848.188	185		
Corrected Total	91.26	184		
R^2 - 0.950				
Adj R^2 - 0.933				

DF: degrees of freedom; Log: logarithmic; K-4-G: kaempferol 3-(2"-hydroxypropionylglucoside)-4'-glucoside; Con: concentration; Temp: Temperature; HPLC: high performance liquid chromatography; R^2: coefficient of correlation; Adj R^2 - adjusted coefficient of correlation

Table 5S1d Effect of particle size, solvent, temperature and concentration on kaempferol 3-[2''-glucosyl-6''-acetyl-galactoside]-7-glucoside (K-7-G) concentration

HPLC Analysis – K-7-G (Log Transformation)	Sum of Squares	DF	Mean Square	Significance
Size	2.85	1	2.85	0.00
Con	20.86	3	6.95	0.00
Size * Con	0.64	3	0.21	0.00
Solvent * Con	13.73	3	4.58	0.00
Con * Temp	9.62	6	1.60	0.00
Size * Solvent	0.63	1	0.63	0.00
Solvent * Temp	1.12	2	0.56	0.00
Size * Solvent * Con	3.21	2	1.61	0.00
Size * Con * Temp	0.56	6	0.09	0.01
Solvent * Con * Temp	3.02	5	0.60	0.00
Size * Solvent * Temp	0.25	2	0.12	0.03
Size * Solvent * Con * Temp	0.35	4	0.09	0.04
Error	4.08	123	0.03	
Total	600.40	167		
Corrected Total	60.71	166		
R^2 - 0.956				
Adj R^2 - 0.941				

DF: degrees of freedom; Log: logarithmic; K-7-G: kaempferol 3-[2''-glucosyl-6''-acetyl-galactoside]-7-glucoside; Con: concentration; Temp: Temperature; HPLC: high performance liquid chromatography; R^2: coefficient of correlation; Adj R^2 - adjusted coefficient of correlation

Table 5S1e Effect of particle size, solvent, temperature and concentration on thomasidioic acid (TA) concentration

HPLC Analysis – TA (SQRT Transformation)	Sum of Squares	DF	Mean Square	Significance
Con	3156.47	3	1052.16	0.00
Temp	143.54	2	71.77	0.00
Con * Temp	839.30	6	139.88	0.00
Solvent * Temp	188.80	2	94.40	0.00
Solvent * Con	2777.30	3	925.77	0.00
Size * Con	394.18	3	131.39	0.00
Size * Temp	81.15	2	40.58	0.00
Size * Solvent * Con	814.61	3	271.54	0.00
Size * Con * Temp	249.71	6	41.62	0.00
Solvent * Con * Temp	580.45	6	96.74	0.00
Size * Solvent * Temp	131.53	2	65.76	0.00
Size * Solvent * Con * Temp	130.10	6	21.68	0.00
Error	698.99	159	4.40	
Total	122771.70	213		
R^2 - 0.956				
Adj R^2 - 0.941				

DF: degrees of freedom; SQRT: square-root; TA: thomasidioic acid; Con: concentration; Temp: Temperature; HPLC: high performance liquid chromatography; R2: coefficient of correlation; Adj R2 - adjusted coefficient of correlation.

Table 5S2 Structural confirmation of major phenolic compounds using LC-MS analysis

Phenolic Compound	Molecular Formula	Monoisotopic Neutral Mass	Theoretical calculated charged masses		Experimented Mass		Error in ppm	
			$[M+H]^+$	$[M+Na]^+$	$[M+H]^+$	$[M+Na]^+$	$[M+H]^+$	$[M+Na]^+$
Thomosidiasic acid	$C_{22}H_{22}O_{10}$	446.1213	447.1286	469.1105	447.1270	469.0829	3.5180	
kaempferol-3-*O*-D-glucoside	$C_{21}H_{19}O_{11}$	447.0927	448.1000	470.0820	448.0974	470.2048	5.8291	
Kaempferol 3-*O*- *β*-sophoroside	$C_{27}H_{30}O_{16}$	610.1528	611.1601	633.1421	611.1574	633.1406	4.4375	2.3012
Kaempferol 3-(2''-hydroxypropionylglucoside)-4'-glucoside	$C_{30}H_{34}O_{18}$	682.1745	683.1818	705.1637	683.1737	705.1640	11.8417	-0.3758
Kaempferol 3-[2''-glucosyl-6''-acetyl-galactoside] 7-glucoside	$C_{35}H_{40}O_{22}$	814.2162	815.2235	837.2054	815.2321	837.2094	-10.5493	-4.7240
Kaempferol 3-*O*-(2”-*O*-Sinapoyl-*β*-sophoroside)	$C_{38}H_{40}O_{20}$	816.2107	817.2180	839.2000	817.2127	839.2026	6.5111	-3.1387

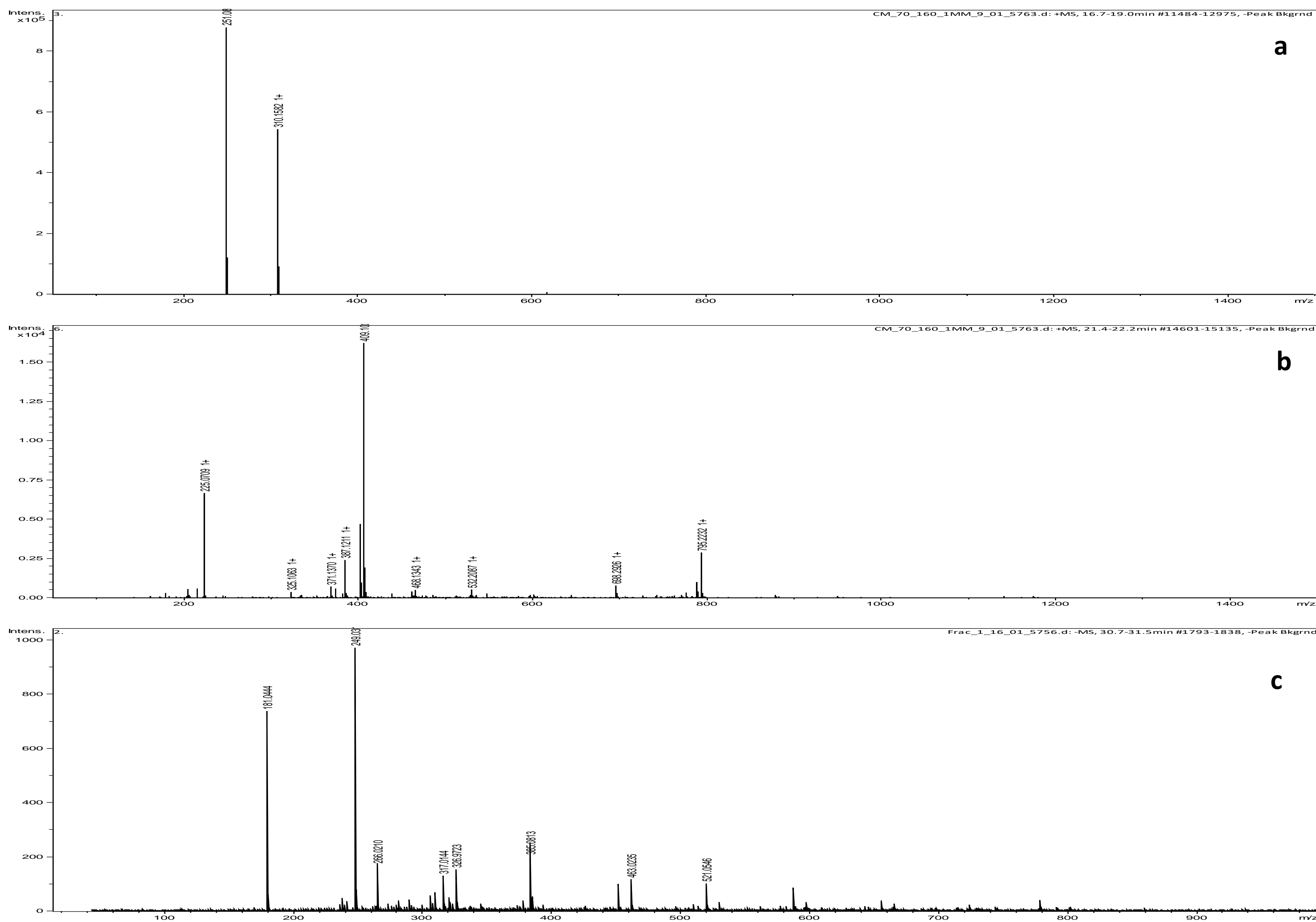

Figure 5S1 Positive electrospray ionization (ESI+) for sinapine (a) sinapic acid (b) and canolol (c) standards

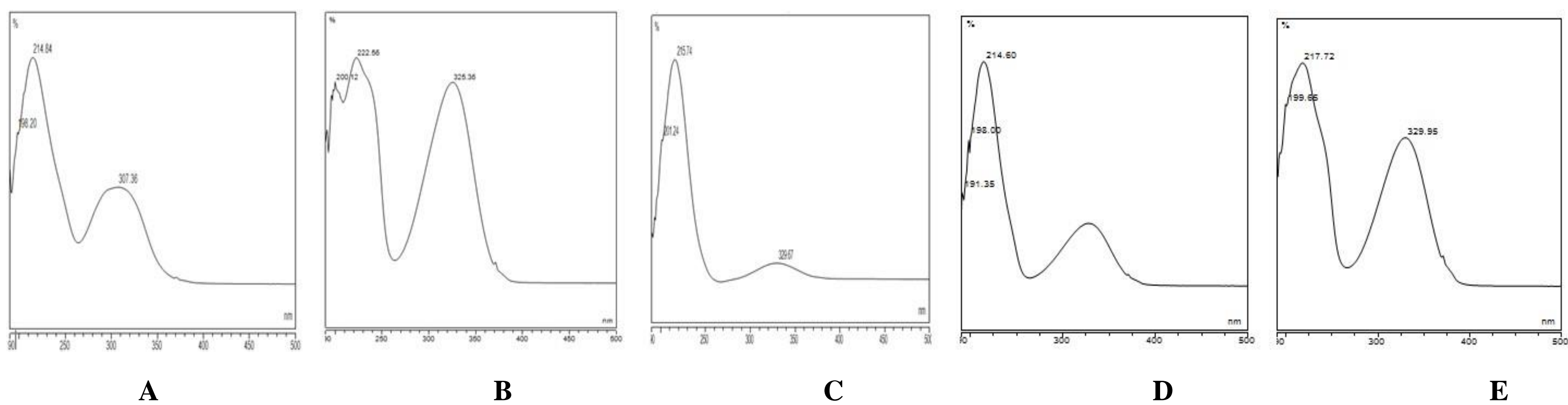

Figure 5S2 UV-Spectra of interested phenolic compounds at 320 nm wavelength

[A- thomasidioic acid, B- kaempferol 3-(2''-hydroxypropionylglucoside)-4'-glucoside, C- kaempferol 3-[2''-glucosyl-6''-acetyl-galactoside]-7-glucoside, D-kaempferol 3-*O*-*β*-sophoroside, E-kaempferol 3-*O*-(2‴-*O*-sinapoyl-*β*-sophoroside)]

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BRIDGE TO CHAPTER 6

Application of high pressure and temperature often requires more sophisticated equipment including ASE. Hence, a need for a simple, yet effective pre-treatment technique warrants in oilseed industry. In previous chapters (chapter 3, 4, and 5) it was discussed the application of ASE on extractability of phenolic compounds with the emphasis of major sinapates and other bitter flavor-active phenolic compounds. Chapter 6 investigates the application of dry heat as a novel pre-treatment method for extracting the major sinapates from canola meal. RapidOxy® 100 equipment was modified to provide inert environment with nitrogen supply to pre-treat the canola meal at different temperature (140, 160, and 180°C) and time settings (2, 5, 10, 15 20 minutes).

AUTHOR CONTRIBUTIONS FOR CHAPTER 6

Rapidoxy® 100: a novel solvent-free pre-treatment for production of canolol. Nandasiri R., Imran, A., Eskin N. A. M., Thiyam-Holländer U. Frontiers in Nutrition, 2021, 8, 687851. Ruchira Nandasiri designed the study, performed the experiments, conducted the HPLC analysis and statistical analysis, and wrote the first draft of the manuscript. Ms Afra Imran helped in extraction of phenolic compounds. Dr. Michael Eskin provided critical feedback on the manuscript with proof reading and assisted with the review comments. Dr. Usha Thiyam-Holländer obtained the funding. (This manuscript was published after the demise of Dr. Usha Thiyam-Holländer).

CHAPTER 6

MANUSCRIPT IV

RAPIDOXY® 100: A NOVEL SOLVENT-FREE PRE-TREATMENT FOR PRODUCTION OF CANOLOL

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Section of the manuscript (abstract and poster) published in Journal of Oil Chemist Society vol 97, 2020, 97
The complete manuscript is published in Frontiers in Nutrition, 2021, 8, 687851.

6.1 Abstract

RapidOxy® 100 is an automated instrument originally designed for measuring the oxidative stability of both solid and liquid samples. The compact and portable design of RapidOxy® 100, and its built-in pressurized heating chamber, provides a suitable environment for studying processing conditions. The feasibility of using oxygen or an inert atmosphere provides the ideal environment to study the effect of dry heat pre-treatment on canola antioxidants. The current study used RapidOxy® 100 to examine the impact of pressurized dry heat pre-treatment, under nitrogen, on the ultrasonic extraction of phenolic compounds. The effect of different pre-treatment temperature-time combinations of 120, 140, 160, and 180°C for 2, 5, 10, 15 20 minutes on the subsequent extraction of canola phenolic compounds was examined. The major sinapates identified by HPLC were sinapine, sinapic acid, canolol. The optimum RapidOxy® condition for the maximum recovery of canolol was 160°C for 10 minutes. RapidOxy® 100 proved to be a novel and versatile instrument for enhancing the extraction of phenolic compounds.

Keywords – RapidOxy® 100, canola meal, canolol, high temperature, and pressure, inert-atmosphere

6.2 Introduction

The *Brassica* family comprise a wide range of horticultural and agricultural crops that are extensively used worldwide. Of these canola (*Brassica napus* L.) and mustard (*Brassica juncea* L.) are among the leading oil crops worldwide. The high demand of oils from these crops produce large amounts of meal and cake by-products. These by-products are limited to the animal industry as feed ingredients and as such are underutilized (Khattab et al., 2010; Naczk et al., 1998). Although, the meal contains many functional ingredients including amino acids, and phenolic compounds it has gained less attention over the last few decades due to many anti-nutritive factors including glucosinolates (Naczk et al., 1998). However, its content of minor components including phenolic compounds are highly valued for their health properties and provide an economic incentive for the greater utilization of canola meal. The meal contains both free and esterified forms of phenolic compounds. Sinapine, an ester of sinapic acid and choline, accounts for over 80% of the phenolic composition (Alu'datt et al., 2017; Quinn et al., 2017). Apart from sinapine, sinapic acid, other flavanol compounds including kaempferol, and kaempferol derivatives (mono-, di-, tri- glucosides) are also present in the meal (Cartea et al., 2011; Li & Guo, 2016a, 2016b; Quinn et al., 2017).

The concentration of phenolic compounds is affected by processing conditions, including high pressure and temperature (Li & Guo, 2016b, 2016a). The application of higher temperatures and pressures often generates novel phenolic compounds with high antioxidant activity such as canolol, its dimers, oligomers, and other breakdown products (Harbaum-Piayda et al., 2010). Moreover, the extractability of major sinapates can be improved with the application of high temperature (~200°C), and pressure (~2000 psi) (Nandasiri et al., 2019). The application of high

pressure and temperature reduces the surface tension and viscosity of the extracting solvents and enhances the solubility and mass transfer of targeted phenolics, a key advantage of the pressurized temperature processing (Nandasiri et al., 2019).

The application of high pressure and temperature, however, requires special equipment and is generally associated with higher operational costs. Hence, there is a need for economical and simple extraction techniques with a higher rate of precision for use by the oilseed processing industry. The extractability of phenolic compounds primarily depends on the polarity of the particular extracting solvent used (Teh & Birch, 2013). Application of pressurized heat was considered a feasible option to extract both sinapine and sinapic acid due to its moderately high H-bonding donor and accepting capability (Li & Guo, 2016a). Previous studies on extractability of phenolic compounds demonstrated that a solvent concentration of 70% (v/v) was the optimum extractant concentration for obtaining phenolic compounds with higher antioxidant activity compared to the corresponding lower extractant concentrations of 60, 40, and 30% (v/v) (Li & Guo, 2016a; Thiyam et al., 2004). Our recent study found that the total phenolic content (TPC) increased with the extraction temperature reaching a maximum at 180ºC ($p > 0.05$) with the 70% (v/v) extractant (20.72 mg SAE/g DM) (Nandasiri et al., 2019).

A newly developed automated instrument RapidOxy® 100 is specifically designed to determine the oxidative stability of various products including foods, cosmetics, flavors and pharmaceutical products in both solid and liquid form. The compact and portable design of RapidOxy® 100, and its built-in pressurized heating chamber, provides the perfect environment for processing conditions. Automated targeted heating (0ºC - 180ºC) and pressurization up to 500 psi provides the ideal setting to pre-treat canola meal prior to the extraction phenolic compounds.

Hence, the substitution of the air supply from oxygen with nitrogen provides an additional advantage by preventing oxidation of the phenolics prior to extraction.

The RapidOxy® 100 instrument provides a unique opportunity to examine the effect of pressure (100 psi) and temperature on the structure-based activity of phenolic compounds. Replacing the air supply with nitrogen (N_2) would provide an inert pressurized environment, as our previous research showed that such an environment with wet heat was favorable for extracting canolol (Nandasiri et al., 2019). Our most recent findings confirmed that 70% methanol (v/v) was optimal for extracting hydroxycinnamic acid derivatives (Nandasiri et al., 2019). The current study examined the structure-based activity of phenolic compounds using RapidOxy® 100 as affected by pressure (100 psi) and temperature. The inert pressurized (100 psi) environment was obtained by replacing the air supply of oxygen with nitrogen. Our previous research demonstrated that an inert pressurized environment with wet heat was favorable for extracting primarily canolol (Nandasiri et al., 2019).

The current study determined the optimum pre-treatment conditions for the dry-heat extraction of the major canola sinapates, sinapine, sinapic acid, and canolol using a pressurized (100 psi) temperature extraction with the aid of RapidOxy® 100. Four different temperatures-time regimens (120, 140, 160, 180ºC and 2, 5, 10, 15 20 minutes) were selected under the same high pressure of 100 psi for the extraction of these major sinapates from both canola (*Brassica napus* L.) and mustard (*Brassica juncea* L.). All solvent extractions were conducted with a 70% (v/v) methanol solution.

6.3 Materials

Mechanically crushed (double expeller pressed) canola meal was provided by Viterra group (St. Agathe, MB, Canada). Both Oriental mustard powder (OMP) and Oriental Mustard Cake (OMC) were provided by G.S Dunn Limited (Hamilton, ON, Canada). Sinapic acid (purity > 98%) was purchased from Fisher scientific Canada Ltd (Ottawa, ON, Canada). Sinapine (purity > 97%) and canolol (purity > 97%) were purchased from ChemFaces® Biochemical Co., Ltd (Wuhan, Hubei, China). All the HPLC grade solvents other chemicals were purchased from Fisher scientific Canada Ltd (Ottawa, ON, Canada).

6.4 Methods

6.4.1 Sample preparation

Canola meal was first ground into powder using a coffee grinder and stored at -20°C until further analysis. Ground canola meal samples were defatted using the Soxtec 2050 as described by Khattab *et al*. (2010) (Foss-Tecator, Foss North America, Eden Prairie, MN, United States).

6.4.2 RapidOxy® 100 pre-treatment

RapidOxy® 100 (Anton Paar Canada Inc., Montreal, QC, Canada) was used to determine the optimum temperature-time, pre-treatment condition to extract antioxidative phenolic compounds. A constant pressure (100 psi) was maintained in a user-defined program throughout the pre-treatment duration. For each pre-treatment regime, 1.0g of defatted canola meal sample was used. Different pre-treatment temperatures (120, 140, 160, and 180°C) were applied to examine the impact of temperature on extractability of phenolic compounds. The test durations (2,

5, 10, 15, and 20 minutes) were defined in the program for each sample at each of the different temperatures. The stability of each sample was monitored through a specific constant induction time period of 5 minutes. The inert environment was maintained throughout the experiment with the continuous supply of N_2 gas to the measuring chamber. Pre-heat-treated canola meal samples were then extracted using the ultrasound for each temperature-time pre-treatment.

6.4.3 Ultrasonic Extraction

The ultrasonic extraction of phenolic compounds was conducted according to the method described in Liang et al. (2018). Briefly, each pre-heat-treated meal sample (1.0 g) was extracted three times with 9.0 mL of methanol (70%, v/v) using a SONOPLUS ultrasonic homogenizer HD 2200 system (BANDELIN electronic GmbH & Co. KG, Heinrichstraβe, Berlin, Germany). The ultrasound extraction was carried out at the power of 40% with a frequency of 20 kHz ± 500 Hz for 1 minute at room temperature (25ºC). After ultrasonic extraction, extracts were centrifuged at 5000 rpm for 15 minutes at 4ºC (Sorvall Biofuge Primo R Centrifuge; Thermo Scientific, Asheville, NC, USA). The extracts obtained from the three extraction steps were combined and made up a total volume of 30.0 mL.

6.4.4 HPLC Analysis

The changes in major sinapates obtained from the pre heat-treated and extracted canola meal samples were evaluated by High Performance Liquid Chromatography (HPLC) described by Nandasiri *et al*. (2019). Phenolic compounds were analyzed by reversed-phase High Performance Liquid Chromatography-Diode Array Detection (HPLC-DAD) (Ultimate 3000, Dionex, Sunnyvale, Torrance, CA, United States). The separation was carried out on a Kinetex® Biphenyl

C_{18} 100 Å RP column (2.6 mm, 150 × 4.6 mm, Phenomenex, Torrance, CA, United States), with flow rate of 0.4mL/min and 10µL injection volume. The column oven was maintained at 30°C. Extract of 70% aqueous methanol was used in the analysis. Using the authentic standards (sinapine, sinapic acid, and canolol) phenolic compounds were identified. The separation was conducted using gradient elution with water (0.1% [v/v] formic acid) as solvent A, and methanol (0.1% [v/v] formic acid) as solvent B. The chromatograms were acquired at 270 nm (canolol) and 330 nm (sinapine and sinapic acid) using Chromeleon software Version 7.2 SR4 (Dionex Canada Ltd., Oakville, ON, Canada).

6.4.5 LC-MS analysis

Structural elucidation of kaempferol-3-*O*-(2‴-*O*-sinapoyl-*β*-sophoroside), kaempferol-3-*O*-sophoroside, syringic acid, sinapic acid dimer, and methyl sinapate were tentatively identified by liquid chromatography with mass spectrometry and tandem mass spectrometry (LC-MS) using the method described by Nandasiri et al. (2021). Cured extracts were dried (N_2) and analyzed by ESI-MS-MS/MS. Positive ion mode (ESI^+) was used, and spectra recorded on a Bruker Compact high resolution quadrupole time of flight mass spectrometer (Q-TOF-MS) (Bruker Daltonics, Billerica, Massachusetts, USA). MS mode was applied during the formula generation and the mass range was from 50 m/z to 2500 m/z was used.

Mass spectrometer was operated at following conditions. The elute pump was operated at a maximum pressure of 10150 psi, with a capillary voltage of 3500V at a dry gas flow rate of 4.0 L/min. Drying temperature was set to 200ºC. MS/MS tuning conditions was carried out with ion energy of 5.0 eV and collision energy of 10.0 eV. The fragmentation patterns obtained were

compared with the literature values (Cai, Arntfield, & Charlton, 1999; Hald et al., 2019; Nandasiri et al., 2019; Rubino, Arntfield, & Charlton, 1996).

6.4.6 Statistical Analysis

All the experiments were conducted in triplicate. Results were presented as mean ± standard deviation of triplicate analysis. Data points were checked for their normality and required transformations were carried out to obtain normalized data (Pallant, 2011). For the current experiment, logarithmic and square-root transformations were conducted accordingly to obtain normalized data (Pallant, 2011). To establish the optimum extraction conditions response surface methodology (RSM) was used. RSM is a well-established statistical technique for obtaining optimal responses with a minimal number of variables (Box & Wilson, 1951; Humbird, David, Fei, 2016). RSM also provides detailed information on interaction effects between individual parameters to discover a stationary point (Box & Wilson, 1951; Humbird, David, Fei, 2016). Hence, the mathematical models proposed by RSM requires analysis of variance (ANOVA) to determine its adequacy and significance. Statistical analysis was performed using the package 'RSM' (Lenth, 2020) within the R statistical software version 3.6.0 (R Core Team, 2019).

6.5 Results and Discussion

6.5.1 Establishing the optimized extraction conditions for major sinapates using RapidOxy® 100 as a pre-treatment method

To establish the optimum pre-treatment time and temperature combinations for the major sinapates including sinapine, sinapic acid, and canolol (**Figure 6.1 a-c**), contour-plots were plotted using response surface methodology. The contour-plots confirmed that both sinapine and sinapic acid concentrations decreased with increase in temperature and time (**Figure 6.2a, b**). However, an inverse trend was observed for canolol which increased with temperature (**Figure 6.2c**). The results further confirmed that sinapine and sinapic acid are the precursors of canolol at higher pressure and temperatures. These results were in agreement with previous results reported by Li and Guo (2016a) and Nandasiri *et al*. (2019). Furthermore, with increase in pre-treatment time at higher temperatures, a reduction in the canolol concentration was observed. This reduction in canolol concentration over the time could be explained by the formation of dimers, oligomers and other degradation products when exposed for longer times at higher temperature pre-treatments (Harbaum-Piayda et al., 2010).

Our previous studies showed that the optimum extraction condition for canolol using accelerated solvent extraction (ASE) was 160°C (Nandasiri et al., 2019). However, the response surface analysis of contour-plots indicated the optimum extraction condition for canolol was between 160°C and 180°C (**Figure 6.2c**). This was further confirmed by HPLC analysis where the highest canolol concentration was recorded at both 180°C for 5-minutes (427.11 ± 7.12 µg/g DW) and 160°C for 10-minutes (453.40 ± 17.66 µg/g DW) pre-treatment time (**Table S1**). Response surface analysis also indicated that the stationary point of response surface for canolol was located

at 173.7ºC at 17.12 minutes. These results showed it was possible to obtain a maximum amount of canolol using the above-described pre-treatment time-temperature combination. Further improvements in the modeling could assist industry to optimize the yield of canolol using the above time-temperature combination for dry heat extraction. Unfortunately, the extractability of both sinapine and sinapic acid, however, were outside the optimized conditions for the current analysis. Response surface analysis indicated that the stationary points for both the sinapine and sinapic acid were located at 115.81ºC and 101.15ºC respectively. Further modeling is required to obtain the optimized extraction conditions for both sinapine and sinapic acid.

In contrast, both sinapine and sinapic acid showed relatively higher correlation coefficient values with the response surface analysis. The adjusted R^2-value for sinapine and sinapic acid was 0.88 and 0.77, respectively. Both the pre-treatment time and the interaction effect of time*temperature had a higher level of significance ($p > 0.001$) over the extractability of sinapine (**Table 6.1a**). However, no significant impact was found in the pre-treatment temperature ($p = 0.399$). Likewise, for sinapic acid both the pre-treatment time ($p > 0.05$) and the interaction effect of time*temperature ($p > 0.01$) were significant. Similarly, pre-treatment temperature had a minimal impact ($p = 0.338$) on the extractability of the sinapic acid (**Table 6.1b**). In contrast, canolol exhibited relatively lower correlation coefficient value with the response surface analysis. The adjusted R^2-value of 0.16, indicated only temperature had an impact ($P > 0.05$) on the canolol concentration. Both pre-treatment time ($P = 0.222$), and time*temperature interaction ($P = 0.295$) showed no significance on the extractability of canolol (**Table 6.1c**). This further suggests that extractability of canolol depended exclusively on the pre-treatment temperature.

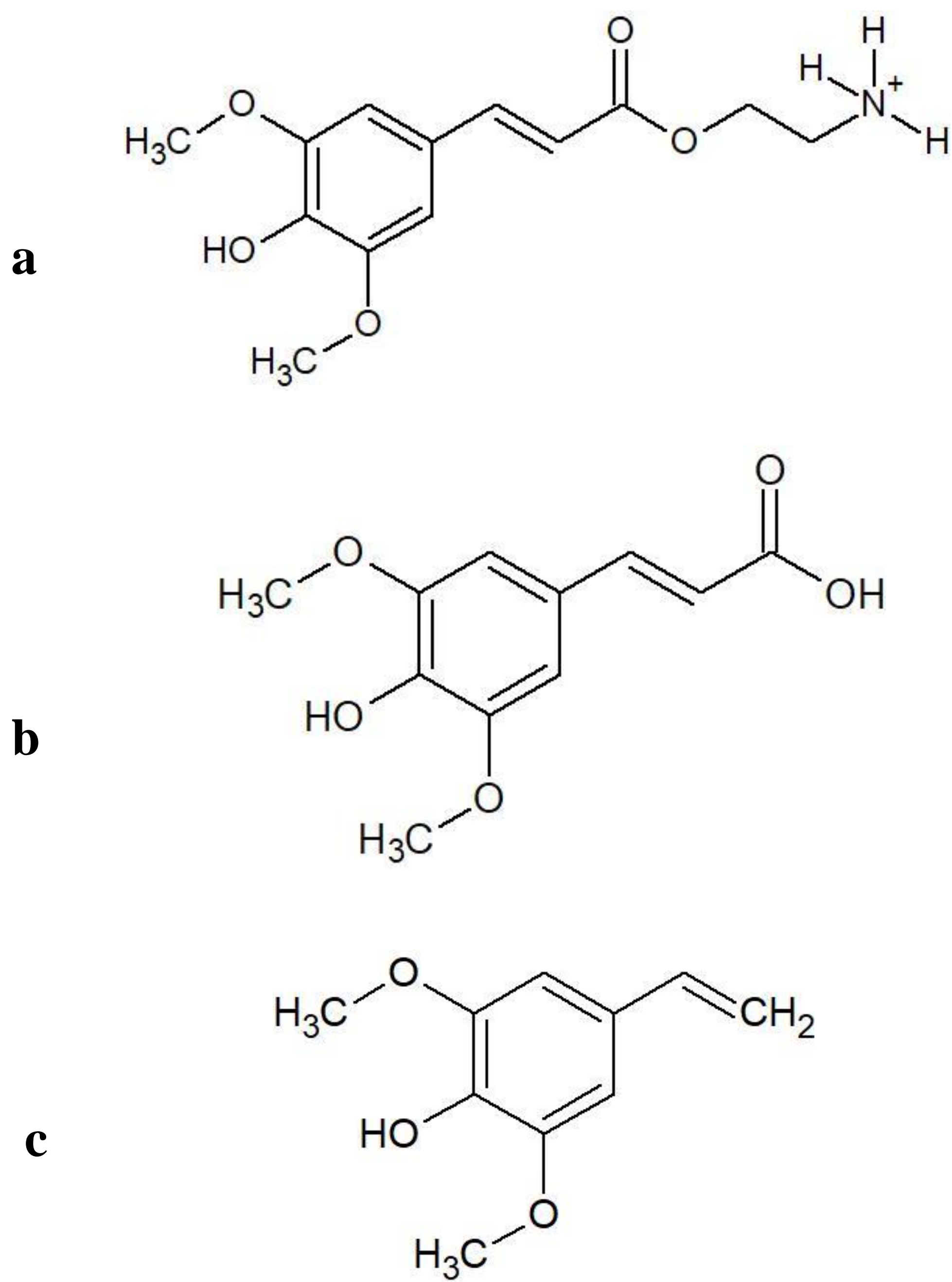

Figure 6. 1 The chemical structures of sinapine (a), sinapic acid (b) and canolol (c)

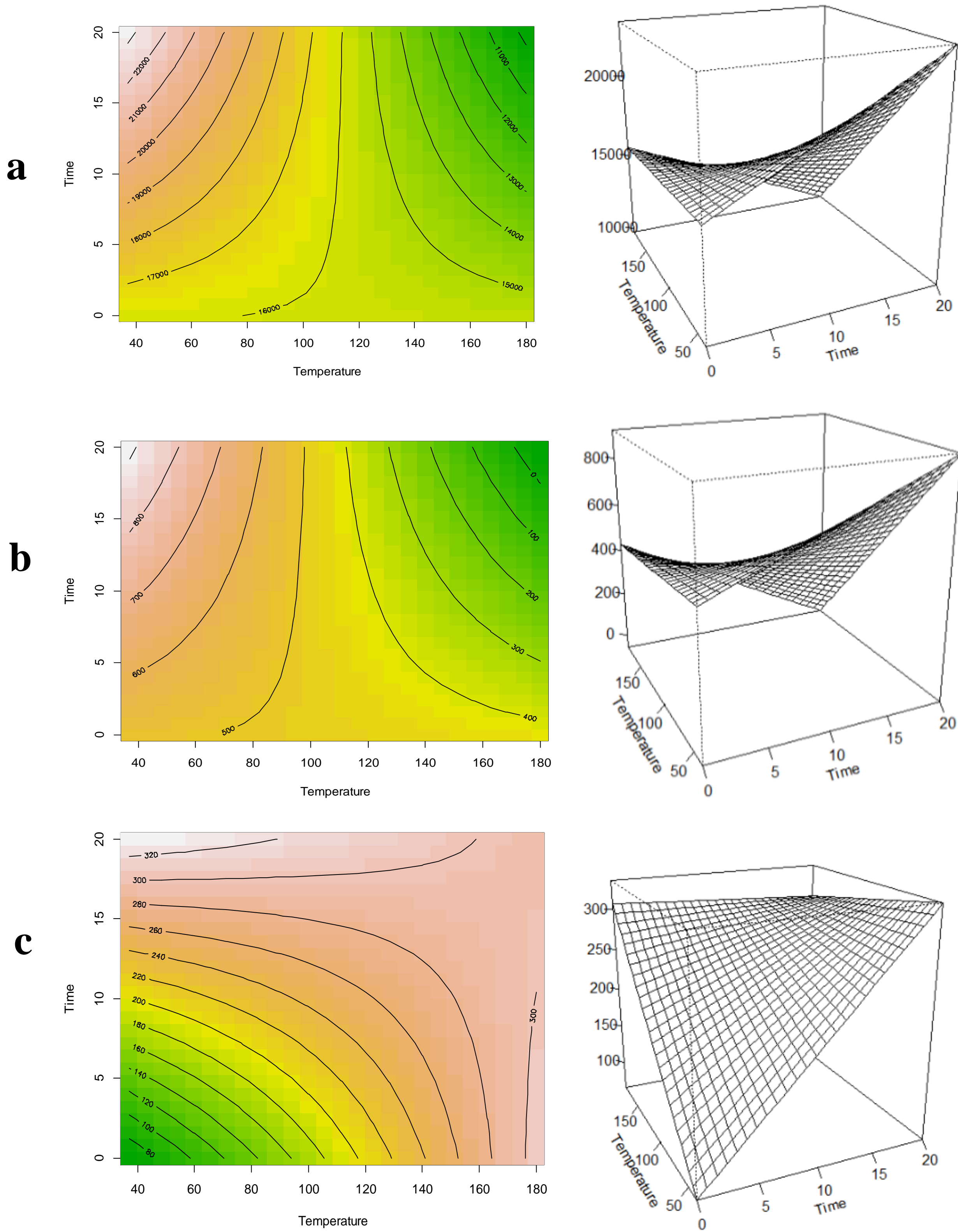

Figure 6.2 Contour-plots and response surface analysis of time (minutes)-temperature (ºC) pre-treatment on Concentration (µg/g DW) of sinapine (a), sinapic acid (b) and canolol (c)

Table 6.1a: Response surface analysis of time-temperature pre-treatment on sinapine

RSM Analysis – SP	Estimate	STD Error	t-value	Significance
Temp	-4.71	5.46	-0.86	0.399
Time	520.27	94.91	5.48	0.000
Time * Temp	-4.49	0.64	-7.00	0.000
R^2 - 0.896				
Adj R^2 - 0.877				

DF: degrees of freedom; STD: standard; SP: sinapine; Temp: Temperature; RSM: response surface analysis; R^2: coefficient of correlation; Adj R^2 - adjusted coefficient of correlation

Table 6.1b: Response surface analysis of time-temperature pre-treatment on sinapic acid

RSM Analysis – SA	Estimate	STD Error	t-value	Significance
Temp	-0.67	0.68	-0.99	0.338
Time	31.18	11.85	2.63	0.017
Time * Temp	-0.31	0.08	-3.85	0.001
R^2 - 0.801				
Adj R^2 - 0.766				

DF: degrees of freedom; STD: standard; SA: sinapic acid; Temp: Temperature; RSM: response surface analysis; R^2: coefficient of correlation; Adj R^2 - adjusted coefficient of correlation

Table 6.1c: Response surface analysis of time-temperature pre-treatment on canolol

RSM Analysis – CL	Estimate	STD Error	t-value	Significance
Temp	1.70	0.78	2.17	0.044
Time	17.26	13.62	1.27	0.222
Time * Temp	-0.10	0.09	-1.08	0.295
R^2 - 0.289				
Adj R^2 - 0.164				

DF: degrees of freedom; STD: standard; CL: canolol; Temp: Temperature; RSM: response surface analysis; R^2: coefficient of correlation; Adj R^2 - adjusted coefficient of correlation

6.5.2 Impact of pre-treatments on extractability of phenolic compounds

The major sinapates present in canola meal, sinapine, sinapic acid and canolol are all thermolabile so that changes in concentrations depends on the pre-treatment and processing conditions (Khattab et al., 2013; Nandasiri et al., 2019; Thiyam et al., 2006). Consequently, the impact on individual compounds differs depending on the pre-treatment time and temperature (**Figure 6.3a-c**). In general, there was a decrease in the concentration of sinapine with increase in the treatment time (**Figure 6.3a**). The lowest sinapine concentration was observed at pre-treatment temperature of 180ºC and a durations time of 20-minutes (4078.58 ± 81.42 µg/g DW) (**Figure 6.3a**). Pre-treatment at 120ºC (7641.86 ± 35.75 µg/g DW) and 180ºC (7622.22 ± 181.27 µg/g DW) showed the highest sinapine content with a treatment time of 2-minutes. Most interestingly the application of a pre-treatment temperature of 180ºC reduced the sinapine concentration by 53% when the pre-treatment time was increased from 2-minutes to 20-minutes (**Table S1**). Sinapine, is known to be the major flavor-active bitter tasting phenolic compound present in canola meal (Thiyam et al., 2009). Thus, the current finding would benefit the canola industry in two ways: by producing a low-bitter flavored canola by-product with a high economic value and concurrently producing a natural side stream phenolic rich extract with potential for the food/feed industry.

Similarly, sinapic acid concentration decreased with the increase in the pre-treatment temperature (**Figure 6.3b**). Thus, the optimum pre-treatment temperature for extraction of sinapic acid was around 120ºC. Nevertheless, with the increase in the temperature as well as pre-treatment time there was a decrease in the sinapic acid concentration. Both 120ºC for 2 minutes (512.97 ± 20.67 µg/g DW) and 120ºC for 5 minutes (504.53 ± 30.02 µg/g DW) reported the highest sinapic acid concentration (**Table S1**). In sharp contrast, both 180ºC and 160ºC pre-treatment temperatures

at 10- (76.78 ± 7.34 µg/g DW) and 15-minutes (75.62 ± 3.69 µg/g DW) pre-treatment times showed the lowest sinapic acid concentration with no significant difference ($p > 0.05$) (**Table S1**). This further suggests the extraction of canolol can be optimized between the pre-treatment temperatures of 160 and 180ºC.

The results from the current study were consistent with previous findings that individual phenolic compounds were temperature dependent (Li & Guo, 2016a; Nandasiri et al., 2019), with the highest concentration of canolol recorded at 180°C ($P > 0.05$) for 5-minutes (427.11 ± 7.12 µg/g DW) and 160ºC for 10-minutes (453.40 ± 17.66 µg/g DW) (**Figure 6.3**). The inert pressurized atmosphere (N_2) provided by RapidOxy® 100 during each pre-treatment produced higher yields of canolol compared to conventional extraction systems. The concentration of canolol, however, was found to decrease over a longer period of time at the higher temperatures. In sharp contrast, the lowest concentrations of canolol, was observed after a 2-minute time period at 120, 140, and 160 (**Figure 6.3c**). These results suggest that longer exposure to higher temperatures, canolol is converted into other phenolic compounds (Harbaum-Piayda et al., 2010). The degradation of canolol at temperatures above 180ºC and elongated pre-treatment times (20 minutes) is attributed to its instability and conversion to dimers and oligomers as well as breakdown products (Kraljić et al., 2015). Apart from these hydroxycinnamic acid derivatives, other phenolic compounds including kaempferol, and kaempferol derivatives, and thermo generative phenolic compounds including thomasidioic acid (TA) are also impacted under the pressurized temperature processing conditions (Cartea et al., 2011; Li & Guo, 2016a, 2016b; Nandasiri et al., 2019; Quinn et al., 2017).

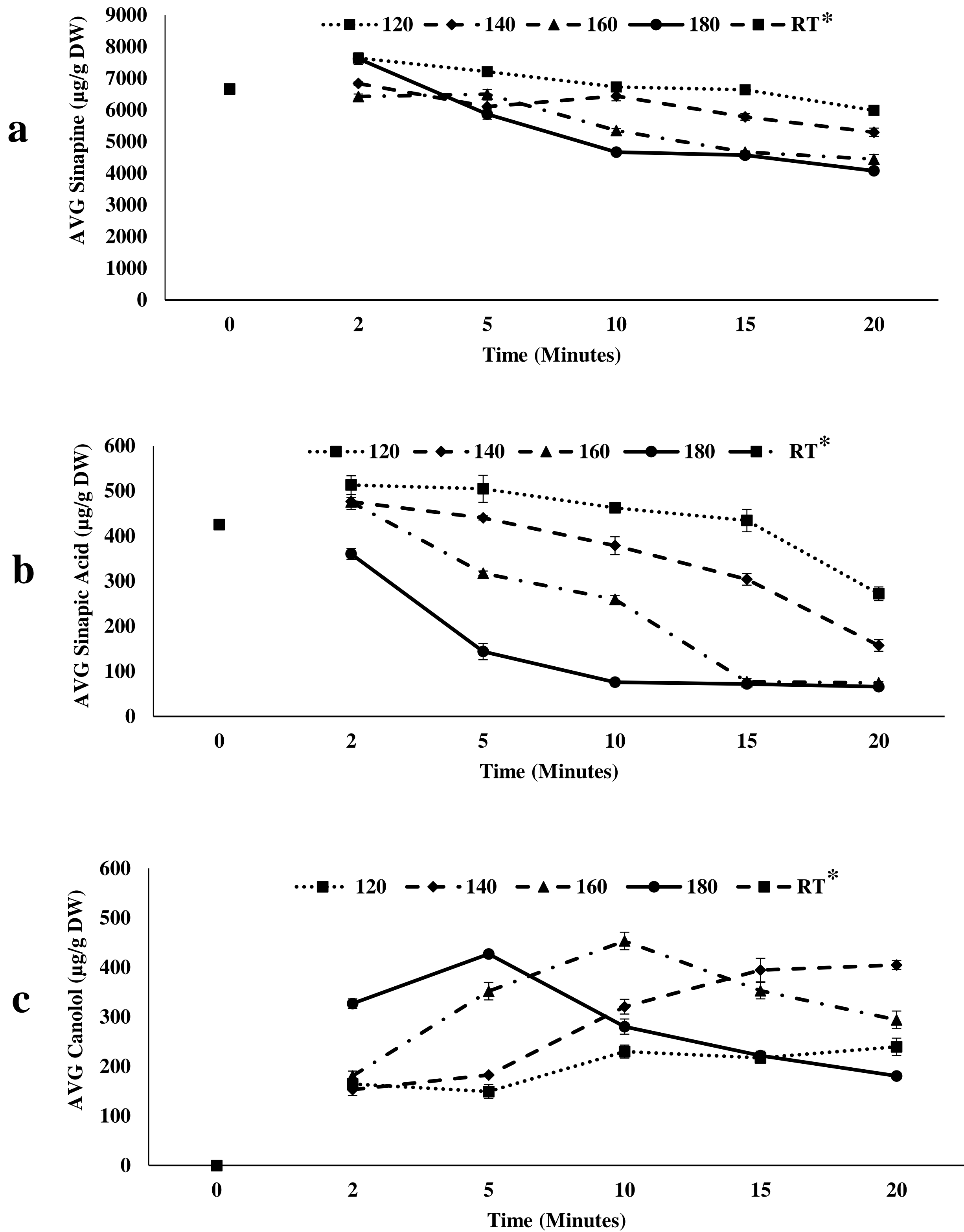

Figure 6.3 changes in sinapine (a) sinapic acid (b) and canolol (c) concentration with the temperature-time pre-treatment for canola meal (*RT- Room Temperature)

6.5.3 Relationship between the Sinapic acid and Canolol

Phenolic compounds have diverse structures often associated with different functions including color, flavor, redox potential, anti-mutagenic activity (Kraljić et al., 2015). High temperature processing is often associated with altering the chemical structures of phenolic compounds through breakage of different type of bonds while impacting the cellular matrix (Teh & Birch, 2014). Hence, it is essential to examine the relationship between sinapic acid and canolol to better understand the effect of the pre-treatments. In fact, the results showed an inverse relationship between sinapic acid and canolol (**Figure 6.4a-d**) at different temperatures (120, 140, 160 and 180°C).

However, at the lower temperatures (120°C) sinapic acid concentration decreased gradually with the increase in the pre-treatment time while the corresponding concentration of canolol increased steadily (**Figure 6.4a**). An inverse relationship was observed for both sinapic acid and canolol at the processing temperature of 140°C (**Figure 6.4b**). At 20-minutes of pre-treatment time canolol showed the highest concentration while sinapic acid was at its lowest. Such changes in the concentration of canolol is directly associated with the degree of decarboxylation of sinapic acid at longer pre-treatment times (Khattab et al., 2010). Nevertheless, with the increase in the treatment temperatures to160^{o}C and 180°C, both compounds showed a decreasing pattern with the longer pre-treatment times (**Figure 6.4c**). The maximum canolol concentration was observed at both 160°C at 10 minutes (453.40 ± 17.66 µg/g DW) and 180°C at 5 minutes (427.11 ± 7.12 µg/g DW). At temperatures above 160°C, however, both sinapic acid and canolol decreased in their concentrations over time (**Figure 6.4 c** and **d**).

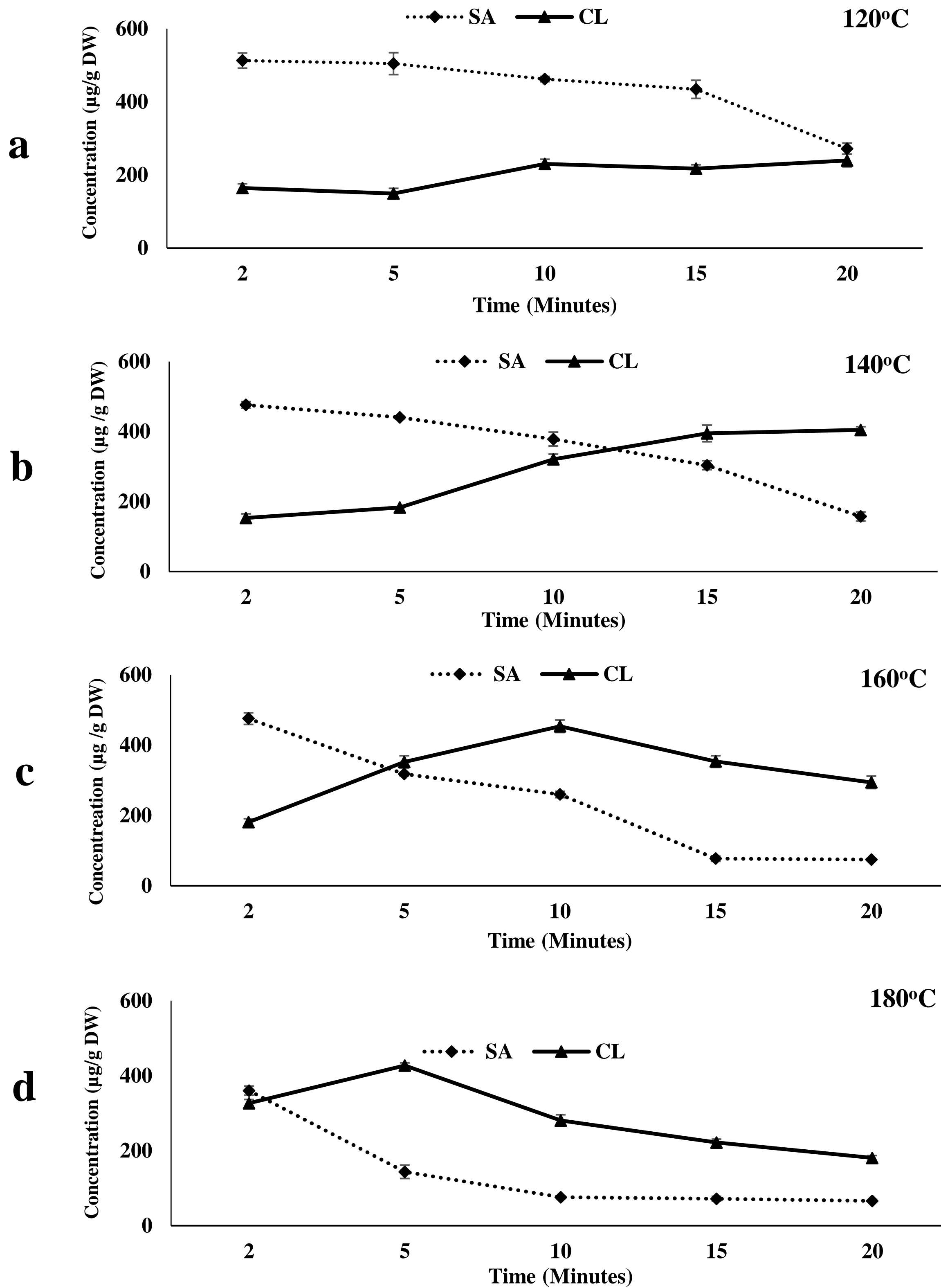

Figure 6.4 The relationship of sinapic acid and canolol for canola meal at (a)120, (b) 140, (c) 160, and (d) 180°C

Such changes could be attributed to structural alterations of canolol at higher temperatures. Both Harbaum-Piayda *et al.* (2010) and Kraljić *et al.* (2015) reported that canolol was converted into different forms including dimers and oligomers at their higher extraction temperatures. In addition, elevated temperatures over 140^oC were reported to impact the concentration of the *cis*-isomer of sinapic acid (Siger, Kaczmarek, & Rudzińska, 2015). The *cis*-isomer was not detectable at temperatures of 160^oC and 180^oC. Consequently, a decrease in both sinapic acid and canolol was observed at the higher temperatures.

To further understand the differences in the concentration of canolol at higher temperatures an experiment was conducted at three different time points (5, 10, 15 minutes) at 180^oC pre-treatment time. HPLC analysis indicated that at higher temperatures with longer exposure times a novel phenolic compound was formed. Further, it was noted that there was an inverse relationship between canolol and the newly formed phenolic compound (**Figure 6.5**). Interestingly the novel phenolic compound (34.4 minutes) had an almost identical retention time to canolol (33.4 minutes). The concentration differences of the novel phenolic compound were also examined in a different matrix using Oriental mustard powder and cake. All three samples canola meal (565.12 ± 11.07 μg/g DW), Oriental mustard cake (99.04 ± 8.11 μg SAE/g DW), and Oriental mustard powder (87.11 ± 14.00 μg SAE/g DW) showed the highest concentration of the novel phenolic compound after 15 minutes extraction time (**Figure 6.5a-c**). In contrast, the concentration of the novel compound was extremely low at the shorter pre-treatment times (5 minutes) suggesting that this compound could be a degraded product of canolol, or a novel phenolic compound formed at higher temperatures in the inert pressurized environment. However, further confirmation studies are needed.

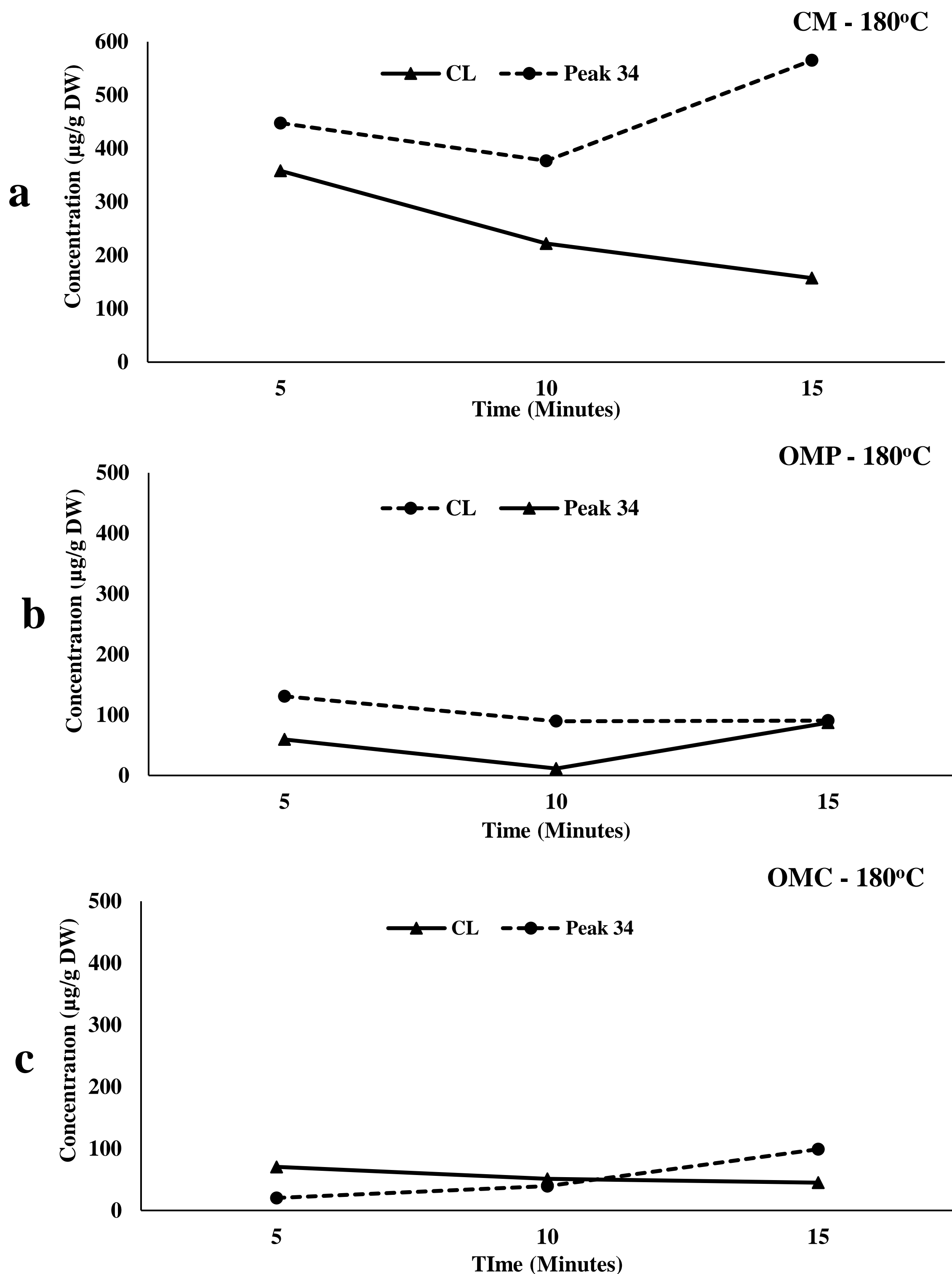

Figure 6.5 The relationship of canolol and peak RT-34 minutes for canola meal (a), oriental mustard powder (OMP) (b), and oriental mustard cake (OMC) (c) at 180ºC

6.5.4 Qualitative analysis of phenolic compounds

Only three major phenolic compounds were identified with the corresponding standards by HPLC. The lower sensitivity, lack of standards, similarity in UV-spectra and retention times between the different phenolic compounds limited the ability of HPLC to identifying other minor components present in the canola meal extracts (Engels, Schieber, & Gänzle, 2012; Hald et al., 2019; Shao et al., 2014; Wolfram et al., 2010). Considering the above limitations of the HPLC, all interpreted signals were labeled as unknowns and subjected to mass spectrometry (MS) analysis for identification as described by Nandasiri *et al.*, (2021). Other key phenolic compounds were tentatively identified using the reference literature mass, fragmentation patterns and relative retention time (**Figure 6.6**). Apart from the major sinapates, nine other phenolic derivatives including syringic acid, methyl sinapates, thomasidioic acid, sinapic acid dimer, kaempferol 3-*O*-(2‴-*O*-sinapoyl-*β*-sophoroside), and kaempferol 3-*O*-*β*-sophoroside were tentatively identified by liquid chromatography mass spectrometry (LC-MS/MS) (**Figure 6.6**). Further purification and fractionation studies are needed for the quantification of these phenolic compounds. Hence, further confirmation of the phenolic structures will require nuclear magnetic resonance (NMR) measurements.

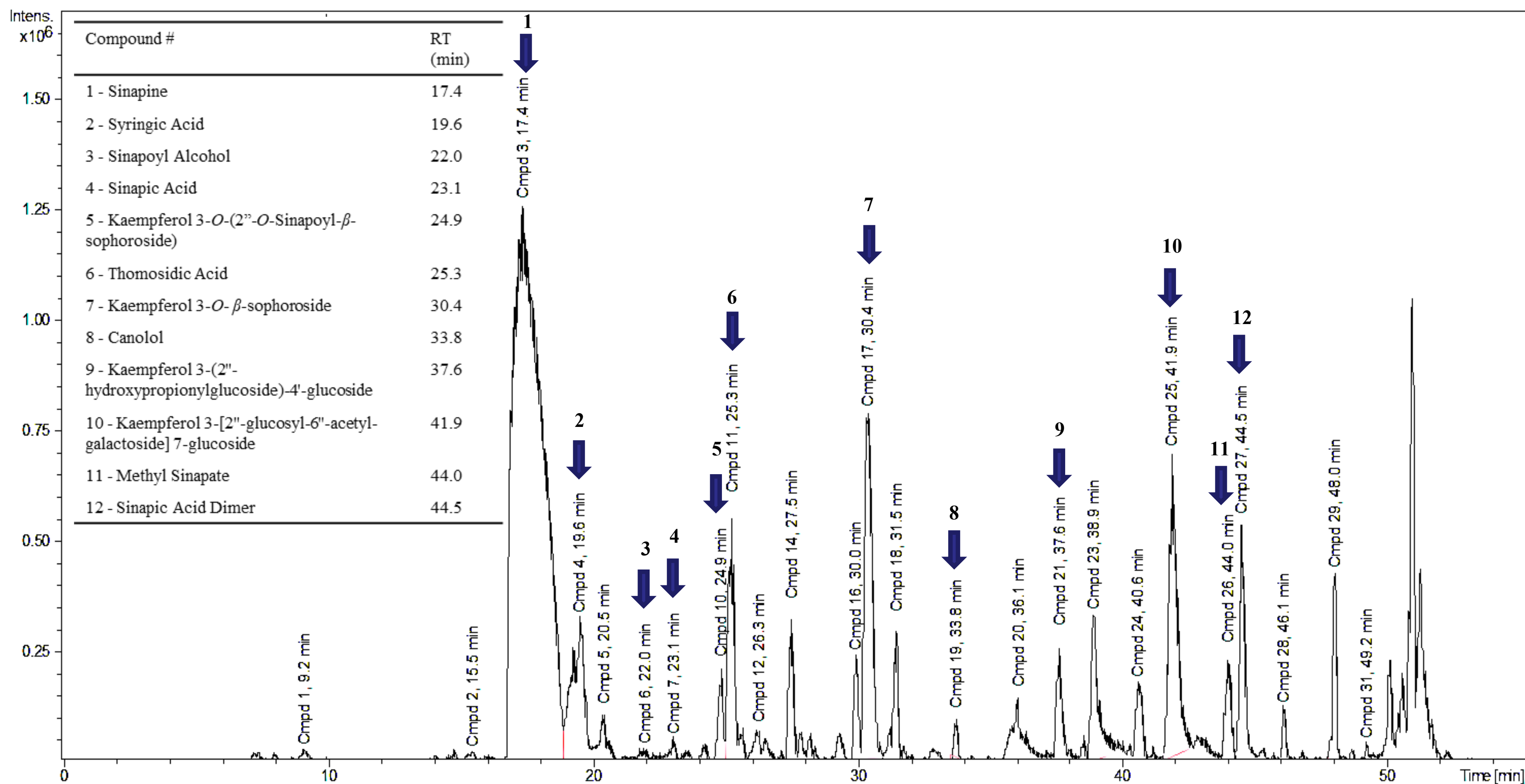

Figure 6.6 Liquid chromatogram of a representative canola meal extract pre heat treated at 160°C with tentative identification of phenolic compounds using LC-MS analysis (RT- retention time, min- minutes)

6.6 Conclusion

Using an inert environment, RapidOxy®100 proved an effective solvent-free dry-heat pre-treatment for enhancing the yield of phenolic compounds by ultrasonic extraction. Its compact and portable design and its ability to modify the gas supply holds considerable potential for enhancing bioactive compounds from underutilized agricultural by-products. To the best of the researchers' knowledge, this is the first report of the novel application of RapidOxy® 100 as a solvent-free pre-treatment prior to the extraction of phenolic compounds from canola and mustard.

6.7 Acknowledgement

This project was funded by NSERC Discovery Grant RGPIN-2015-03809. The grant holder, Dr. Usha Thiyam-Holländer, sadly passed away on December 24th, 2020, and this publication is one several manuscripts submitted after her death. Dr. Michael Eskin was a close collaborator of Dr. Thiyam-Holländer. The University of Manitoba's GETS scholarship was awarded to Ruchira Nandasiri is kindly acknowledged. The authors kindly acknowledge Anton Paar Canada Inc. and Mr. Matthew Taron for providing RapiOxy® 100 to conduct the pre-treatment experiments. Ms. Emy Komatsu, Technician, Department of Chemistry, University of Manitoba is acknowledged for her technical support on mass spectrometry. In addition, we would like to acknowledge Dr. Anas Mohamad, Post-Doctoral researcher, Department of Biology, University of Regina for his statistical advice.

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Supplementary Materials

Table S1 Effect of pre-treatment time and temperature on changes in major sinapates

Temperature (°C)	Time (Min)	Sinapine (µg/g [Ψ]DW)	Sinapic Acid (µg/g DW)	Canolol (µg/g DW)
37°C		6667.52 ± 149.47	425.16 ± 7.58	135.63 ± 17.30
120°C	2	7641.86 ± 35.75	512.97 ± 20.67	164.43 ± 11.59
	5	7213.59 ± 102.47	504.53 ± 30.02	149.23 ± 14.24
	10	6725.75 ± 125.27	462.42 ± 7.27	229.97 ± 13.07
	15	6640.56 ± 113.27	434.23 ± 24.79	217.48 ± 10.57
	20	5985.38 ± 60.66	271.99 ± 15.12	239.67 ± 17.38
140°C	2	6837.13 ± 52.27	476.01 ± 9.42	153.06 ± 11.78
	5	6109.96 ± 207.10	440.29 ± 4.86	182.86 ± 2.65
	10	6445.03 ± 154.32	378.59 ± 19.79	320.65 ± 14.85
	15	5782.76 ± 103.93	303.89 ± 12.82	394.59 ± 23.72
	20	5296.80 ± 130.91	157.37 ± 12.90	404.82 ± 8.97
160°C	2	6427.37 ± 78.65	475.18 ± 16.53	180.77 ± 9.82
	5	6499.69 ± 150.71	317.37 ± 4.99	351.97 ± 17.64
	10	5350.97 ± 60.46	259.45 ± 8.93	453.40 ± 17.66
	15	4667.24 ± 39.53	76.78 ± 7.34	353.01 ± 16.58
	20	4449.88 ± 147.75	74.41 ± 2.80	294.08 ± 17.73
180°C	2	7622.22 ± 181.27	360.19 ± 12.04	326.80 ± 9.70
	5	5870.31 ± 161.53	143.68 ± 17.90	427.11 ± 7.12
	10	4667.09 ± 79.23	75.62 ± 3.69	280.41 ± 15.38
	15	4577.56 ± 101.27	71.97 ± 6.23	221.85 ± 8.91
	20	4078.58 ± 81.42	65.96 ± 2.06	180.75 ± 6.24

results are expressed as mean values ± standard deviations.
Min: minutes; µg: microgram; g: gram; [Ψ]DW: dry weight; °C: centigrades

CHAPTER 7

CONCLUSION

Canola meal is a rich source of phenolic antioxidants. In addition to flavor-active phenolic compounds, the presence of high fiber, antinutritional components, and precursors of glucosinolates have all limited its use to the feed industry. This thesis targeted the extraction of the flavor-active phenolic compounds by pressurized heat treatment. Two extraction conditions (wet heat and dry heat) were optimized removing the targeted flavor-active phenolic compounds. The application of pressurized heat, through accelerated solvent extraction (ASE), was conducted to assess the effectiveness of the wet heat method. Two different particle sizes (0.5 and 1.0 mm), four different solvent concentrations (30, 40, 60, 70 % (v/v)), three different solvents (water, methanol, and ethanol) and three different temperatures (140, 160 and 180°C) were examined for their effect on the extractability. Methanol and ethanol at 70% (v/v) were both effective in extracting the targeted phenolic compounds. In contrast to wet extraction, an accelerated oxidation measuring commercial instrument RapidOxy® 100 was modified with N_2, to provide an inert environment, for use as a dry heat pre-treatment method for the extraction of targeted phenolic molecules.

The impact of particle size, extraction temperature, solvent concentration, and solvent type on the extraction of total phenolic content (TPC), total flavonoid content (TFC) and its antioxidant activity was examined. The total phenolic content increased with both the extraction temperature (180°C) and solvent concentration (70% (v/v)). However, particle size had no impact on the extractability. Ethanol extractant (70% (v/v)) produced the highest TPC for both particle sizes 24.71 (0.5 mm) and 16.40 mg SAE/g DM (1.0 mm),

respectively. A similar trend was observed for TFC, FRAP, DPPH, and metal ion-chelation activity. The results confirmed 70% ethanol as an ideal green extractant for extracting phenolic compounds via ASE. Targeted phenolic extraction was investigated by HPLC-DAD with the major flavor-active sinapates identified as sinapine, sinapic acid and canolol. Higher processing temperatures (180°C) attenuated the concentration of major sinapates, sinapine, sinapic acid and canolol. The improved extraction of these flavor-active phenolic antioxidants by ASE resulted in the production of phenolic rich canola by-products with enhanced antioxidative properties. In addition, the optimized extraction condition for canolol was found to be 160°C with both solvents (ethanol and methanol) at 70% (v/v). Attenuation of these flavor-active phenolic compounds would benefit both the oilseed industry and food industry by providing alternative phenolic rich extracts with improved antioxidative properties. These co-streams of natural phenolic antioxidants may be beneficial to canola protein industries by meeting the ongoing strong demand for alternative plant-based natural preservatives.

In addition to the major sinapates, other phenolic compounds also impart a bitter flavor in canola by-products. Identification and quantification of these flavor-active phenolic compounds were conducted using LC-MS/MS. Kaempferol 3-*O*-(2‴-*O*-sinapoyl-*β*-sophoroside), kaempferol 3-*O*-sophoroside, kaempferol 3-(2″-hydroxypropionylglucoside)-4′-glucoside, and kaempferol 3-[2″-glucosyl-6″-acetyl-galactoside]-7-glucoside were identified using their retention time, UV-spectra, fragmentation patterns, and literature values. The targeted extraction of these compounds is important by producing phenolic rich by-products with the potential application in the nutraceutical industry. A complete understanding of the flavor-active phenolic profile of

canola meal is important for developing new canola varieties with attenuated bitter flavor-active molecules.

The optimized solvent level (70 % (v/v)) obtained by ASE was applied in a different extraction medium using a commercial instrument, RapidOxy® 100 as a pre-treatment to the extraction of canolol and other sinapates. Response surface methodology (RSM) demonstrated that the optimum conditions for extracting canolol required a pre-treatment temperature of 173.7°C along for 17.12 minutes. Extraction of canolol was exclusively dependent on extraction temperature ($P > 0.05$). Increasing the time from 2-minutes to 20-minutes at a pre-treatment temperature of 180°C reduced the sinapine concentration in the meal by over 53%. Attenuation of the sinapine concentration at the higher pre-treatment temperatures (180°C) would produce a natural phenolic-rich mainstream extract. An inverse relationship between sinapic acid and canolol was observed at all pre-treatment temperatures (120, 140, 160 and 180°C). The optimum extraction condition for canolol was observed at both 160°C at 10 minutes (453.40 ± 17.66 μg/g DW) and 180°C at 5 minutes (427.11 ± 7.12 μg/g DW). At the higher pre-treatment temperature (180°C), a novel phenolic compound was formed with a retention time similar to canolol and exhibited an inverse relationship with increase of pre-treatment time.

To authors knowledge this is the first reported extensive study of pressurized heat processing in which all extraction parameters were examined at a one high pressure setting using accelerated solvent extraction (ASE). Furthermore, this is the first study to tentatively identify both kaempferol 3-(2″-hydroxypropionylglucoside)-4′-glucoside (K-4-G) and kaempferol 3-[2″-glucosyl-6″-acetyl-galactoside]-7-glucoside (K-7-G) in the extracts

obtained from pressurized heat treatment of canola meal. In addition, this is also the first study adapting the commercial RapidOxy® 100 instrument as a dry pre-heat treatment step for extracting flavor-active sinapates including canolol. The improved extraction of flavor-active phenolic compounds represents additional value-added by-products from canola meal as promising source of nutraceuticals. The current findings will open up new opportunities for the greater utilization of the canola meal by-products as a source of natural antioxidative and anti-cancer phenolic compounds by the food/feed and nutraceutical industries.

MAJOR CONTRIBUTIONS

The empirical findings confirmed that pressurized heat via ASE was an ideal method for extracting thermo generative phenolic compounds, including canolol, from canola meal extracts. Both 70% (v/v) aqueous ethanol and methanol extractants were very effective in extracting phenolic compounds. From a health and environmental perspective, ethanol would be the preferred solvent of choice.

The short extraction time, smaller sample size, and the use of lower solvent volumes are the major advantages of ASE. Consequently, operating the high pressure of ASE surged the solubility of the targeted phenolic compounds along with improved mass transfer while permitting the organic solvents to remain at a liquid state. Thus, ASE could be introduced as a green extraction method for extracting targeted phenolic compounds.

Extractability of phenolic compounds was primarily dependent on the extraction temperature and solvent concentration. Particle size of the meal did not significantly affect the extractability of the phenolic compounds. Similarly, the antioxidant activity was also dependent on the extraction temperature as well as the solvent concentration.

ASE effectively extracted the bitter flavor-active phenolic compounds including sinapine, sinapic acid, canolol, kaempferol derivatives and TA. The combination of extractant concentration of 70% (v/v) and the temperature of 180ºC significantly ($p<0.05$) extracted the largest amount of flavor-active phenolic compounds. These new co-processing streams can help to meet the ongoing strong demand for alternative plant-based natural antioxidants and anti-cancer agents.

The formation and extraction of major flavor-active phenolic compounds were extensively studied using ASE. Extraction temperature and solvent type were the key parameters important for the extraction of TA and KS. Particle size and concentration of the solvents were important for the extraction of K-7-G while only particle size was important for the extraction of K-4-G. The extractability of KSS, however, was dependant on all extraction parameters.

To authors knowledge this is the first study to tentatively identify both kaempferol 3-(2′′-hydroxypropionylglucoside)-4′-glucoside (K-4-G) and kaempferol 3-[2′′-glucosyl-6′′-acetyl-galactoside]-7-glucoside (K-7-G) as present in canola meal extracts. A complete understanding of these individual flavor-active phenolic compounds is necessary to develop better quality varieties with attenuated bitter flavor and nutritive value.

The operation of conditions with low pressurized heat was examined for the extractability of phenolic compounds using a modified RapidOxy® 100 method. The built-in pressurized heating chamber, and the feasibility of replacing the air supply with nitrogen (N_2), provided an ideal inert environment for studying the effect of pressurized dry-heat treatment on the extraction of canola phenolic antioxidants.

The maximum recovery of canolol was obtained at the pre-treatment condition of 160°C for 10 minutes confirming the previous findings using ASE. The extractability of canolol at lower pressure with RapidOxy® 100, however, was comparatively lower compared to similar conditions with ASE. Such differences indicate the significant impact that the higher pressure had on the extractability of the phenolic compounds. To the best

of the researchers' knowledge, this is the first report of the novel application of RapidOxy® 100 as a pressurized solvent-free pre-treatment for dry-extraction of phenolic compounds.

FUTURE PROSPECTS

To further enhance the application of canola meal by-products after the removal of phenolic compounds, by examining the efficacy of the extracted phenols as natural antioxidants and anti-mutagenic agents.

(1) While the antioxidant properties of major canola meal phenolics have been confirmed in this thesis, further structure-based antioxidant activity of the other minor components is required to better understand the impact of pressurized heat processing on the canola meal matrix.

(2) Organic synthesis of novel phenolic compounds and improved fractionation studies are needed for the preliminary identification. The application of nuclear magnetic resonance (NMR) coupled with mass spectrometry (MS) is required to definitively establish for the structure of the thermo generative novel phenolic compounds.

(3) Further statistical modeling is required to understand the optimal thermal condition for canolol formation using the different processing methods. A lab scale optimized extraction setting is needed on the bench scale for conversion to industrial scale to establish an economic method for extracting the major sinapates including canolol.

(4) More investigations are needed to monitor the stability of the extracted flavor-active phenolic compounds in different atmospheric settings with different gases including

compressed air, oxygen, helium, or carbon dioxide in both pressurized wet and dry extraction systems.

(5) The antioxidant activity should re-assess using both chemical *in-vitro* cell-based assays and further expanded to include *in-vivo* animal based models to assess bioavailability and mechanisms. Cytotoxicity of each bitter flavor-active phenolic compound is also recommended in future studies.

(6) The bitter flavor-active phenolic compounds should then be studied in food systems for their antioxidant and antimicrobial properties. Sensory analysis will be required to ensure that these bitter flavor-active phenolic compounds do not negatively impact the palatability in food/feed ingredients in which they are added.

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Appendix A: Analytical Methods

Appendix A1: Defatting of canola meal using soxtec extraction

Principle:

Solvent extraction of canola meal samples was conducted using solid–liquid extraction methodology. The classic technique for solid–liquid extraction methodology is named as Soxhlet extraction. This was considered as a standard technique for over a century. The most serious drawbacks of Soxhlet extraction as compared to other techniques for solid sample preparation are the long time required for extraction and the large amount of extractant wasted, which is not only expensive to dispose off, but also the source of additional, environmental problems. Samples are usually extracted at the solvent boiling point over long periods, which can result in thermal decomposition of thermolabile target species. Also, a conventional Soxhlet device provides no agitation, which would help to expedite the process. In addition, the large amounts of extractant used call for an evaporation–concentration step after extraction.

Automation of Soxhlet extraction was initially implemented on the commercial equipment Soxtec® System HT, which provided substantial savings in time and extractant. This apparatus uses a combination of reflux boiling and Soxhlet extraction (both assisted by electrical heating) to perform two extraction steps (boiling and rinsing), followed by extractant recovery. Exchange from one to another step is achieved by switching the lever. Soxtec Systems have been used in officially approved methods such asAOAC2003.05 and 2003.06 (crude fat in feed, cereal grain and forage using diethylether and hexane extraction methods). The oil content is defined as the whole of the substances extracted under operating conditions specified in this method and expressed as percentage by weight of the product or relative to the dry weight.

Apparatus:

- Analytical balance
- Grinding mill
- Extraction thimble and cotton wool
- Desiccator
- Soxtec 2050 and accessories

Reagent:

- n- hexane (optima)

Protocol:

Step I	Grind the sample and pass through the required sieve size (0.5 mm and 1.0 mm). Weigh 15.0 g of the sample and transfer to the thimble. Close the mouth of the thimble with a cotton plug which was pre-soaked for 20 min in n-hexane. This will remove any soluble particles present on cotton.
Step II	Accurately weigh the clean & dried oil collection cups (W1)
Step III	Place the thimbles into the Soxtec 2050 system, followed by inserting the pre-weighed empty oil collection cups
Step IV	Add 60 mL of n-hexane to each sample, using a dispenser
Step V	Start the program (Boiling: 15 min, Rinsing: 60 min & Recovery: 20 min)
Step VI	After the specific run time, carefully remove the cotton from the thimbles
Step VII	Keep oil collection cups containing oil in the fume hood for 15 h and measure the weight of the cup (W2) after the specified time. The meal should be transferred to an aluminum foil and kept in the fume hood for 15 h. After 15 h, take a portion of the sample, weigh and follow moisture content analysis.
Step VIII	**Calculation**

	Oil content, % mass as received = (W2 –W1) X 100 / weight of the sample (15.0 g) Oil content, % dry basis = (H X 100) / (100 – M) Where, H = mass % oil content of the product as received U = mass % of moisture content
Step IX	Remaining portion of the meal is kept in a desiccator for 12 h followed by refrigeration (4ºC)

Appendix A2: Phenolic extraction protocol for canola meal via accelerated solvent extractor (ASE)

Principle:

The phenolics in the canola meal samples were extracted using an Accelerated Solvent Extractor (ASE) (ASE 300, Dionex). ASE is an automated extraction technique that rapidly performs solvent extraction using a combination of high temperature (140, 160, and 180°C) and high pressure (1500 psi). The high pressure (~1500 psi) and temperature (up to 200°C) of ASE facilitates the removal of the aglycone moieties attached to phenolic compounds by hydrolysis with minimal interference on its original composition. Furthermore, the high pressure of ASE increases the solubility of the targeted compounds and the diffusion rates as well as the mass transfer rates of the solutes.

Equipment and Materials

ASE 300 (Dionex)
Cell 33 mL & 66 mL
Ottawa sand (Fisher Sci)
Cellulose filters (Thermo-fisher Sci)
250 mL pressurized bottles with caps and septa

Procedure

Step I	Both sieved samples were mixed with Ottawa sand in a ratio of 1:5 to optimize extraction efficiency
Step II	Extraction was carried out using 33 mL cells (5.0g of sample and 25.0g of sand)
Step III	Cellulose filter paper (Thermo Scientific) was placed at the bottom of each cell followed by filling it with samples
Step IV	Hand tightens the cells and place them in the machine
Step V	Setup the machine for required temperature (100 to 200°C), required solvents (methanol, ethanol, isopropanol, hexane), and static cycles
Step VI	The final extracts will be concentrated using the rotary evaporator (BÜCHI Rotavapor®)
Step VII	Concentrated extracts will be freeze-dried at -50°C for 36 to 48 hours
Step VIII	All freeze-dried samples were reconstituted (30.0 mL) in methanol (≥ 99.9% v/v)

Notes

Fixed extraction conditions include

Static time: 5 min.

Static cycles: 3

Flush volume: 60%

Purge time: 60 seconds

Pressure: 1500 psi

Preheat: 5-7 min

Appendix A3: Synthesis and purification of canolol

Principle:

This method uses the procedure described by Simpson et al. (2005) and Zago et al. (2015) where vinyl phenols were synthesized from hydorxybenzaldehydes by Knoevenagel condensation.

Equipment:

- Conical Flask: 200 mL
- Analytical balance
- Pipette: 5 mL capacity
- Heated Magnetic stirrer
- Glass column
- Magnet
- Rotary Evaporator
- TLC plates

Reagents:

- Methanol (analytical grade).
- Silica Gel (60 Å)
- Syringaldehyde (8.23 µmol) - 6.0 mg
- Malonic acid (12.35 µmol) - 5.2 mg
- Piperidine (41.17 µmol) - 16.28 mL
- Toluene - 84 mL
- N-hexane
- Ethyl acetate
- Formic acid

- Saturated Sodium chloride (NaCl)

Protocol:

Step I	Turn on the heated magnetic stirrer and set the temperature to 115°C
Step II	Carefully weigh 6.0 mg of Syringaldehyde, 5.2 mg of Malonic acid and 16.28 mL of Piperidine to 500 mL flat bottom flask
Step III	Add 84.0 mL of toluene to the reaction mixture and keep the reaction mixture at 115°C with continuous stirring at 200 rpm (3.5 -4 hrs)
Step IV	The conversion of Syringaldehyde will be monitored using TLC plates
Step V	When all the Syringaldehyde was totally consumed (4 hrs) the reaction medium will be cooled down to room temperature (25°C) using ice bath
Step VI	Cooled reaction mixture will be evaporated under the vacuum using the roto evaporator (at 40°C)
Step VII	To remove/eliminate traces of Piperidine the above step will be repeated twice with 80 mL toluene
Step VIII	The final precipitate was dissolved in 80 mL of ethyl acetate and washed three times with saturated NaCl solution in a separatory funnel
Step IX	The organic phase was dried (sodium sulfate), filtered, concentrate and finally fractionated using column chromatography
Step X	The organic phase will be purified using a glass column filled with silica gel 60 Å as the stationary phase and n-hexane/ethyl acetate (70/30, v/v) as the mobile phase`
Step XI	Fraction separation will be followed applying drops of each collector tube to TLC plates which were developed with n-hexane/ethyl acetate/formic acid (70/30/1, v/v/v), dried and directly analyzed using a UV lamp

The maximum wavelength for Syringaldehyde (310 nm) was deduced from its spectrum in the UV-visible domain.

Appendix A4: Ultrasound extraction of canola meal

Principle:

This method extracts the canola meal using methanol (70 % (v/v)) by the ultrasound probe. In this method, the canola meal, the by-product of expeller pressed canola oil was treated with ultrasound assisted extraction.

Equipment:

- Analytical balance
- 50 mL centrifuge tubes
- Pipette: 5 mL capacity
- Vortex Mixer
- Ultrasound probe (ultrasound homogenizer)
- Centrifuge
- Volumetric flask: 10 mL

Reagents:

- Methanol (optima grade).

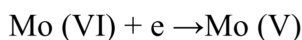
Protocol:

Step I	Carefully weigh 1.0 g of canola meal sample in a 50 mL centrifuge tube. Add 9.0 mL 70% (v/v) methanol
Step II	Vortex the tube for 30 seconds
Step III	Turn on the Ultrasound probe and set the level of power to 40%
Step IV	Extract the samples for 1 minute at 40% power using an ultrasound probe
Step V	Centrifuge the homogenate at 5000 RPM for 15 minutes at 4℃
Step VI	Pipette out the supernatant into a graduated tube (50 mL centrifuge tube)
Step VII	Add another 9.0 mL 70% (v/v) methanol to the residue and repeat the extraction procedures (Step II & V). Repeat the extraction twice
Step VIII	Combine the methanolic extracts into the same tube (50 mL centrifuge tube) and make up the total volume up to 25 mL using 70% methanol
Step IX	Store the phenolic extract at -20 ºC for further analysis
Step X	Each sample should be carried out at least in triplicates

Appendix A5: Determination of total phenolic content (TPC) of canola meal extracts using Folin-Ciocalteu (FC) assay

Principle:

The chemistry behind the FC assay relies on the transfer of electrons in alkaline medium from phenolic compounds and other reducing species to molybdenum, forming blue complexes that can be detected spectrophotometrically at 750-765nm. In essence, it is believed that the molybdenum is easier to be reduced in the complex and electron-transfer reaction occurs between reductants and Mo (VI). Generally, Gallic acid is used as the reference standard compound and results are expressed as Gallic acid equivalents (mg/L).



$$Mo\ (VI) + e \rightarrow Mo\ (V)$$

Equipment & Glassware:

- Ash-less Filter Paper (Whatman NO.42)
- Balance
- Micro-pipette tips (200, 1000, 5000 µL)
- Weighing Dish
- Vortex (VWR)
- Spectrophotometer (DU 800 Beckman)
- Magnetic Stirrer (VWR)
- Ultrasound bath (VWR)
- Conical Flask (10.0 mL, 25.0 mL)

Reagents & Samples:

1. **19% Sodium carbonate solution**

Weigh 1.9 g of sodium carbonate (Sigma-Aldrich) in a weighing dish and transfer the sodium carbonate into a 10 mL standard volumetric flask. Add approximately 5

mL of distilled water into the weighing dish, rinse, and transfer to the volumetric flask containing sodium carbonate. Dissolve the sodium carbonate using a magnetic stirrer; by adding minimum quantity of distilled water (it should not cross the marking in the flask). After the complete dissolving of sodium carbonate, fill the flask with distilled water till the marking. Filter the sodium carbonate mixture through an ash-less filter paper. Label name, date. Prepare fresh solution every time.

2. Folin-Ciocalteu's reagent

Always use FC reagent from Sigma-Aldrich unless specified otherwise.

3. Sinapic acid and gallic acid standard (1.0 mg/mL)

Weigh 10.0 mg of sinapic acid in weight boat and transfer to a 10.0 mL standard volumetric flask by using a funnel. Make up to 10.0 mL using 100% optima methanol. Mix the sample by using Ultrasound bath, for 20 seconds at room temperature. Keep this stock solution at 4°C in a refrigerator until use. Similarly, use gallic acid for making 1.0 mg/mL standard solution.

Protocol:

Step I	Perform at least duplicate for each sample. Exactly measure 100 µL of sample (methanolic extracts) into a 10.0 mL conical flask, followed by addition of 400 µL of distilled water to the sample. Vertex the flask for 5 seconds. Now add 4.5 mL of distilled water and vertex the flask for another 5 seconds.
Step II	Add 0.5 mL Folin-Ciocalteu's phenol reagent to the above reaction mixture and vortex for 10 seconds. Keep for 3 minutes.
Step III	Add 1.0 mL 19% Sodium Carbonate and vertex the flask. Now add distilled water to make the total volume 10.0 mL (= Add 3.5mL of distilled water).
Step IV	Store the flask with reaction mixture in dark cabinet for 60 minutes with an intermittent shaking (shake the flask using hand, after 30 minutes for 5 seconds).
Step V	Measure the absorption using the Spectrophotometer at 750 nm. The **blank** is prepared in the same way (Step I-IV) by replacing 200 µL of sample with 200 µL of distilled water.
Step VI	Calculate the content of total phenolics in the extracts (using the absorbance values) as mg sinapic acid equivalents per kg of the sample by using sinapic acid calibration curve.

Sinapic acid calibration curve

Step I	Prepare different concentration of sinapic acid/gallic acid solution using the stock (1.0 mg/mL) (Pipette 20-180 µL of stock sinapic acid solution and make up to 500 µL using distilled water). Transfer 500 µL of sinapic acid into a 10.0 mL volumetric flask. Vertex the flask for 5 seconds. Now add 4.5 mL of distilled water and vertex the flask for another 5 seconds.
Step II	Add 0.5 mL Folin-Ciocalteu's phenol reagent to the above reaction mixture and vortex for 10 seconds. Keep for 3 minutes. Then follow steps III-V as mentioned above. Repeat the same procedure for different concentrations of sinapic acid.
Step III	Prepare a standard graph in Excel plotting concentration against absorbance.

Appendix A6: Determination of total flavonoid content (TFC) of canola meal extracts

Principle:

Diphenylboric acid-2-aminoethyl ester forms acid labile complexes with the ortho-dihydroxyl groups in the A- or B-ring of flavonoids. Quercetin is reported to be suitable for building the calibration curve. Therefore, standard Quercetin solutions of various concentrations were used to build up the calibration curve.

Equipment & Glassware:

- Balance
- Micro-pipette tips (200, 1000, 5000 µL)
- Weighing Dish
- Vortex (VWR)
- Spectrophotometer (DU 800 Beckman)
- Conical Flask (10.0 mL, 25.0 mL)

Reagents & Samples:

1. **Quercetin Standard Solution (1.0 mM)**

   The standard solution of quercetin will be made using respective dilutions of quercetin standard from the range of 0.01 mM to 0.5 mM concentration.

2. **Diphenylboric acid-2-aminoethyl ester Solution (1% v/v)**

   1.0 mg of diphenylboric acid-2-aminoethyl ester will be added to 100.0 mL volumetric flask and distilled water will be added to the mark to make it a 1% (v/v) solution.

Protocol:

Step I	Prepare the Fresh diphenylboric acid-2-aminoethyl ester solution (1% v/v).
Step II	Mix 1.0 mL of canola meal extract with 3.0 mL of water and add 100 µL of diphenylboric acid-2-aminoethyl ester solution (1% v/v). Measure the absorbance of the colored complex at 404 nm.

Quercetin calibration curve

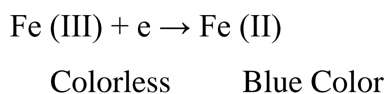
Step I	Prepare different concentration of quercetin solution using the stock (1.0 mM).
Step III	Prepare a standard graph in Excel plotting concentration against absorbance.

Standard Concentration (mM)	Quercetin (mL)	Methanol (mL)
0.1	1.0	9.0
0.2	2.0	8.0
0.4	4.0	6.0
0.6	6.0	4.0
0.8	8.0	2.0
1.0	10.0	0.0

Appendix A7: Determination of antioxidant activity of canola meal extracts using ferric reducing ability of plasma assay (FRAP)

Principle:

The FRAP assay uses antioxidants as reductants in a redox-linked colorimetric method employing an easily reduced oxidant, Fe(iii). Reduction of ferric tripyridyltriazine complex to ferrous-(2,4,6-tripyridyl-s-triazine)$_{2}$. This can be monitored by measuring absorbance at 593 nm. The reading of the absorbance is related to the reducing power of the electron-donating antioxidants present in the test compound. Generally, Trolox is used as the reference standard compound and results are expressed as Trolox equivalents (mg/L).



Equipment & Glassware:

- Balance
- Micro-pipette tips (200, 1000, 5000 µL)
- Weighing Dish
- Vortex (VWR)
- Spectrophotometer (DU 800 Beckman)
- Magnetic Stirrer (VWR)
- Water bath (37oC) (VWR)
- Conical Flask (10.0 mL, 25.0 mL)
- Timer

Reagents & Samples:

1. **Acetate Buffer Solution (300 mM, pH - 3.6)**

Weigh 3.1 g of sodium acetate (Sigma-Aldrich) in a weighing dish and transfer the sodium acetate into a 1.0 L standard volumetric flask. Dissolve the sodium acetate using a magnetic stirrer; by adding 50.0 mL of distilled water. Add 16.0 mL of glacial acetic acid (Fisher-Sci) into the volumetric flask and keep stirring. After the complete dissolving of sodium acetate, fill the flask with distilled water till the marking. Check the pH and keep the solution at 4^oC. (solution is good for one year)

2. **HCl Solution (40 mM)**

Take 1.0 L volumetric flask and add 50.0 mL of distilled water. Add 1.46 mL of hydrochloric acid (Fisher-Sci) into the volumetric flask and fill the flask with distilled water till the marking. Keep the solution at 4^oC. (solution is good for one year)

3. **TPTZ solution (10 mM)**

The working TPTZ (2,4,6-tri[2-pyridyl]-s-triazine) solution will be made using the following way. Weigh 0.031 g of TPTZ in to a 10.0 mL volumetric flask. Dissolve the TPTZ using 40 mM HCl solution. Fill the flask with HCl solution till the marking. To dissolve the TPTZ completely use the water bath (50^oC). Make every day a fresh solution

4. **Ferric Chloride Solution (20 mM)**

The working ferric chloride ($FeCl_3.6H_2O$) solution will be made using the following way. Weigh 0.054 g of $FeCl_3.6H_2O$ in to a 10.0 mL volumetric flask. Dissolve the $FeCl_3.6H_2O$ using distilled water and fill the flask with water till the marking. Make every day a fresh solution

Protocol:

Step I	Prepare the Fresh FRAP reagent using 200.0 mL acetate buffer, 20.0 mL TPTZ solution, 20.0 mL $FeCl_3$ solution. Mix all three solvents (10:1:1) well and keep the solvent mixture at 37^oC. The mixed solution should be straw colored. If it is tinged with blue color, discard and make a fresh solution.
Step II	Run a set of blanks first. Add 1.0 mL of distilled water to 2.0 mL of working FRAP reagent. Keep the samples in dark for 30 minutes and read the absorbance at 593 nm.
Step III	For samples, mix 100 µL of the sample with 900 µL of distilled water. Add 2.0 mL of FRAP reagent and measure the absorbance at 593 nm after keeping in dark for 30 minutes.
Step IV	Perform at least duplicate for each sample.

Ferrous Sulphate Standard Solution (1.0 mM)

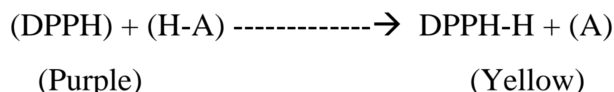
The working ferrous sulphate ($Fe_2SO_4.7H_2O$) solution will be made using the following way. Weigh 0.278 g of $Fe_2SO_4.7H_2O$ in to a 1.0 L volumetric flask. Dissolve the $Fe_2SO_4.7H_2O$ using distilled water and fill the flask with water till the marking. Make every day a fresh solution. Make a series of standards using the following:

Standard Concentration (mM)	$Fe_2SO_4.7H_2O$ (mL)	Distilled Water (mL)
0.1	1.0	9.0
0.2	2.0	8.0
0.4	4.0	6.0
0.6	6.0	4.0
0.8	8.0	2.0
1.0	10.0	0.0

Appendix A8: Determination of antioxidant activity of canola meal extracts using DPPH assay: (2, 2-diphenyl-1-picrylhydrazyl)

Principle:

In this assay, the purple chromogen radical 2,2-diphenyl- 1-picrylhydrazyl (DPPH•) is reduced by antioxidant/reducing compounds to the corresponding pale-yellow hydrazine. The scavenging capacity is generally evaluated in organic media by monitoring the absorbance decrease at 515–528 nm until the absorbance remains constant or by electron spin resonance. The scavenging reaction between (DPPH.) and an antioxidant (H-A) can be written as:



Antioxidants react with DPPH, which is a stable free radical and is reduced to the DPPHH and as consequence the absorbance's decreased from the DPPH radical to the DPPH-H form. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability. Despite some of the limitations, the DPPH• radical is stable, commercially available, and does not have to be generated before assay like ABTS•+. Therefore, it is considered an easy and useful spectrophotometric method regarding screening/measuring the antioxidant capacity of both pure compounds and complex samples.

Free radical scavenging activity of canola extracts will be assayed using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) according to the procedure of Hou, Chen, Chen, Lin, Yang & Lee (2001) with some modification. To determine the DPPH radicals scavenged, 50 µl of canola extract will be dissolved in 2.95 ml of 0.1 mM DPPH solution in methanol and differences before (t=0) and after sample injection (t=10) will be read at 516 nm using the UV/Visible Spectrophotometer.

Materials and Equipment:

- 100 mL volumetric flask;
- 20-200 µL pipette, 500-5000 µL pipette;
- 4.5 mL disposal plastic cuvettes and disposal cuvette stirrers;
- 200 mL glass bottle with cap and covered with aluminum foil

Reagents:

- Absolute Ethanol

DPPH reagent

Prepare 0.1 mM (0.1 mmol/L) solution of the DPPH in absolute ethanol. This can be done by dissolving 3.94 mg DPPH (394.32 g/mol) in ethanol and by making up to 100 mL and stir for 10 min. Since the reagent is light sensitive, the flask must be covered. Measure the absorbance of the obtained solution at 516 nm. The appropriate absorbance for fresh DPPH solution (0.1mM) should be around 1.1-1.2.

Notes:

- Time management is very important
- Do not uses the ultrasound bath to dissolve DPPH
- Use disposable plastic stirrers to make sure of mixing the materials
- The assay should be conduct without light (use covered tubes and glassware)
- The appropriate absorbance for fresh DPPH solution (0.1mM) should be around 1.1-1.2

Protocol:

Step I	In spectrophotometer cuvettes (three for each sample) add 50 µL of the phenolic extract.
Step II	To each cuvette add 2.95 mL of the DPPH solution. Mix the solution well using disposable plastic stirrers.
Step III	Make three cuvettes for the control and add to each of them only 2.95 mL of the DPPH solution and 50 µL of methanol (solvent control). See note 1
Step IV	Cover and leave the cuvettes to stand in the dark for exactly 10 min.
Step V	Read the absorbance at 516 nm using absolute ethanol as blank.
Step VI	Use the following formula to calculate the scavenging activity: Scavenging Effect (%) = $\frac{(A_c - A_s) \times 100}{A_c}$ Where A_c is absorbance of solvent control A_s is absorbance of sample
Note 1	**Which solution should be used as solvent control?** The same solvent used to extract the sample (sample solvent) should be added to the DPPH instead of the sample. So, the blank will contain everything (DPPH and sample solvent) except the sample itself. If we are investigating phenolic extracts (70% methanolic extracts) and adding 50 µL of the extract to 2950 µL of the DPPH solution, so the solvent control is 2950 µL DPPH + 50 µL of 70% methanol/70% ethanol.
Note 2	To calculate the E_{50} concentration, use different concentrations of 100 µL sample [20, 40, 60, 80, 100 µL of the sample and all of them were made to 100 µL using 80, 60, 40, 20 & 0 µL of methanol]

Appendix A9: Determination of antioxidant activity of canola meal extracts using metal ion chelation

Principle:

Chelating agents reduce the concentration of metal ions available for catalyzing peroxidation and thus are known to serve as effective secondary antioxidants. In this assay ferrous ions form a complex with Ferrozine, and the intensity of the purple color of the complex decreases in the presence of chelating agents.

Equipment & Glassware:

- Balance
- Micro-pipette tips (200, 1000, 5000 µL)
- Weighing Dish
- Vortex (VWR)
- Spectrophotometer (DU 800 Beckman)
- Conical Flask (10.0 mL, 25.0 mL)

Reagents & Samples:

1. **Ferrozine Solution (5.0 mM)**

   Ferrozine solution (5.0mM) will be prepared using dissolving 24.6228 mg of ferrozine in 10.0 mL of distilled water.

2. **Ferrous Chloride ($FeCl_2.4H_2O$) Solution (2.0 mM)**

   Ferrous Chloride ($FeCl_2.4H_2O$) solution (2.0 mM) will be prepared using dissolving 3.9762 mg of ferrous chloride in 10.0 mL of distilled water.

Protocol:

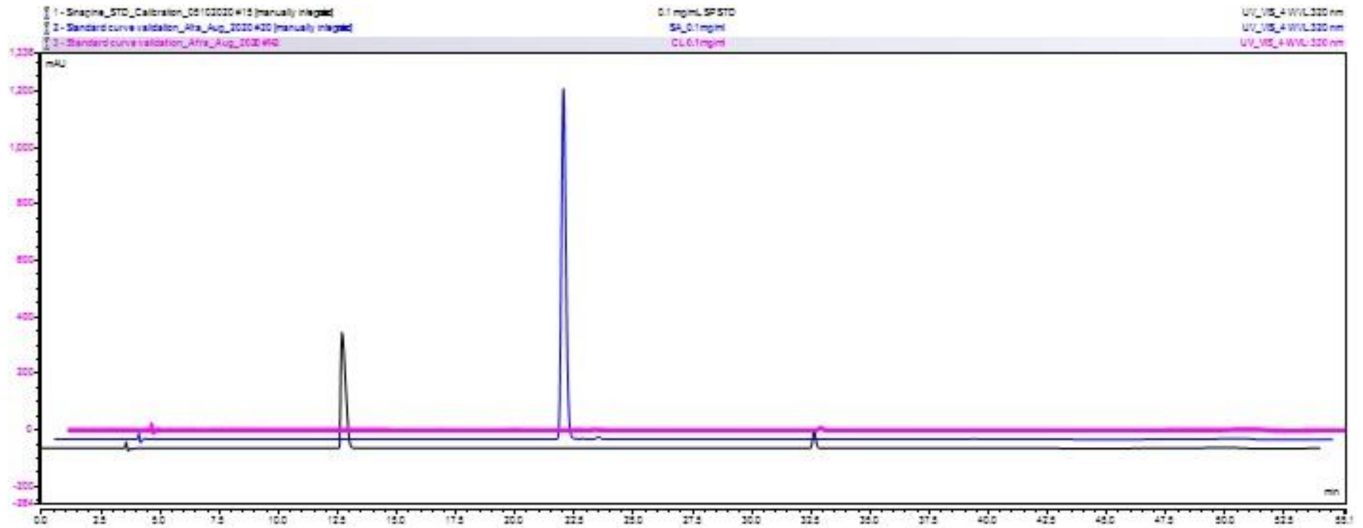
Step I	Prepare the fresh Ferrozine (5.0 mM) and Ferrous Chloride (2.0 mM) Solutions
Step II	Mix 1.0 mL of canola meal extract with 50 µL of ferrous chloride and add 1.85 mL of distilled water.
Step III	To the sample mixture add 0.1 mL of 5.0 mM Ferrozine solution and make the total volume up to 3.0 mL
Step IV	Let the sample mixture stand for 10 minutes at the room temperature
Step V	Measure the absorbance of the sample mixture at 562 nm
Step VI	Blanks will be prepared using 0.4 mL of the sample and 3.6 mL of distilled water
Step VII	Different concentrations (0.05-2 mM) of Na_3EDTA will be used to prepare the standard curve

Metal ion chelation percentage will be calculate using the following formula:

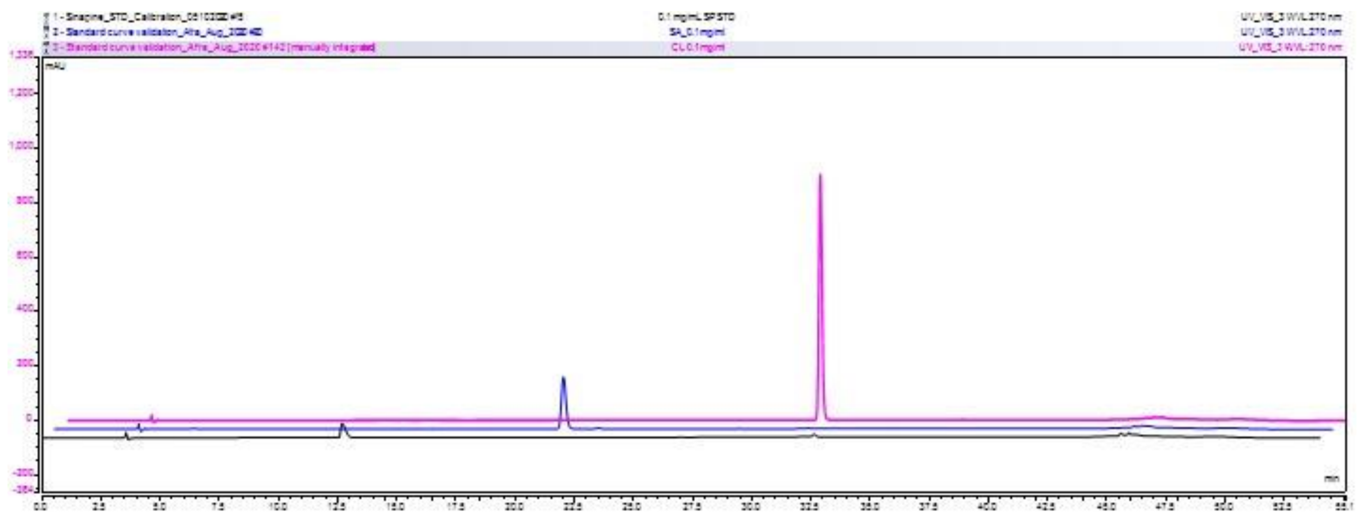
metal chelating effect (%) = [1 - (absorbance of the sample - absorbance of the control)] 100

Appendix B: HPLC chromatograms for ASE extracts

Appendix B1: HPLC chromatograms of standard compounds (A-320 nm, B-270 nm)



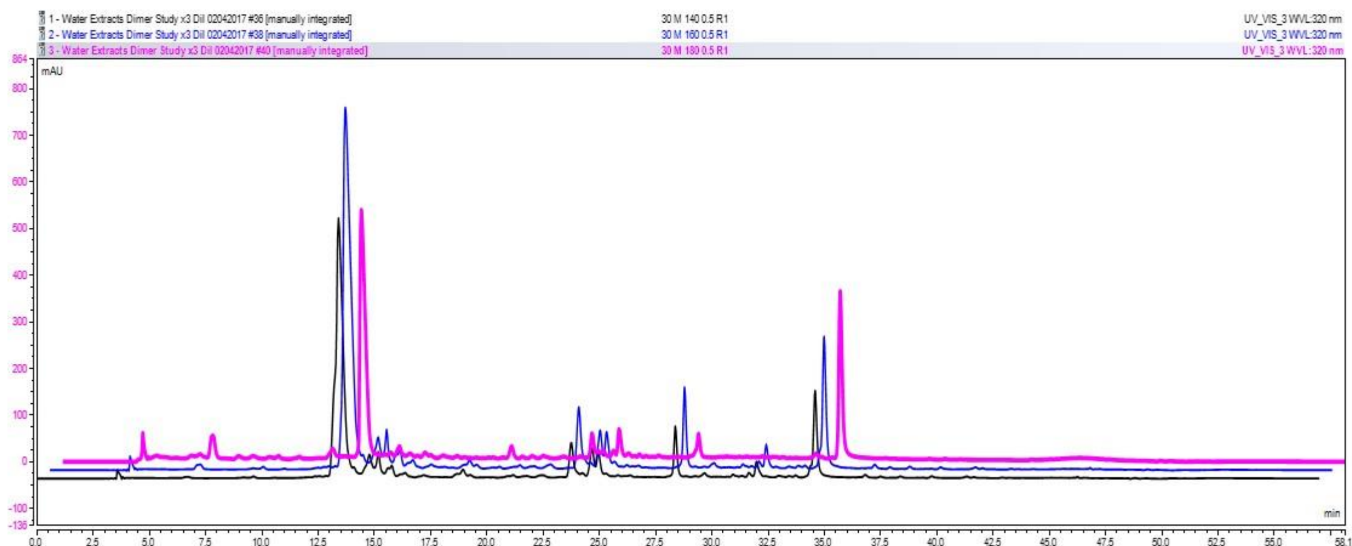
A - HPLC chromatogram of sinapine (black), sinapic acid (blue) and canolol (pink) (320 nm)



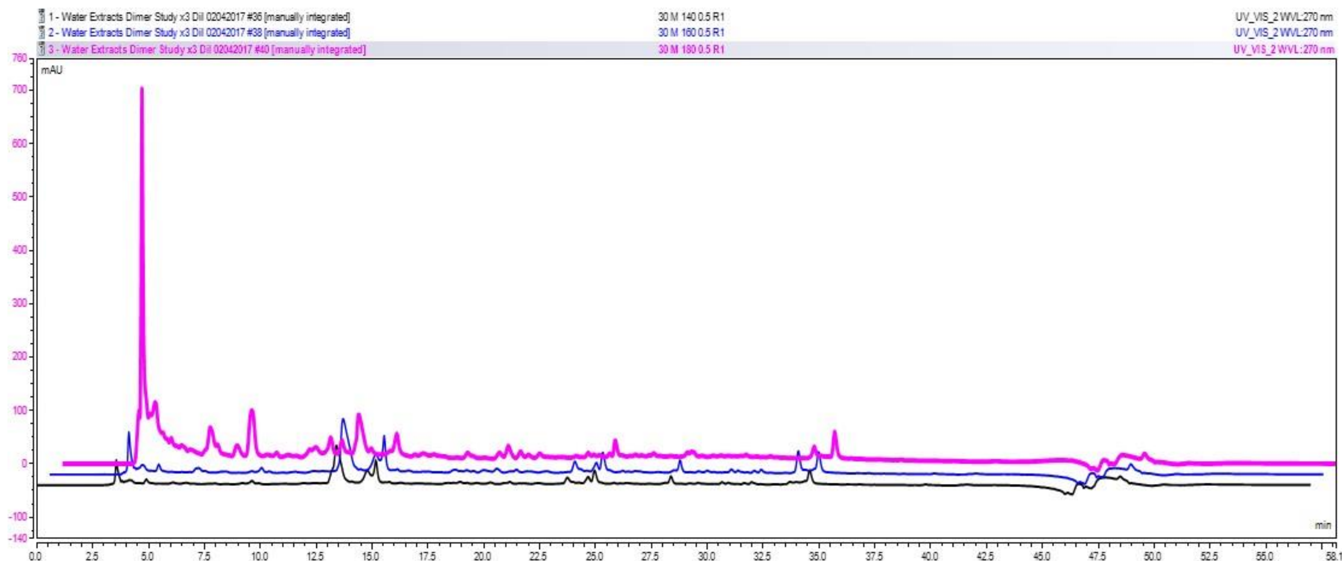
B - HPLC chromatograms of sinapine (black), sinapic acid (blue) and canolol (pink) (270 nm)

Appendix B2: HPLC chromatogram of methanol extracts (A-320 nm, B-270 nm) for 0.5 mm particle size

Appendix B2.1: 30% (v/v) methanol extracts at different processing temperatures

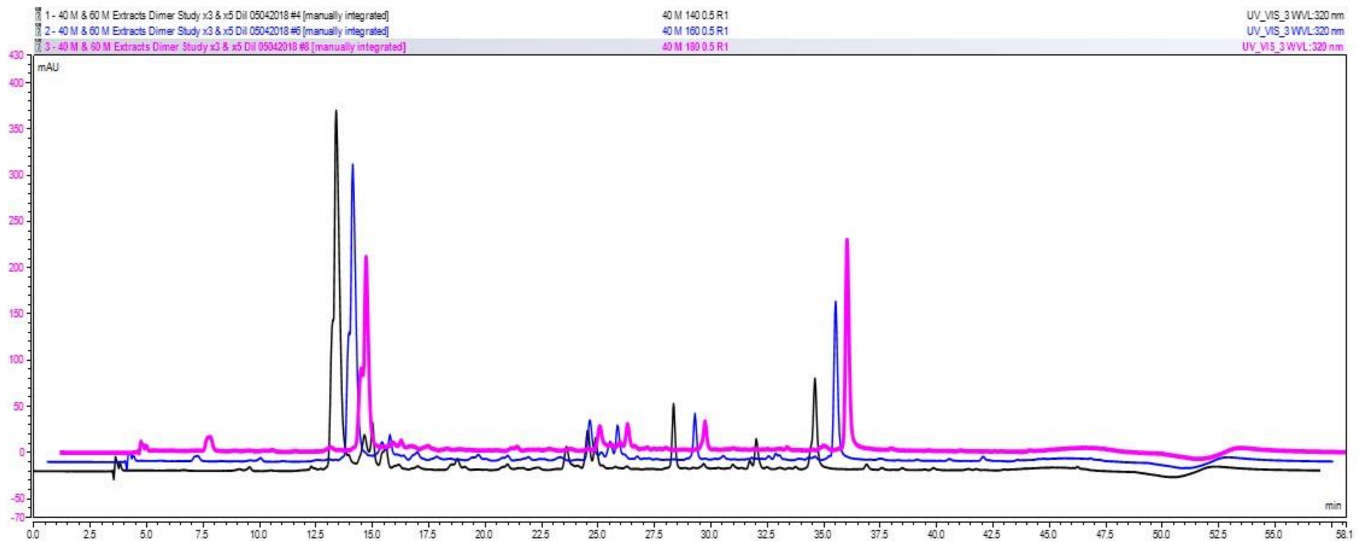


A - HPLC chromatogram of 30% (v/v) methanol extracts at 140°C (black), 160°C (blue) and 180°C (pink)

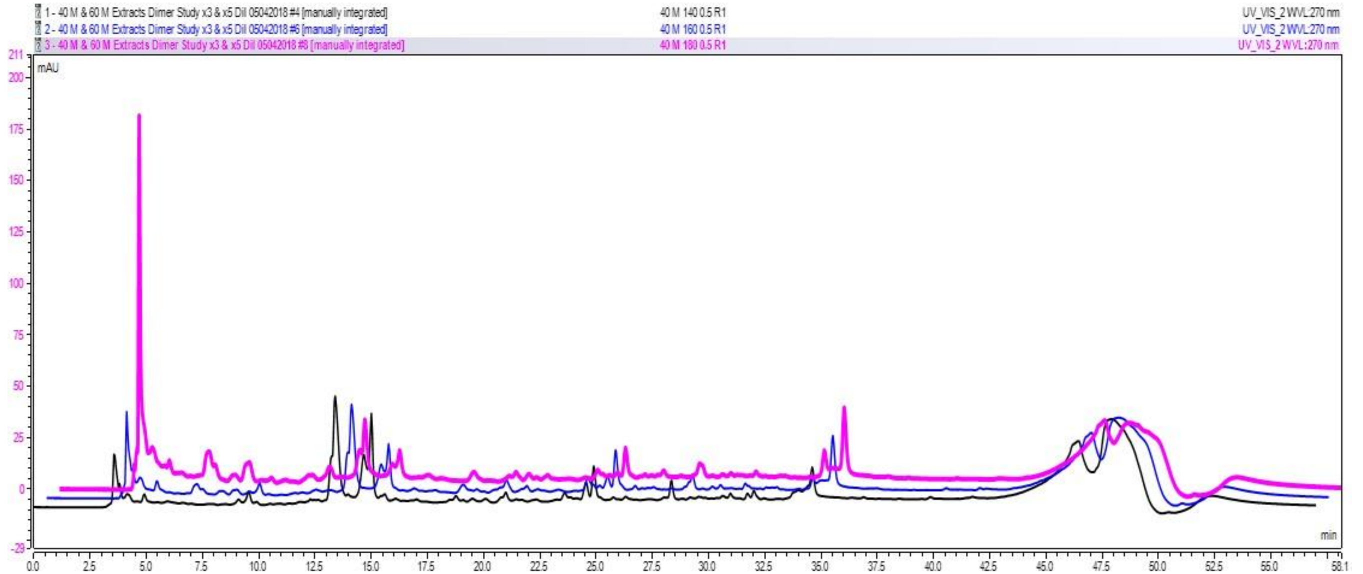


B - HPLC chromatogram of 30% (v/v) methanol extracts at 140°C (black), 160°C (blue) and 180°C (pink)

Appendix B2.2: 40% (v/v) methanol extracts at different processing temperatures

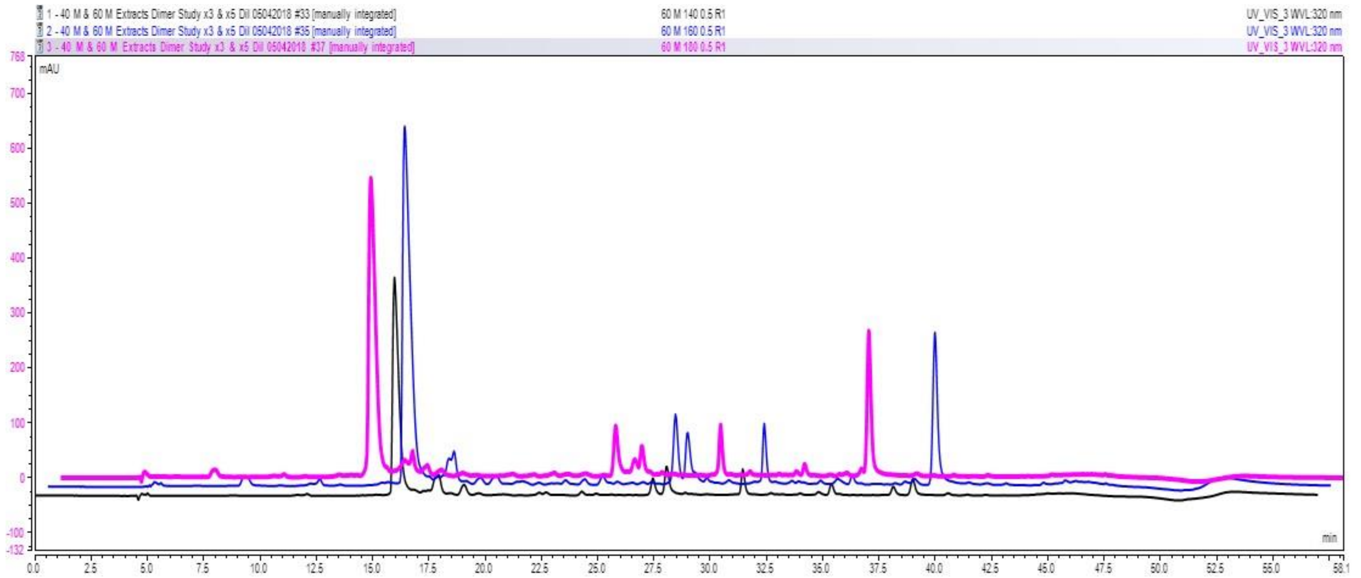


A - HPLC chromatogram of 40% (v/v) methanol extracts at 140ºC (black), 160ºC (blue) and 180ºC (pink)

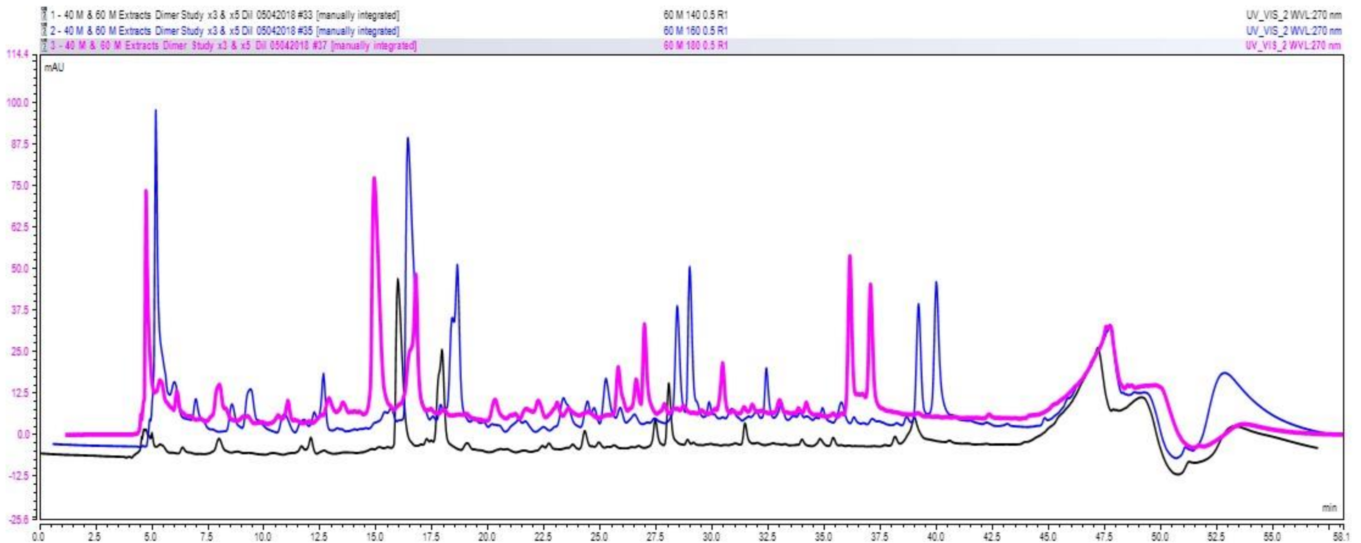


B - HPLC chromatogram of 40% (v/v) methanol extracts at 140ºC (black), 160ºC (blue) and 180ºC (pink)

Appendix B2.3: 60% (v/v) methanol extracts at different processing temperatures

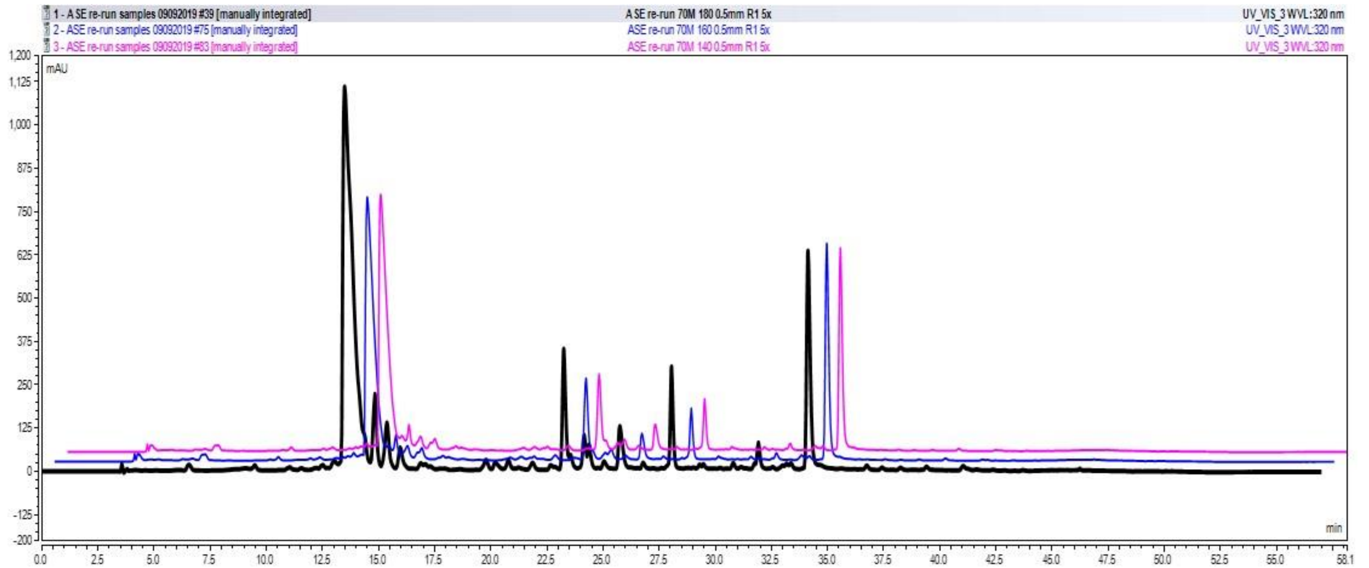


A - HPLC chromatogram of 60% (v/v) methanol extracts at 140ºC (black), 160ºC (blue) and 180ºC (pink)

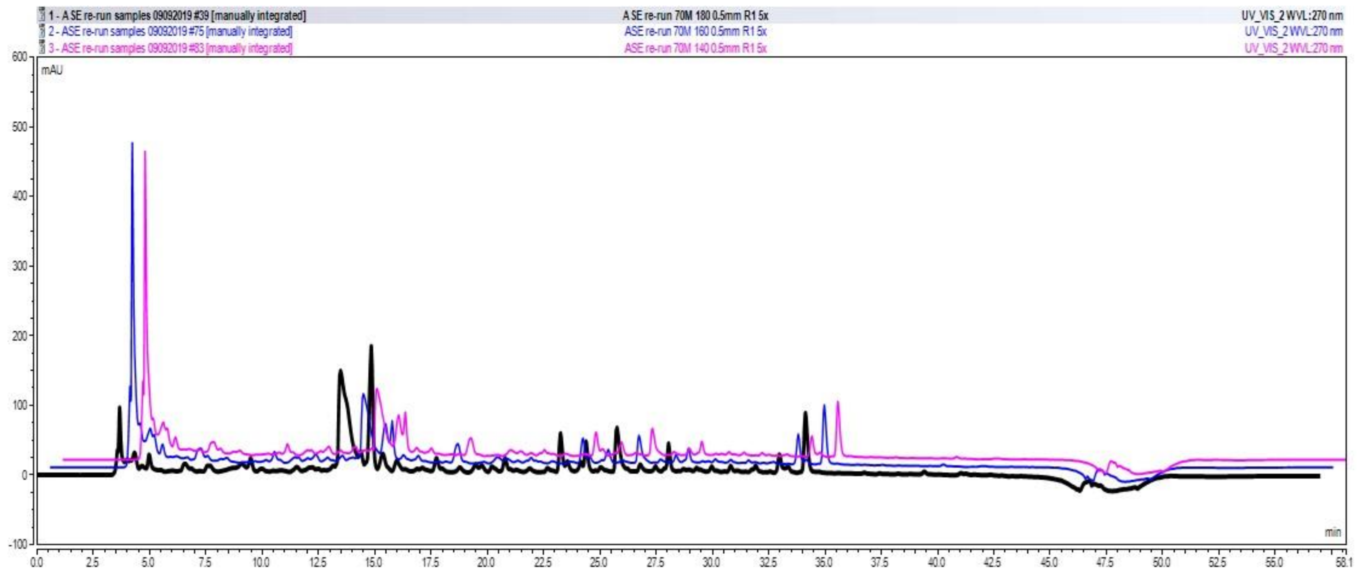


B - HPLC chromatogram of 60% (v/v) methanol extracts at 140ºC (black), 160ºC (blue) and 180ºC (pink)

Appendix B2.4: 70% (v/v) methanol extracts at different processing temperatures



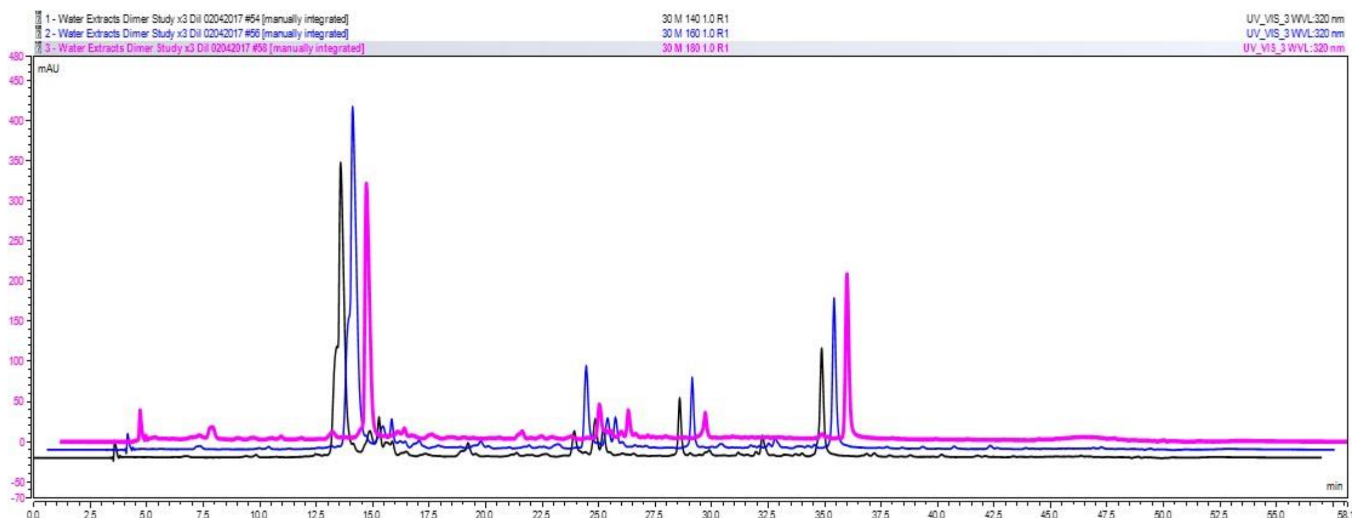
A - HPLC chromatogram of 70% (v/v) methanol extracts at 140ºC (pink), 160ºC (blue) and 180ºC (black)



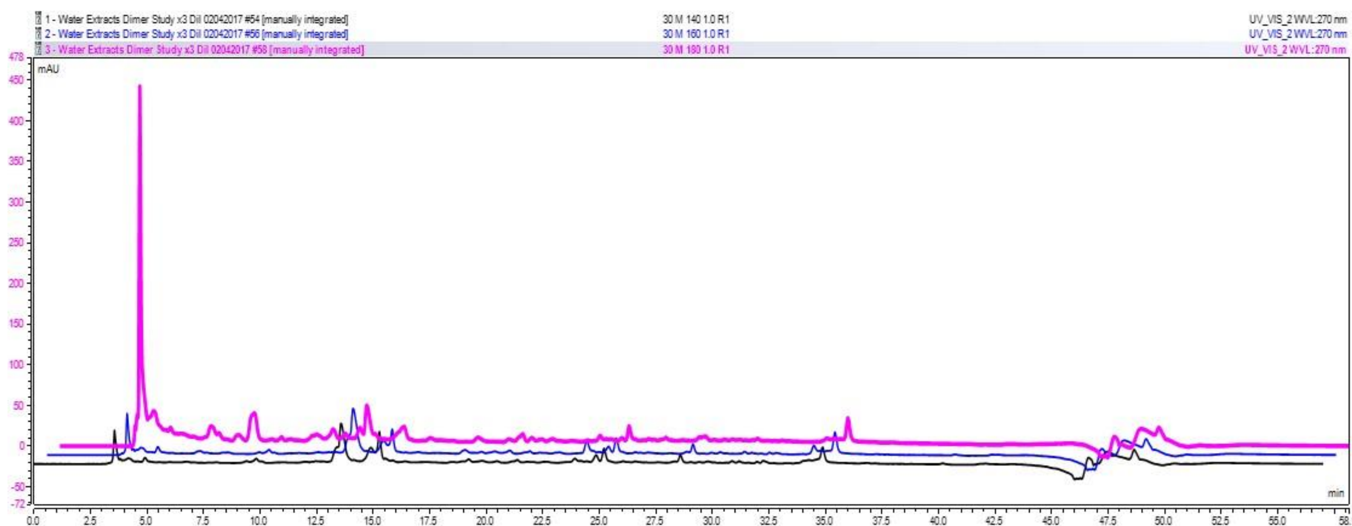
A - HPLC chromatogram of 70% (v/v) methanol extracts at 140ºC (pink), 160ºC (blue) and 180ºC (black)

Appendix B3: HPLC chromatogram of methanol extracts (A-320 nm, B-270 nm) for 1.0 mm particle size

Appendix B3.1: 30% (v/v) methanol extracts at different processing temperatures

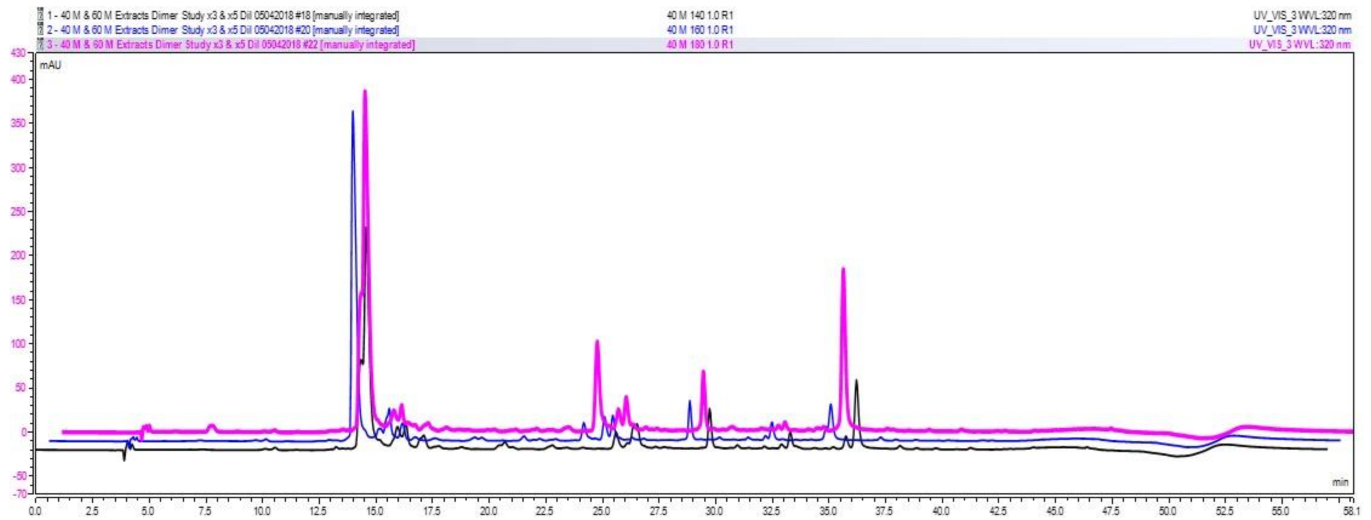


A - HPLC chromatogram of 30% (v/v) methanol extracts at 140ºC (black), 160ºC (blue) and 180ºC (pink)

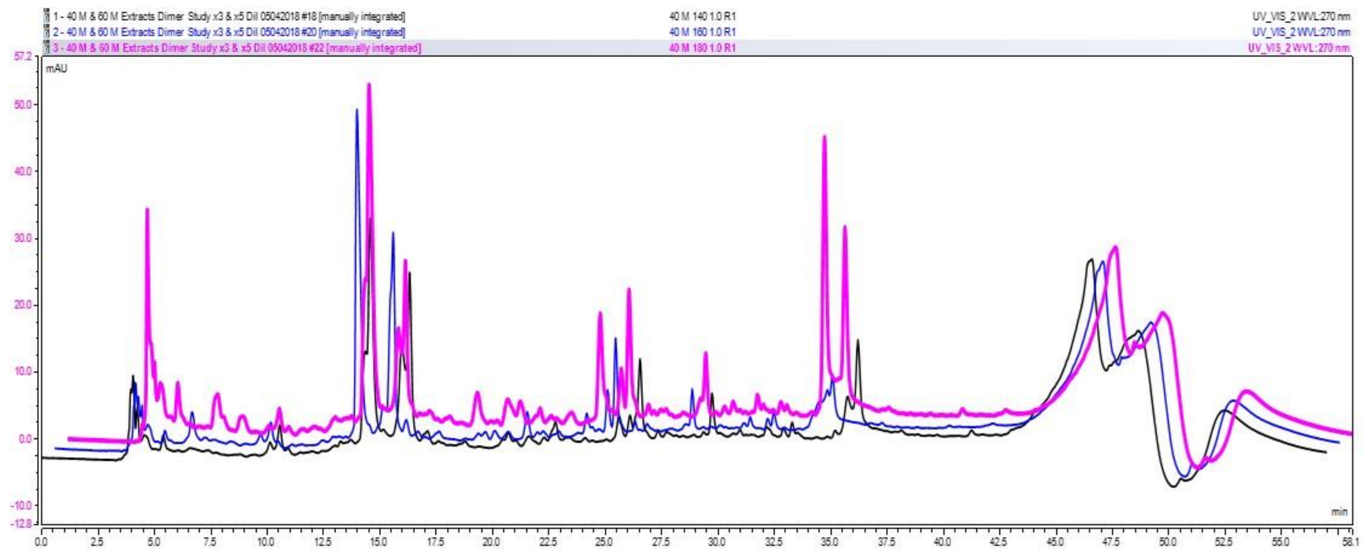


B - HPLC chromatogram of 30% (v/v) methanol extracts at 140ºC (black), 160ºC (blue) and 180ºC (pink)

Appendix B3.2: 40% (v/v) methanol extracts at different processing temperatures

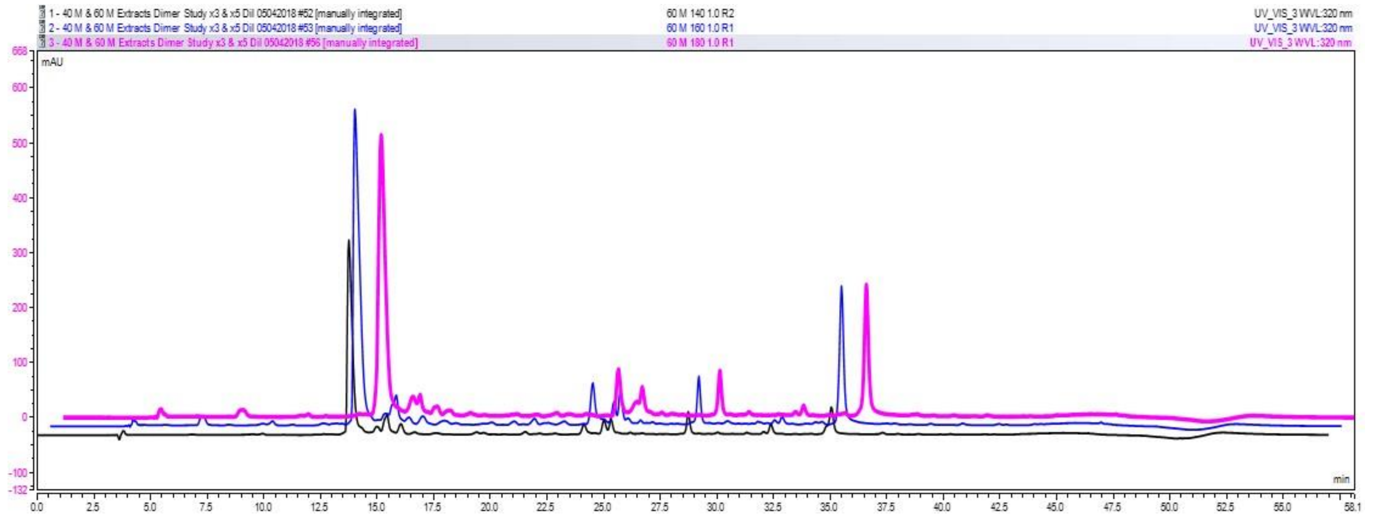


A - HPLC chromatogram of 40% (v/v) methanol extracts at 140ºC (black), 160ºC (blue) and 180ºC (pink)

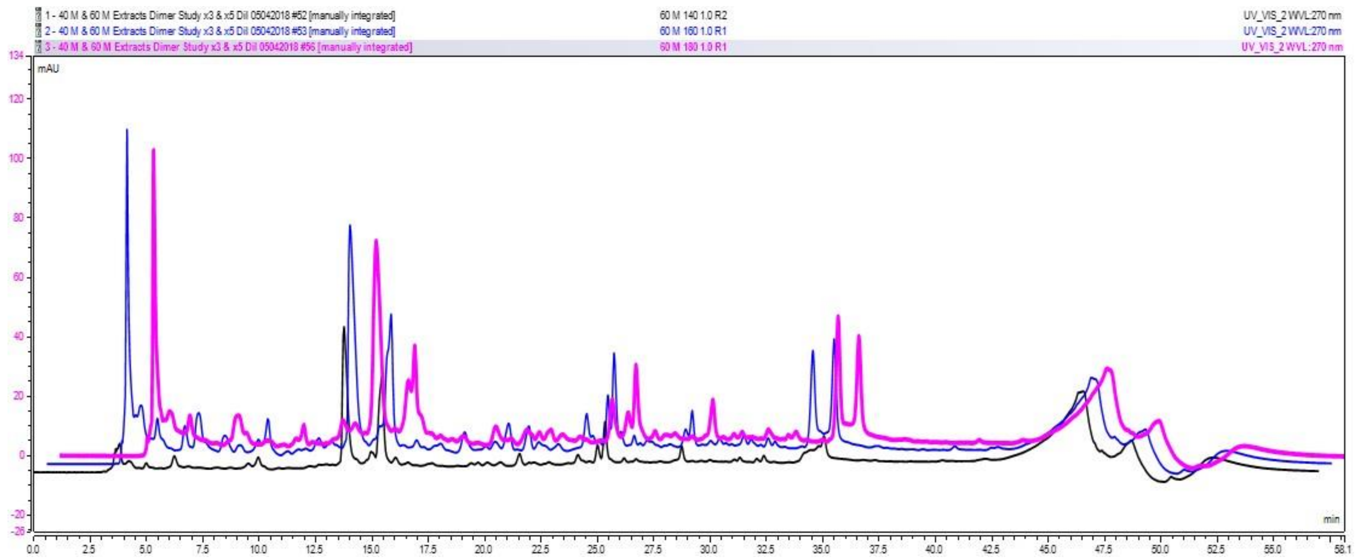


B - HPLC chromatogram of 40% (v/v) methanol extracts at 140ºC (black), 160ºC (blue) and 180ºC (pink)

Appendix B3.3: 60% (v/v) methanol extracts at different processing temperatures

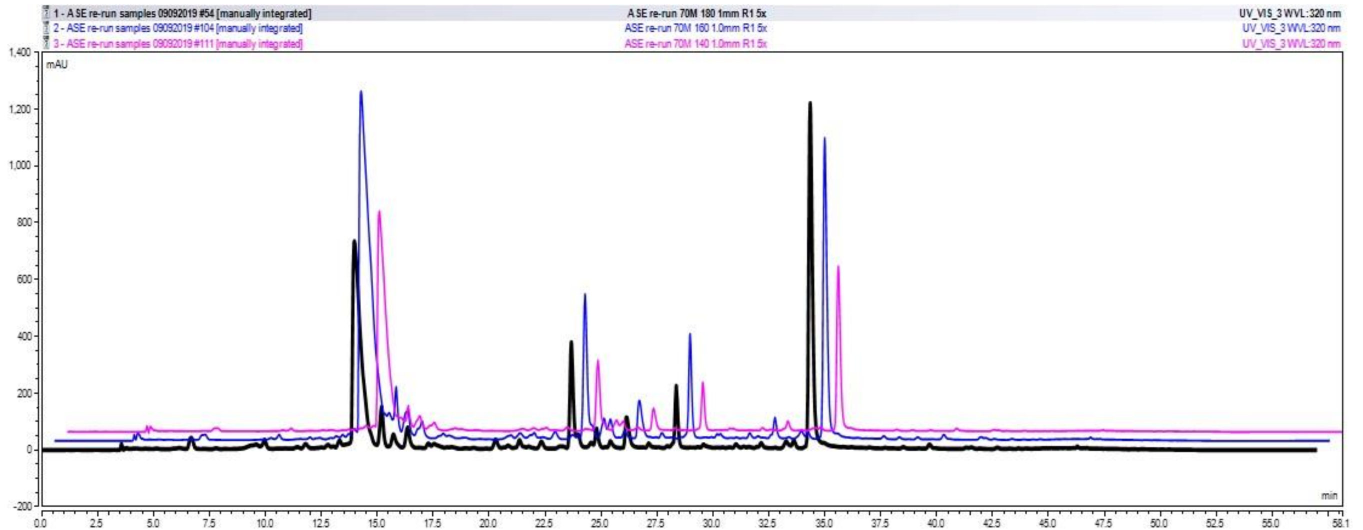


A - HPLC chromatogram of 60% (v/v) methanol extracts at 140°C (black), 160°C (blue) and 180°C (pink)

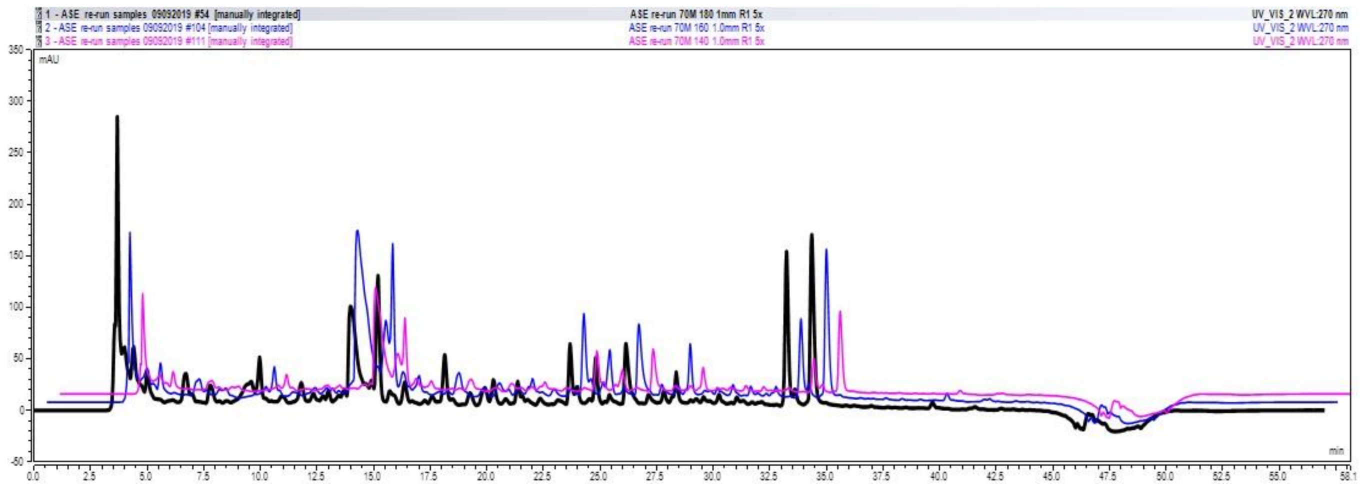


B - HPLC chromatogram of 60% (v/v) methanol extracts at 140°C (black), 160°C (blue) and 180°C (pink)

Appendix B3.4: 70% (v/v) methanol extracts at different processing temperatures



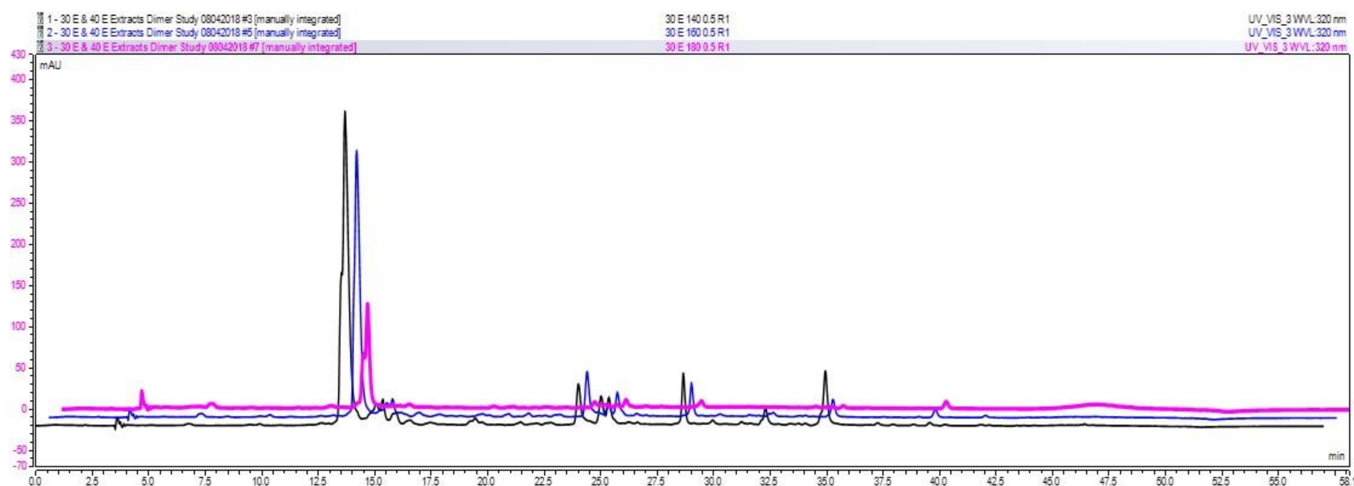
A - HPLC chromatogram of 70% (v/v) methanol extracts at 140ºC (pink), 160ºC (blue) and 180ºC (black)



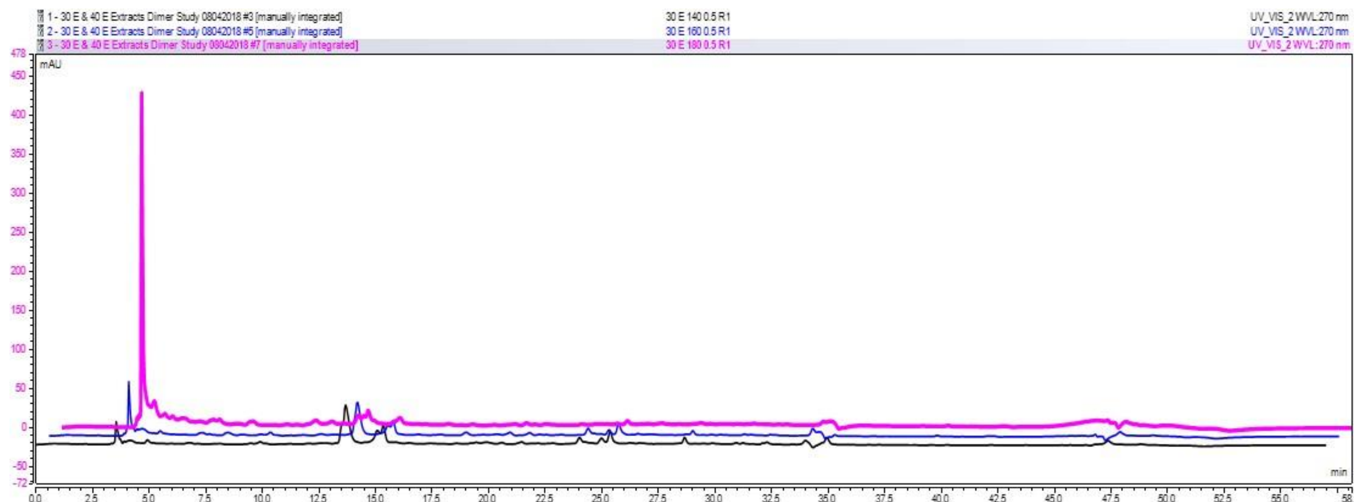
B - HPLC chromatogram of 70% (v/v) methanol extracts at 140ºC (pink), 160ºC (blue) and 180ºC (black)

Appendix B4: HPLC chromatogram of ethanol extracts (A-320 nm, B-270 nm) for 0.5 mm particle size

Appendix B4.1: 30% (v/v) ethanol extracts at different processing temperatures

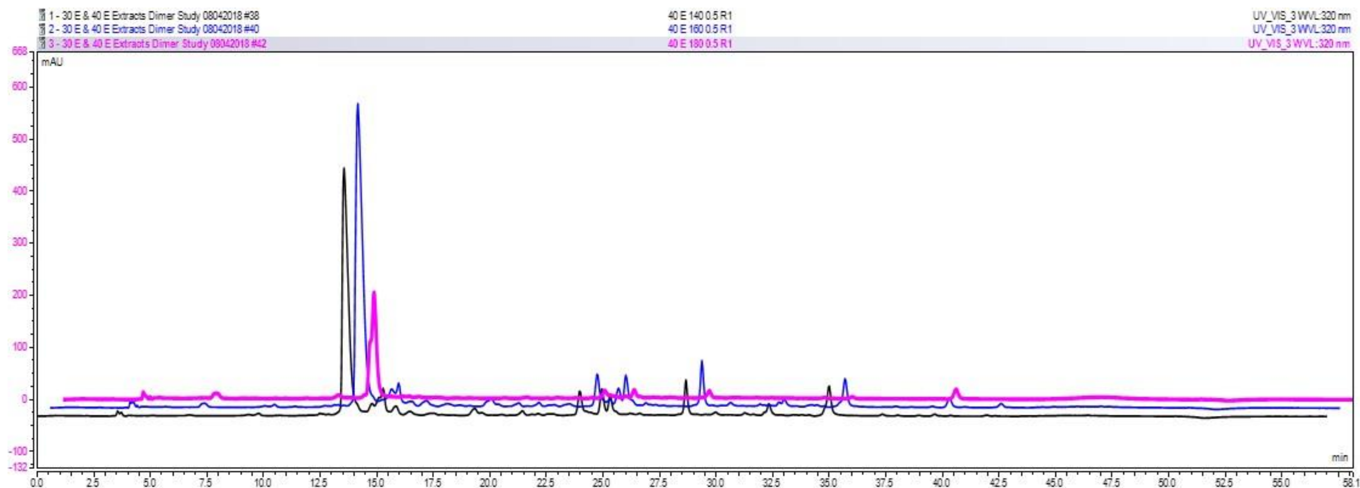


A - HPLC chromatogram of 30% (v/v) ethanol extracts at 140ºC (black), 160ºC (blue) and 180ºC (pink)

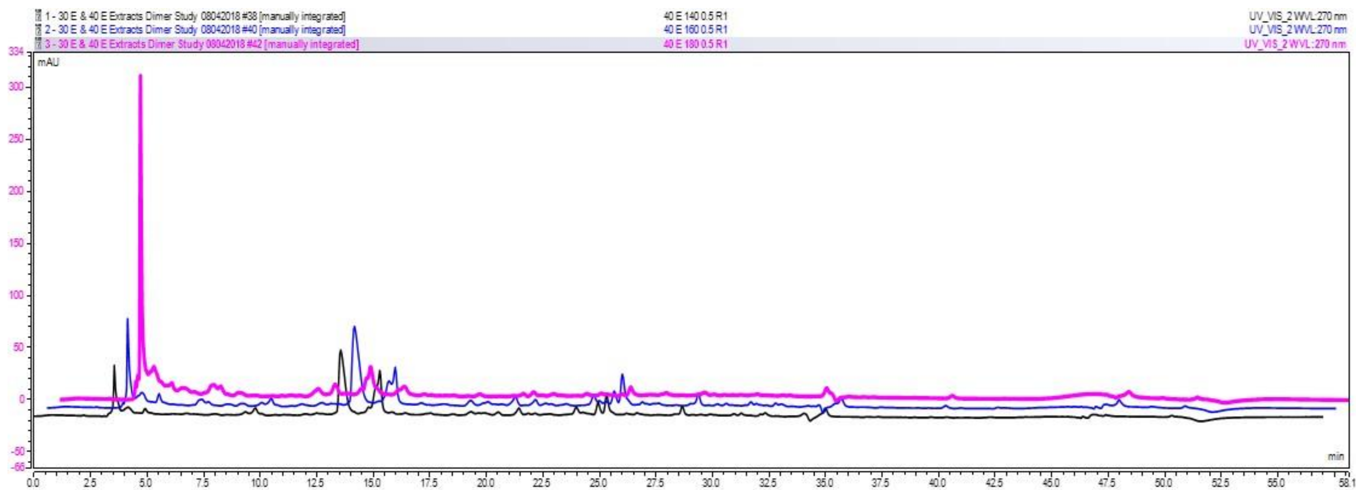


B - HPLC chromatogram of 30% (v/v) ethanol extracts at 140ºC (black), 160ºC (blue) and 180ºC (pink)

Appendix B4.2: 40% (v/v) ethanol extracts at different processing temperatures

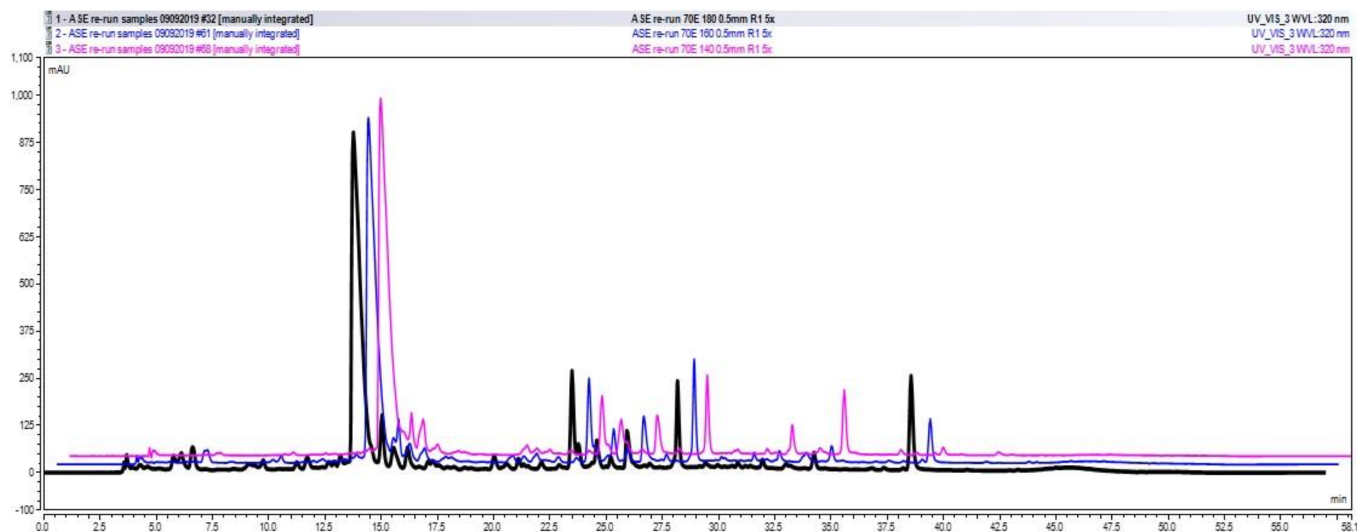


A - HPLC chromatogram of 40% (v/v) ethanol extracts at 140ºC (black), 160ºC (blue) and 180ºC (pink)

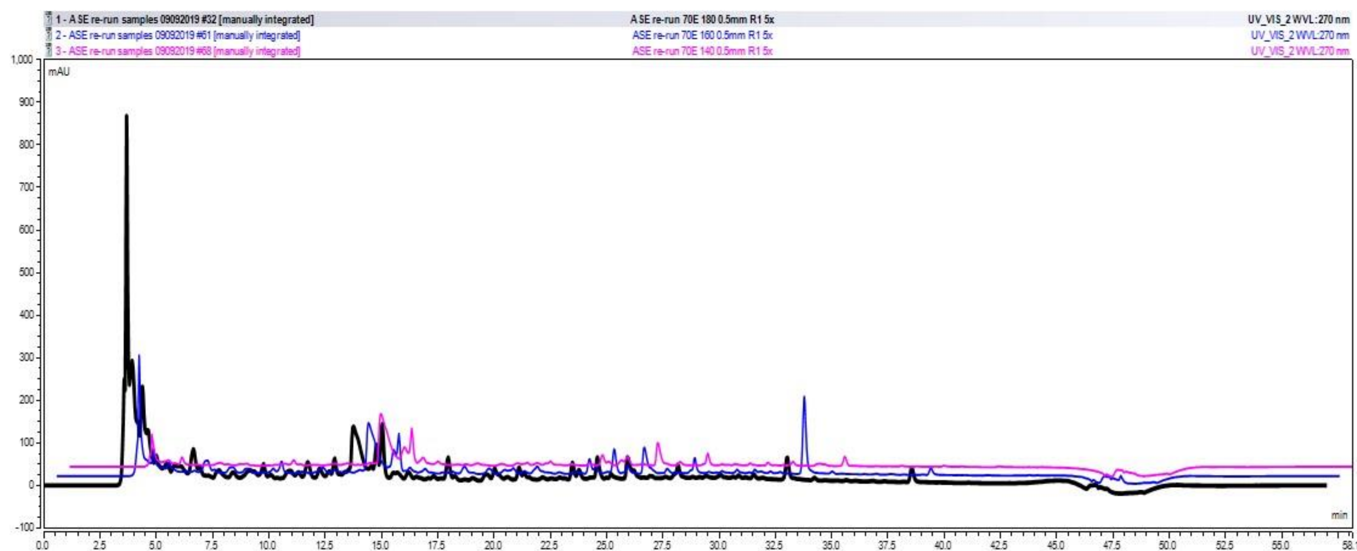


B - HPLC chromatogram of 40% (v/v) ethanol extracts at 140ºC (black), 160ºC (blue) and 180ºC (pink)

Appendix B4.3: 70% (v/v) ethanol extracts at different processing temperatures



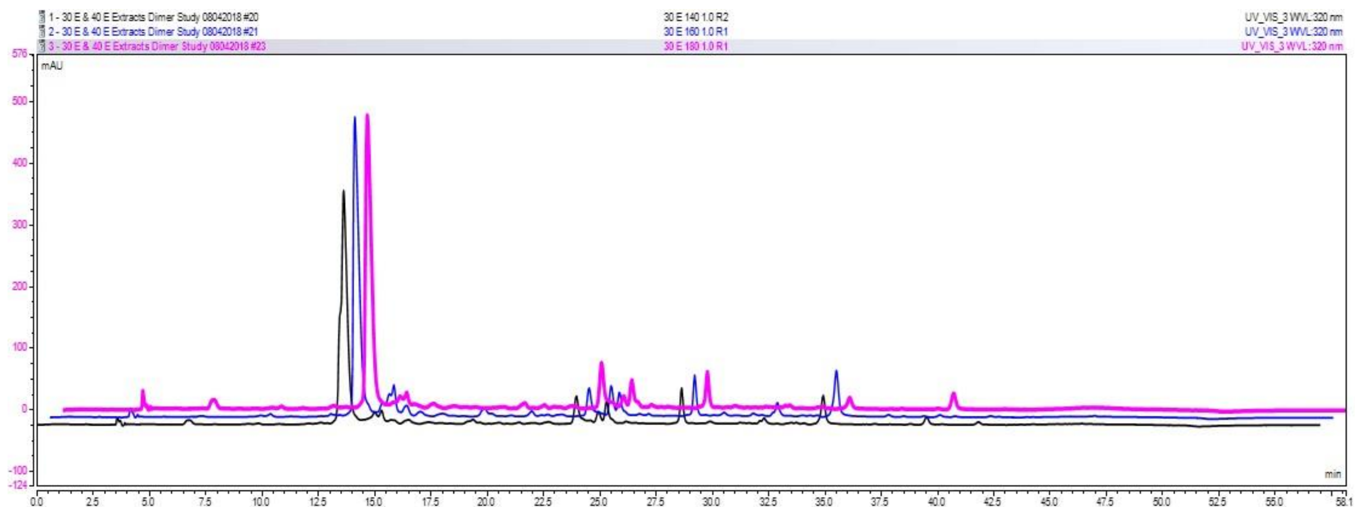
A - HPLC chromatogram of 70% (v/v) ethanol extracts at 140ºC (pink), 160ºC (blue) and 180ºC (black)



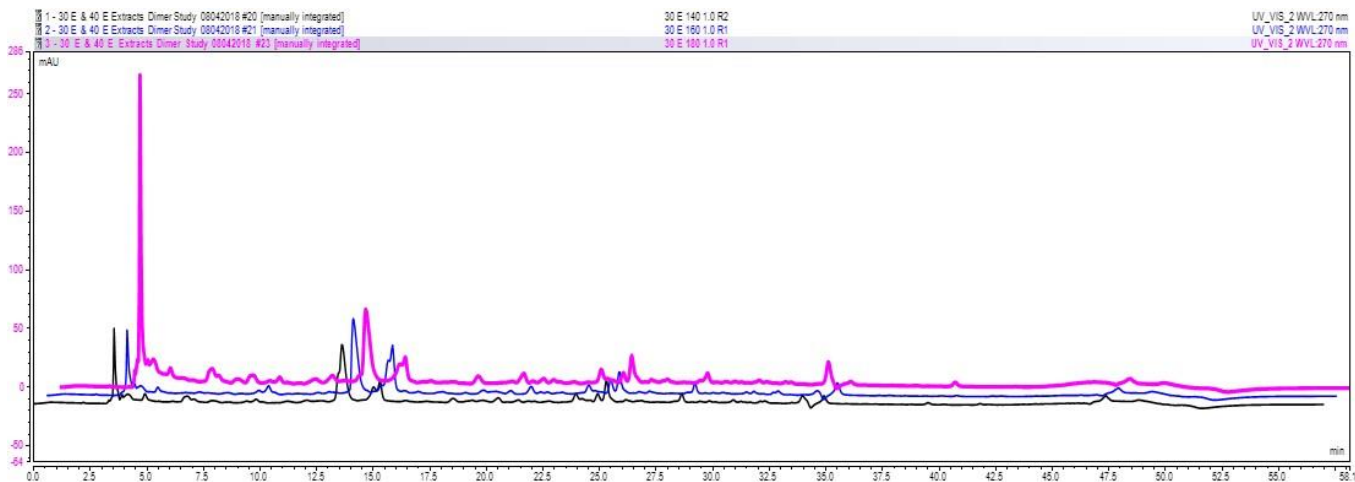
A - HPLC chromatogram of 70% (v/v) ethanol extracts at 140ºC (pink), 160ºC (blue) and 180ºC (black)

Appendix B5: HPLC chromatogram of ethanol extracts (A-320 nm, B-270 nm) for 1.0 mm particle size

Appendix B5.1: 30% (v/v) ethanol extracts at different processing temperatures

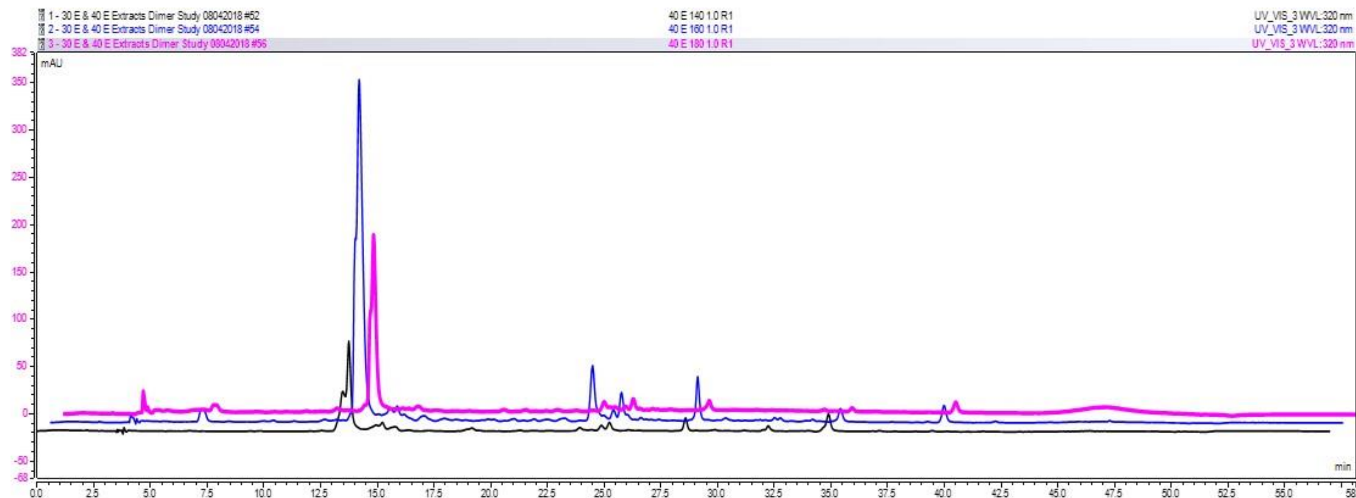


A - HPLC chromatogram of 30% (v/v) ethanol extracts at 140ºC (black), 160ºC (blue) and 180ºC (pink)

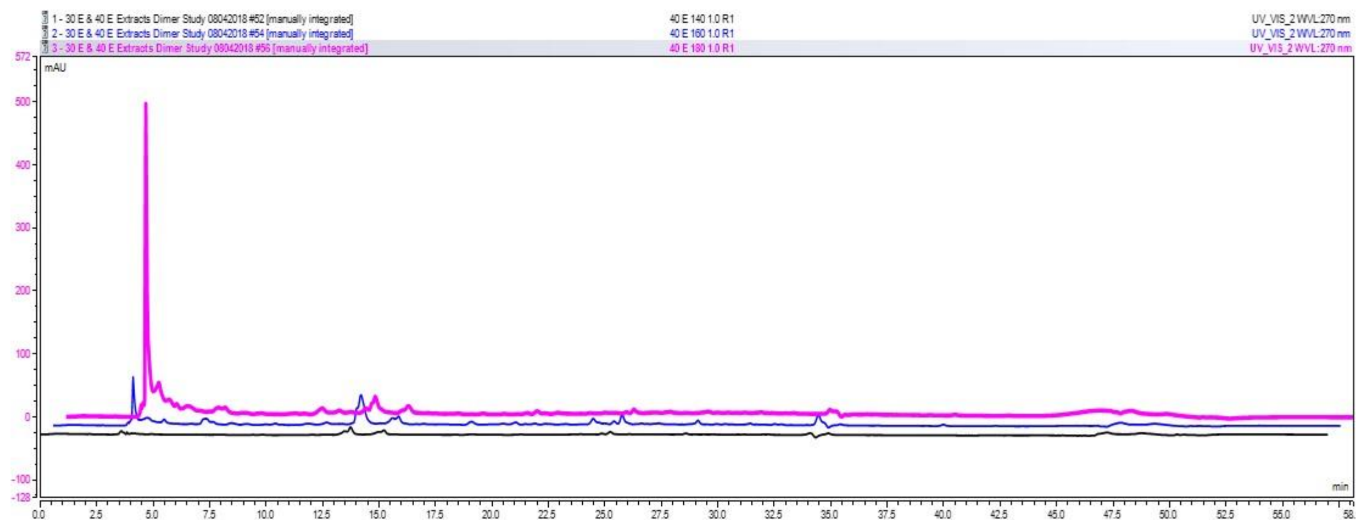


B - HPLC chromatogram of 30% (v/v) ethanol extracts at 140ºC (black), 160ºC (blue) and 180ºC (pink)

Appendix B5.2: 40% (v/v) ethanol extracts at different processing temperatures

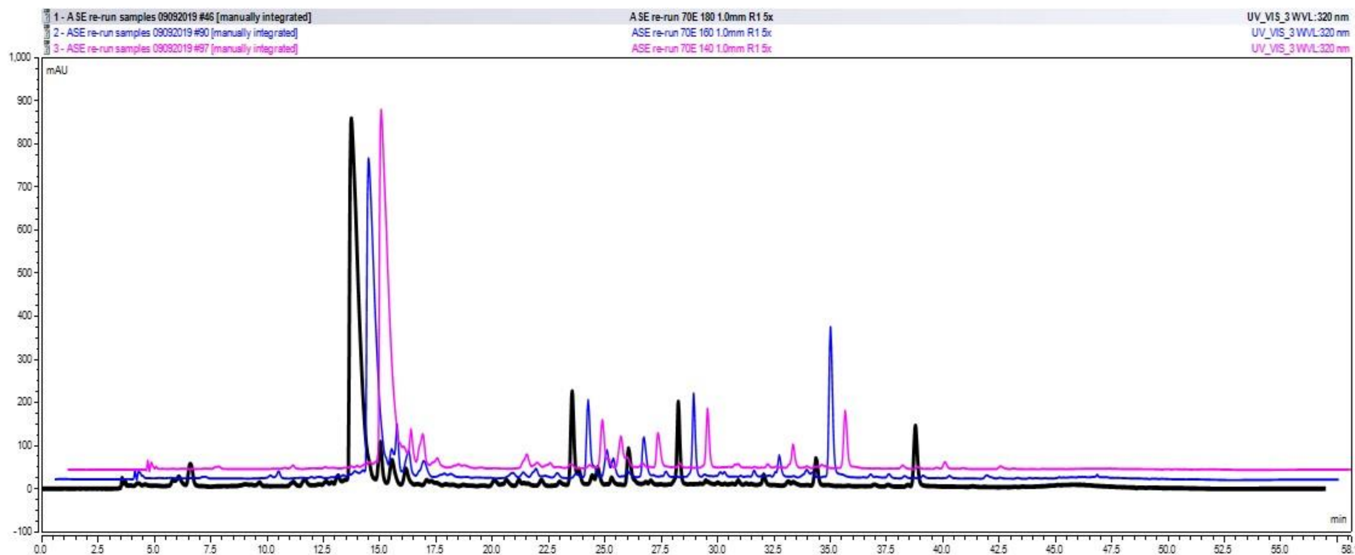


A - HPLC chromatogram of 40% (v/v) ethanol extracts at 140ºC (black), 160ºC (blue) and 180ºC (pink)

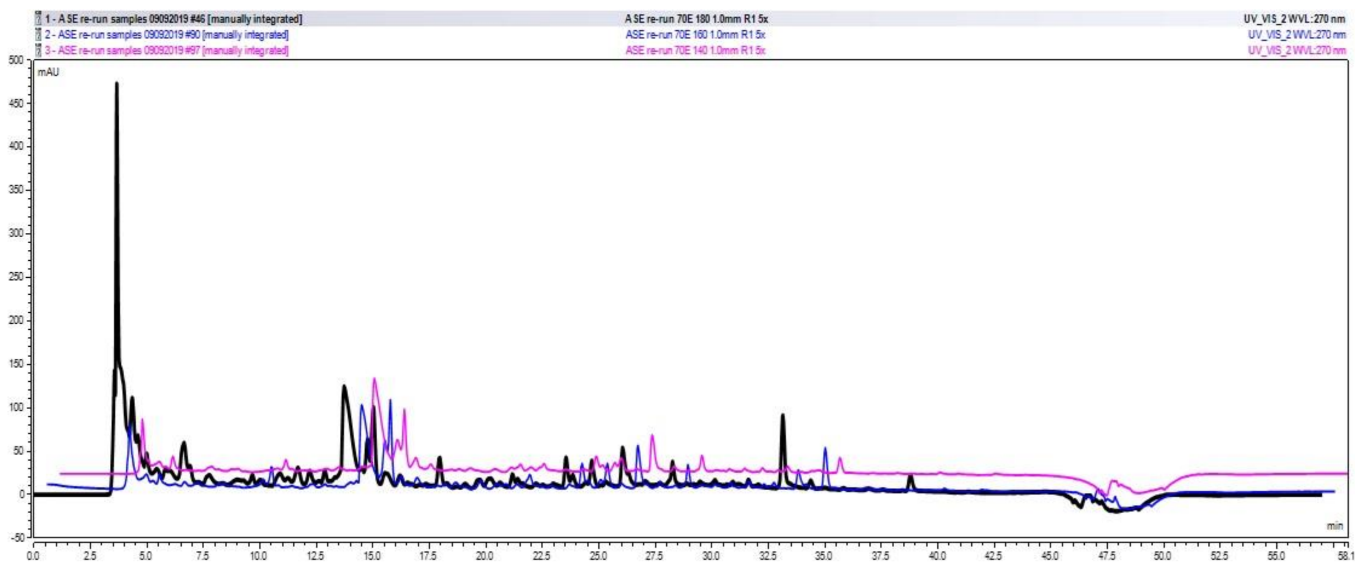


B - HPLC chromatogram of 40% (v/v) ethanol extracts at 140ºC (black), 160ºC (blue) and 180ºC (pink)

Appendix B5.3: 70% (v/v) ethanol extracts at different processing temperatures



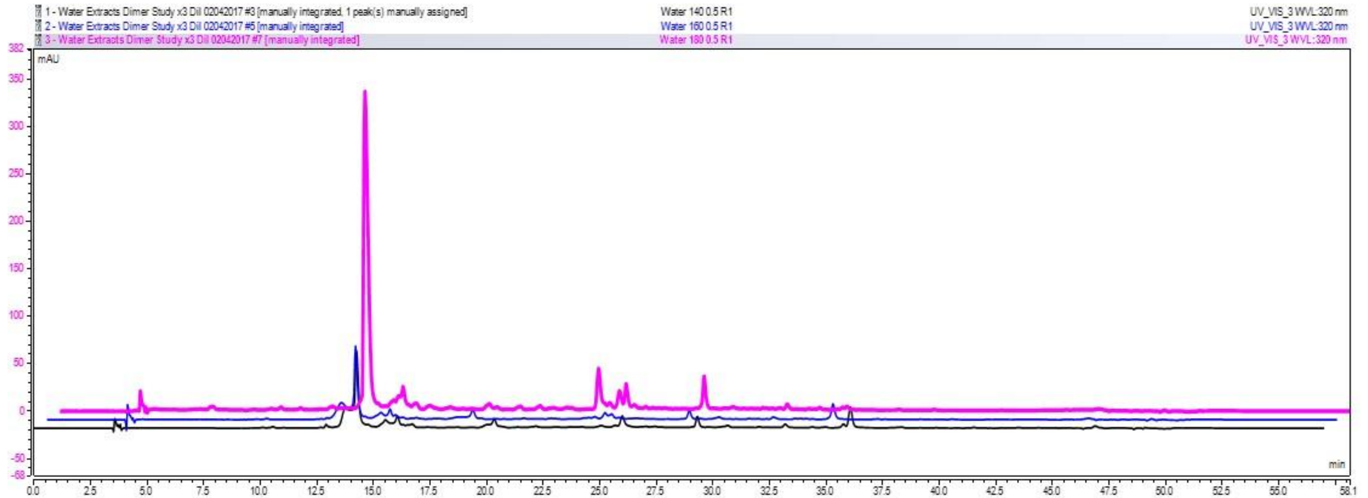
A - HPLC chromatogram of 70% (v/v) ethanol extracts at 140ºC (pink), 160ºC (blue) and 180ºC (black)



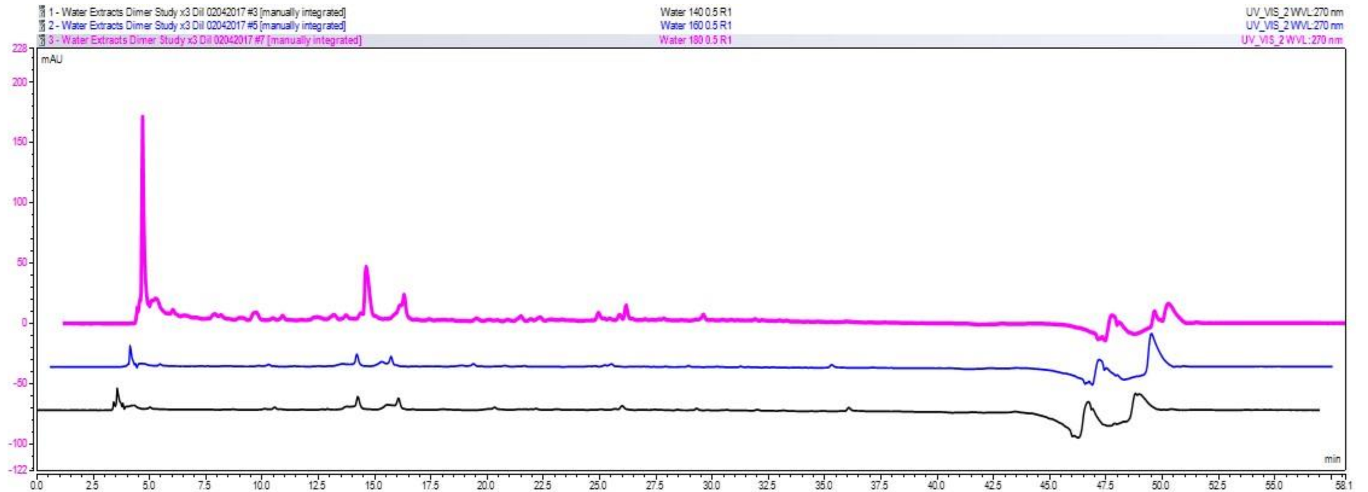
B - HPLC chromatogram of 70% (v/v) ethanol extracts at 140ºC (pink), 160ºC (blue) and 180ºC (black)

Appendix B6: HPLC chromatogram of water extracts (A-320 nm, B-270 nm) for 0.5 mm and 1.0 mm particle size

Appendix B6.1: water extracts at different processing temperatures (0.5 mm)

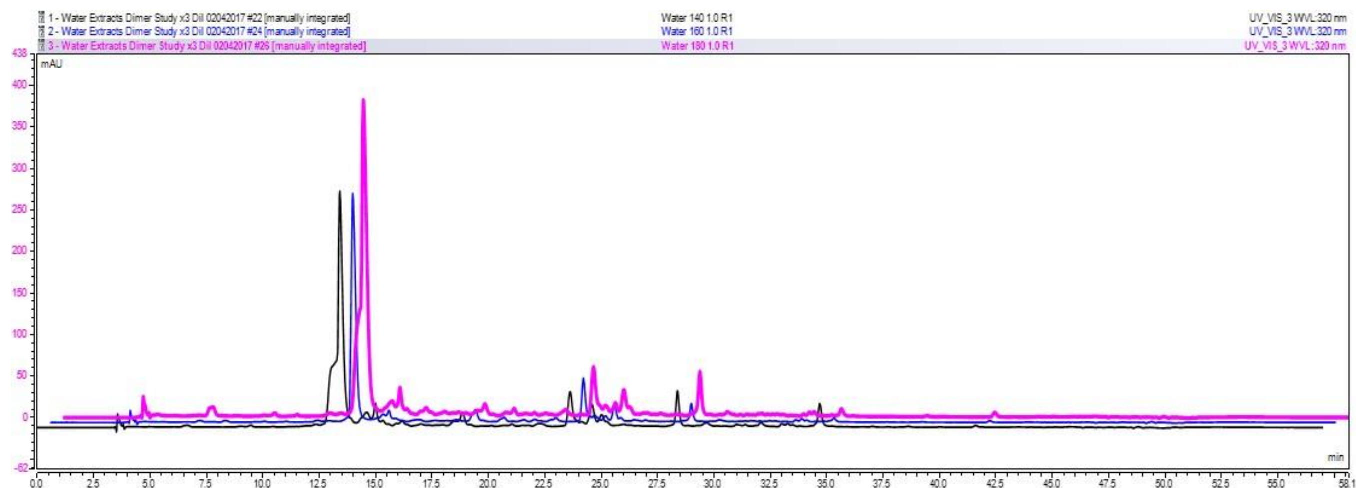


A - HPLC chromatogram of water extracts at 140ºC (pink), 160ºC (blue) and 180ºC (black)

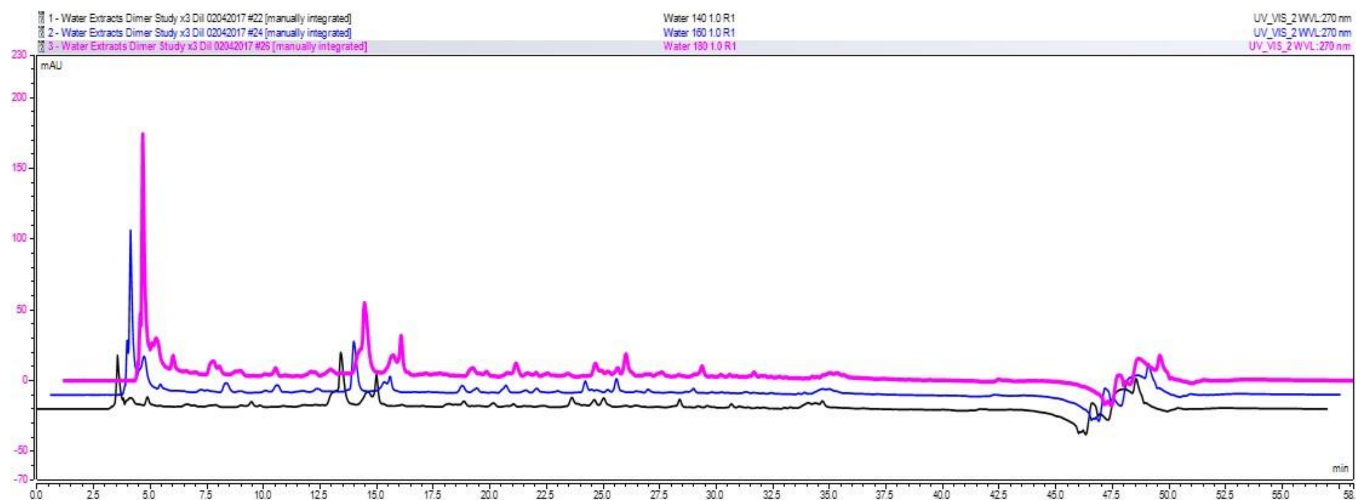


B - HPLC chromatogram of water extracts at 140ºC (pink), 160ºC (blue) and 180ºC (black)

Appendix B6.2: water extracts at different processing temperatures (1.0 mm)



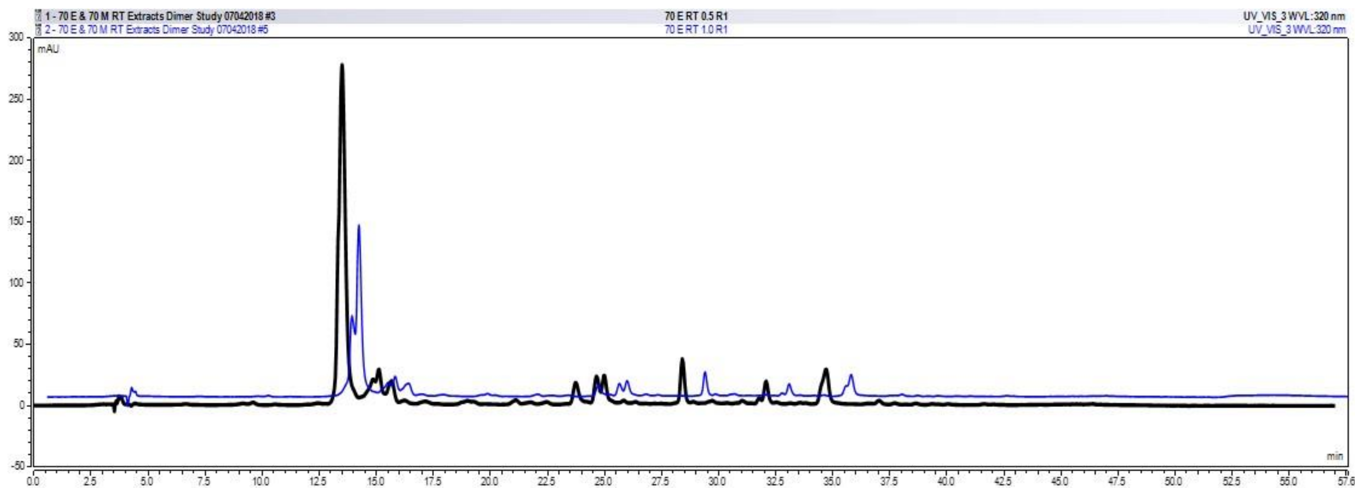
A - HPLC chromatogram of water extracts at 140ºC (pink), 160ºC (blue) and 180ºC (black)



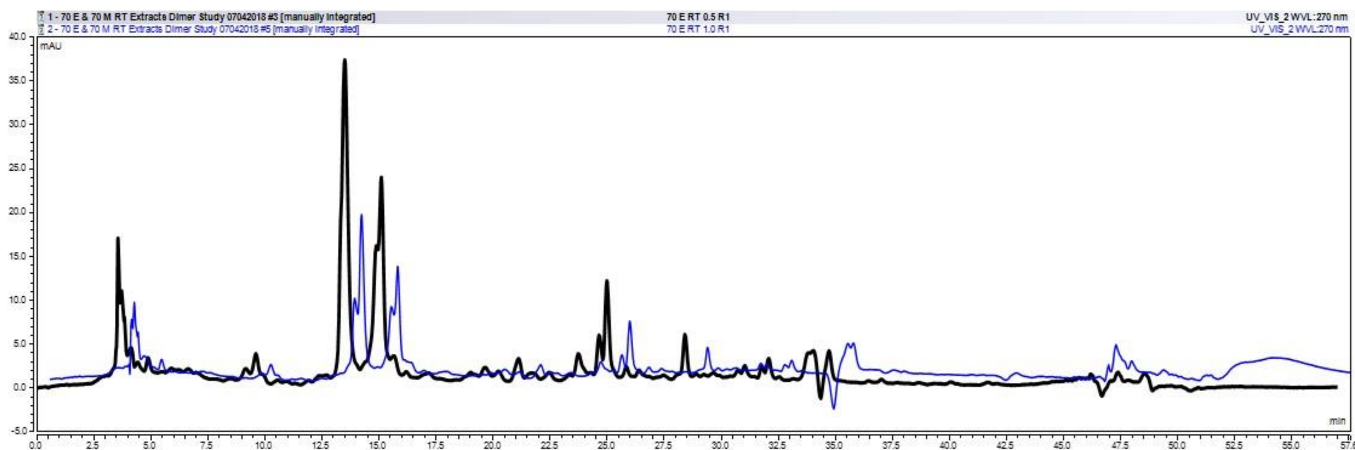
B - HPLC chromatogram of water extracts at 140ºC (pink), 160ºC (blue) and 180ºC (black)

Appendix B7: HPLC chromatogram of room temperature extracts (A-320 nm, B-270 nm) of 70% (v/v) methanol and ethanol extracts for 0.5 mm and 1.0 mm particle size

Appendix B7.1: 70% (v/v) ethanol extracts for room temperature extracts

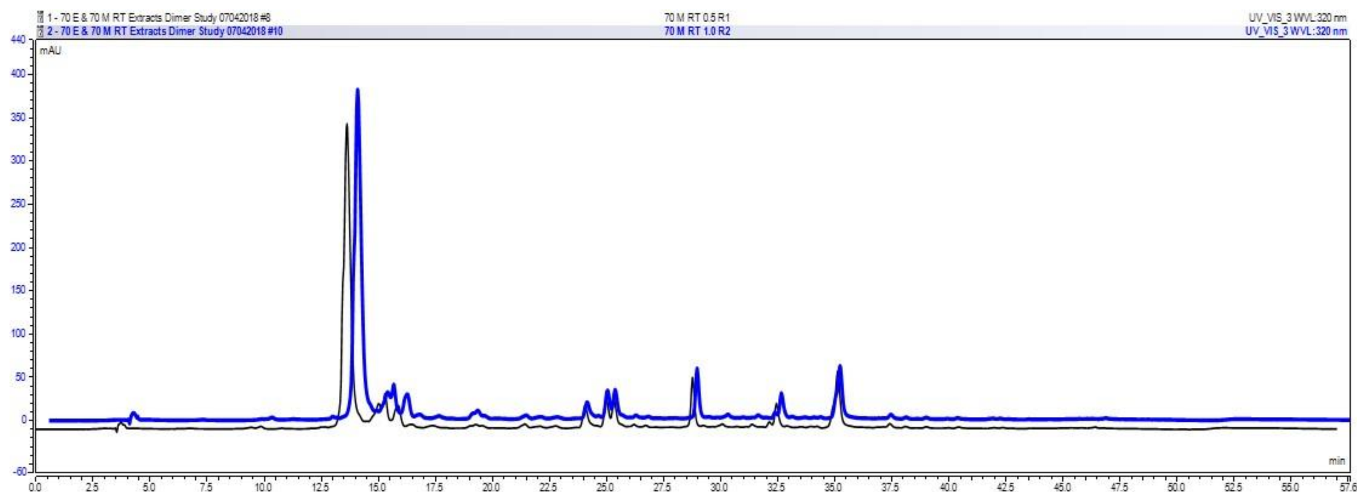


A - HPLC chromatogram of room temperature 70% ethanol extracts 0.5 mm (black) 1.0 (blue)

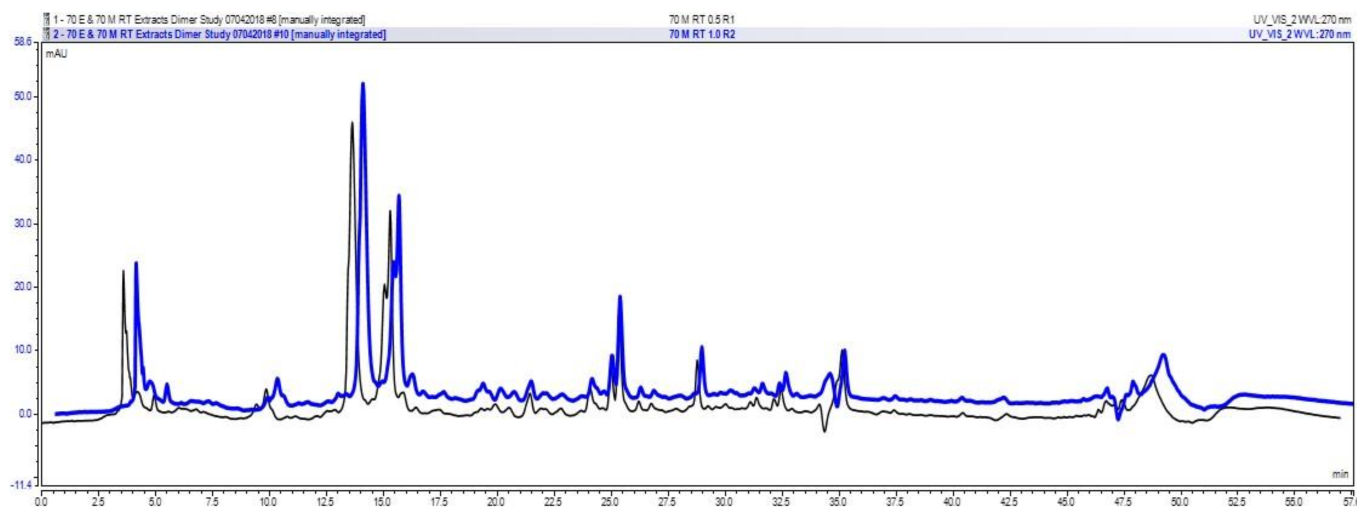


B - HPLC chromatogram of room temperature 70% ethanol extracts 0.5 mm (black) 1.0 (blue)

Appendix B7.2: 70% (v/v) methanol extracts for room temperature extracts



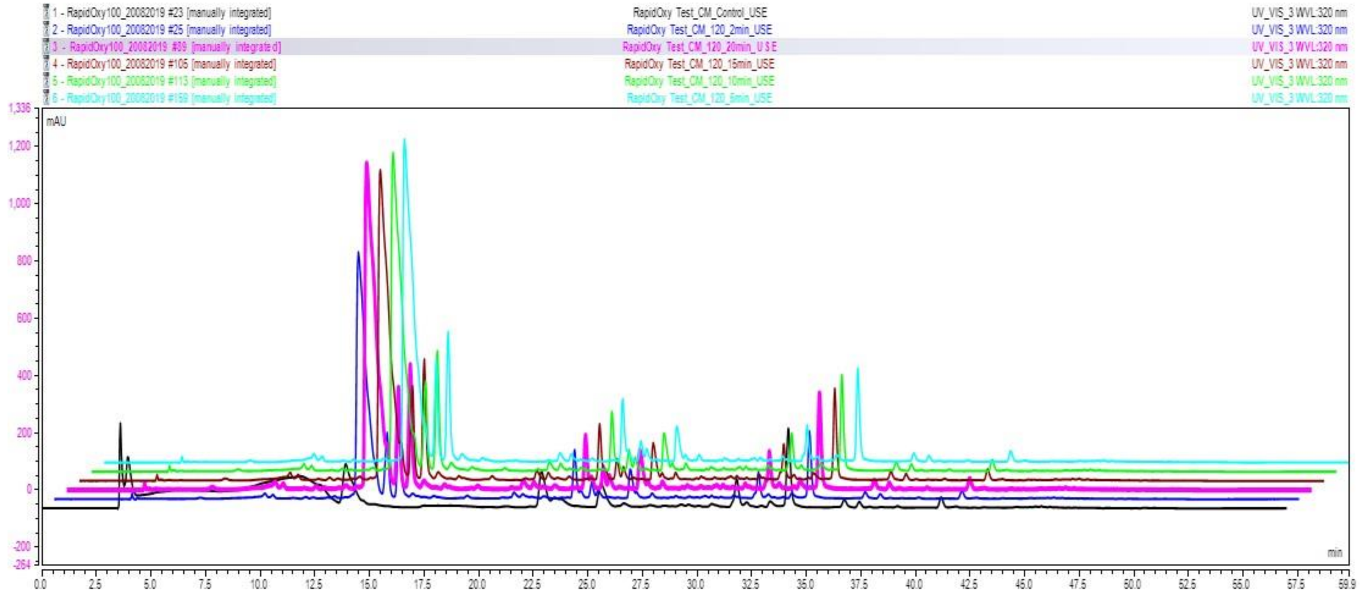
A - HPLC chromatogram of room temperature 70% methanol extracts 0.5 mm (black) 1.0 (blue)



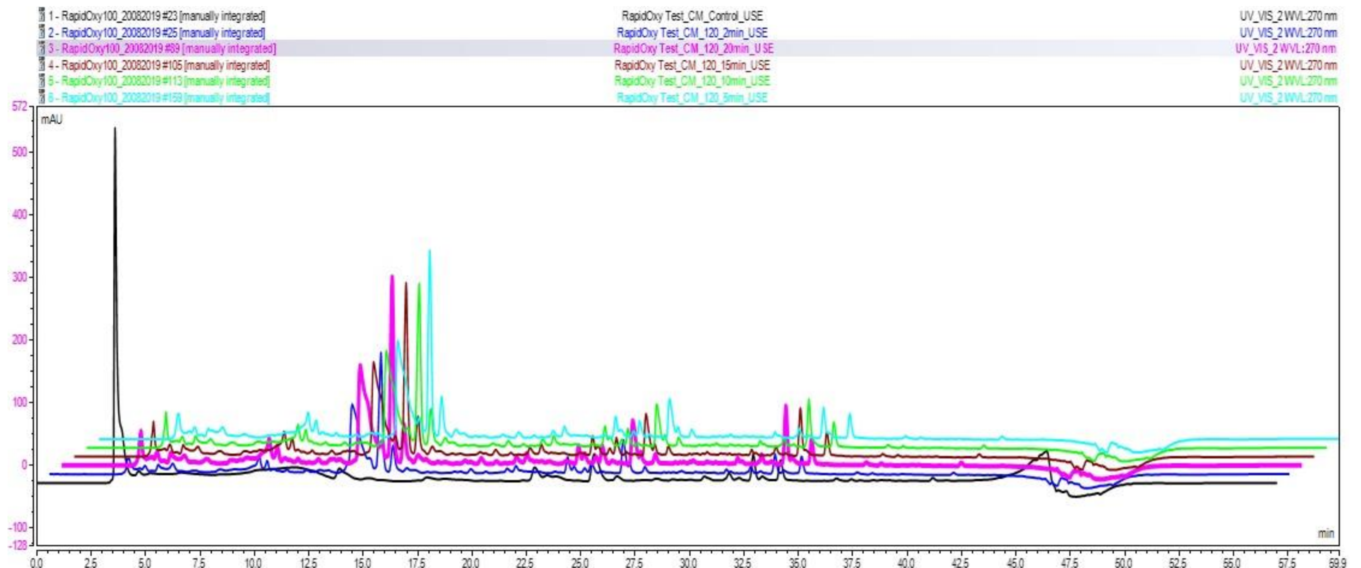
B - HPLC chromatogram of room temperature 70% methanol extracts 0.5 mm (black) 1.0 (blue)

Appendix C: HPLC chromatograms for RapidOxy® 100 extracts

Appendix C1: HPLC chromatograms of ultrasound assisted extracts at 120°C for 2, 5, 10, 15- and 20-minutes pre-treatment (A-320 nm, B-270 nm)

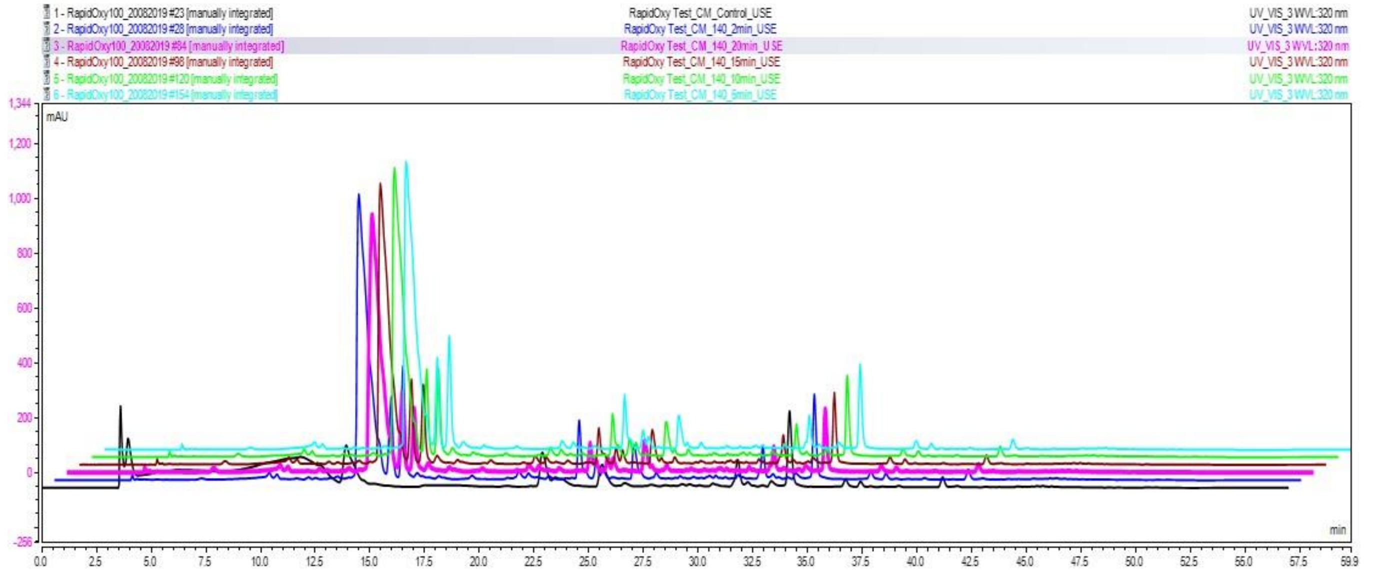


A - HPLC chromatogram of ultrasound assisted extracts at 120°C at 2 min (blue), 5 min (light blue), 10 min (green), 15 min (brown), 20 min (pink), and control 37°C (black)

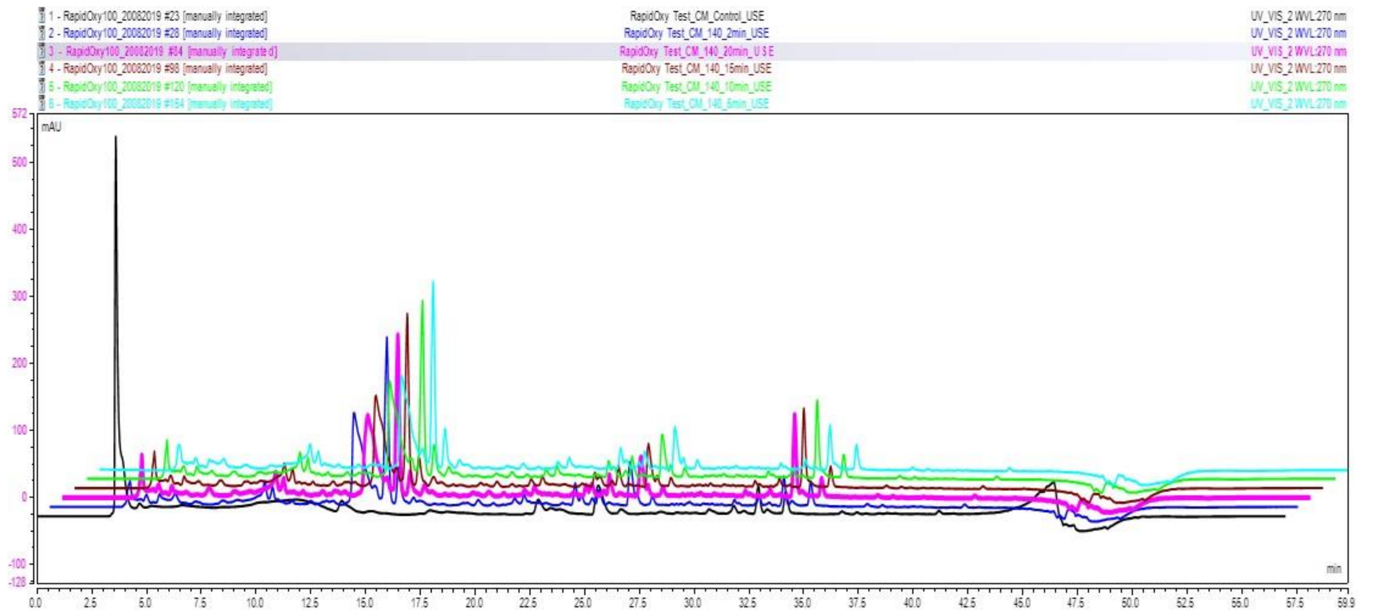


B - HPLC chromatogram of ultrasound assisted extracts at 120°C at 2 min (blue), 5 min (light blue), 10 min (green), 15 min (brown), 20 min (pink), and control 37°C (black)

Appendix C2: HPLC chromatograms of ultrasound assisted extracts at 140°C for 2, 5, 10, 15- and 20-minutes pre-treatment (A-320 nm, B-270 nm)

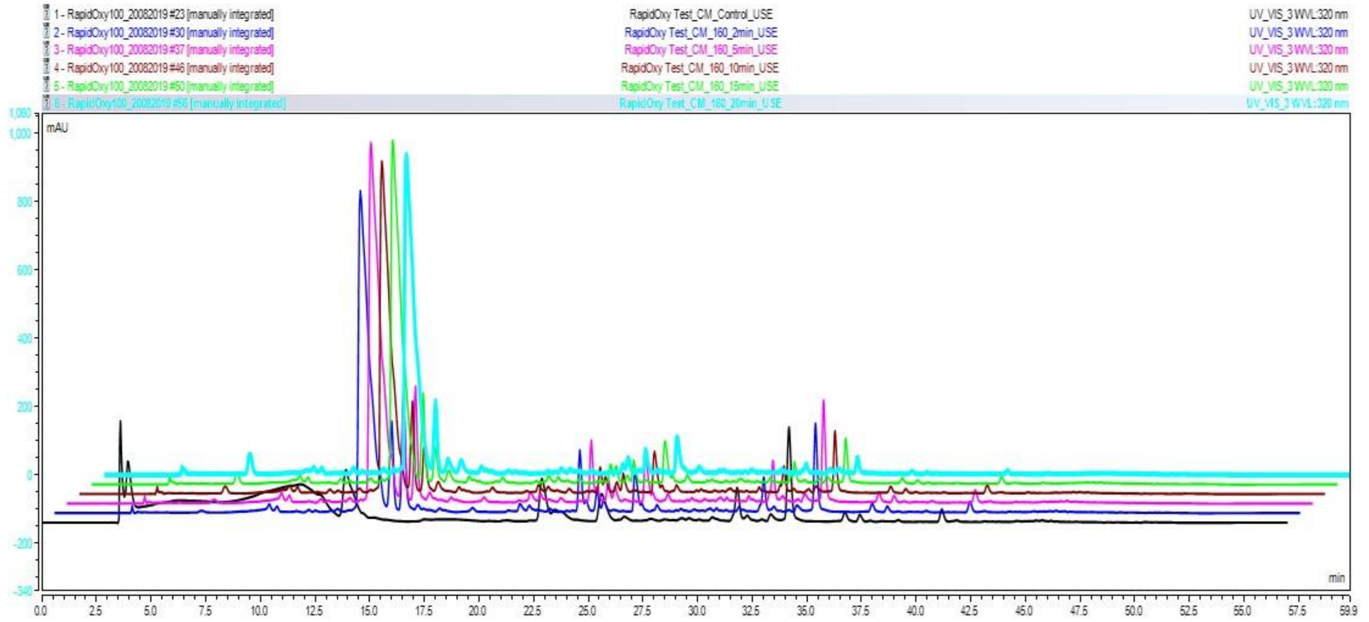


A - HPLC chromatogram of ultrasound assisted extracts at 140°C at 2 min (blue), 5 min (light blue), 10 min (green), 15 min (brown), 20 min (pink), and control 37°C (black)

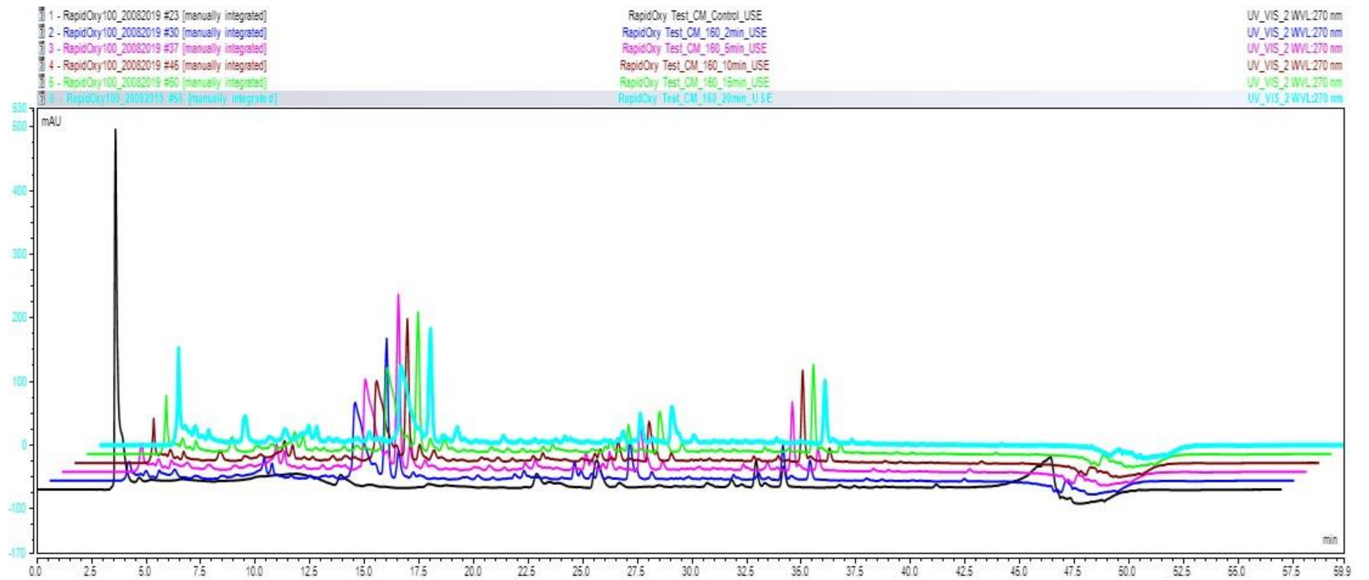


B - HPLC chromatogram of ultrasound assisted extracts at 140°C 2 min (blue), 5 min (light blue), 10 min (green), 15 min (brown), 20 min (pink), and control 37°C (black)

Appendix C3: HPLC chromatograms of ultrasound assisted extracts at 160°C for 2, 5, 10, 15- and 20-minutes pre-treatment (A-320 nm, B-270 nm)

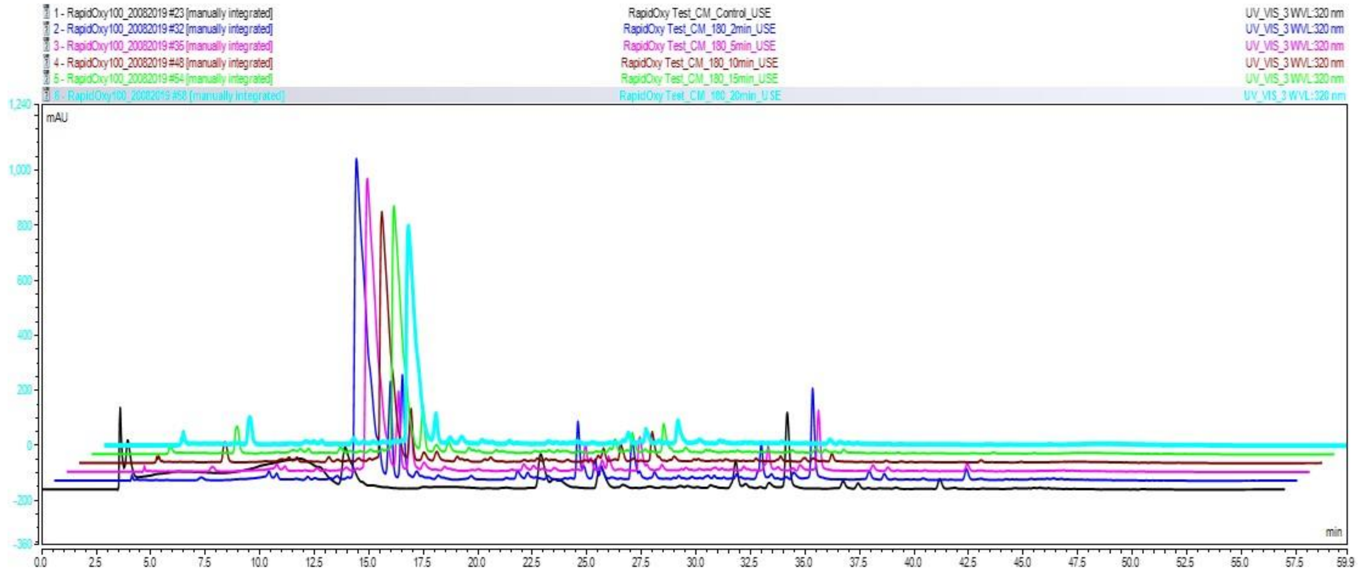


A - HPLC chromatogram of ultrasound assisted extracts at 160°C at 2 min (blue), 5 min (light blue), 10 min (green), 15 min (brown), 20 min (pink), and control 37°C (black)

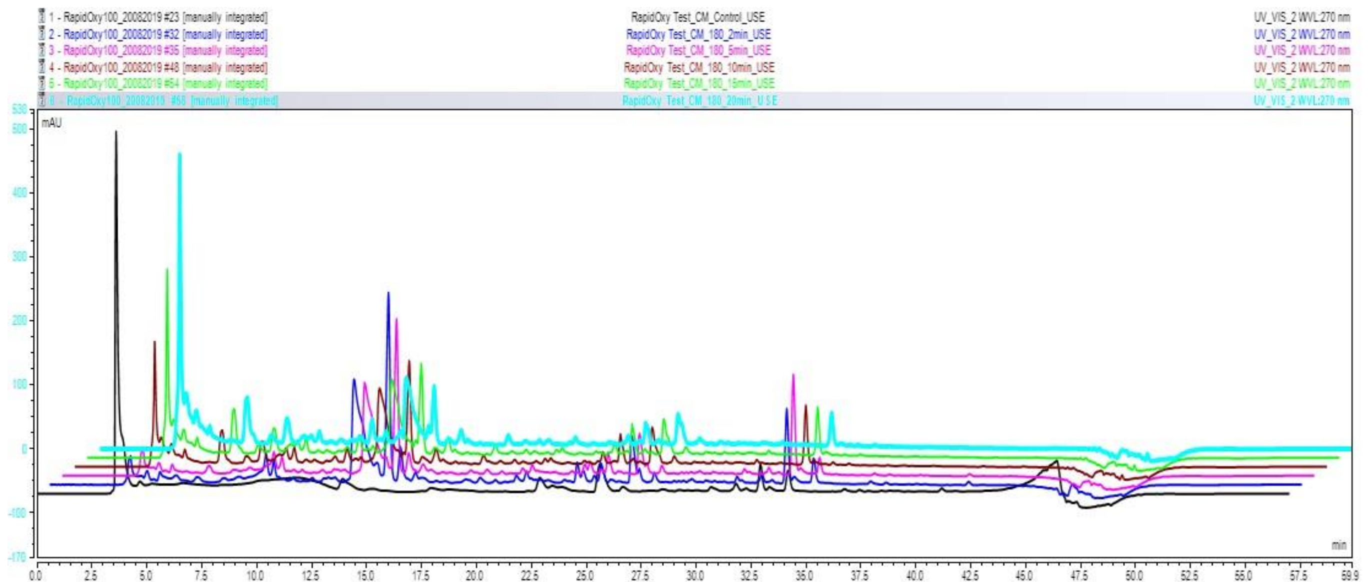


B - HPLC chromatogram of ultrasound assisted extracts at 160°C at 2 min (blue), 5 min (light blue), 10 min (green), 15 min (brown), 20 min (pink), and control 37°C (black)

Appendix C4: HPLC chromatograms of ultrasound assisted extracts at 180°C for 2, 5, 10, 15- and 20-minutes pre-treatment (A-320 nm, B-270 nm)



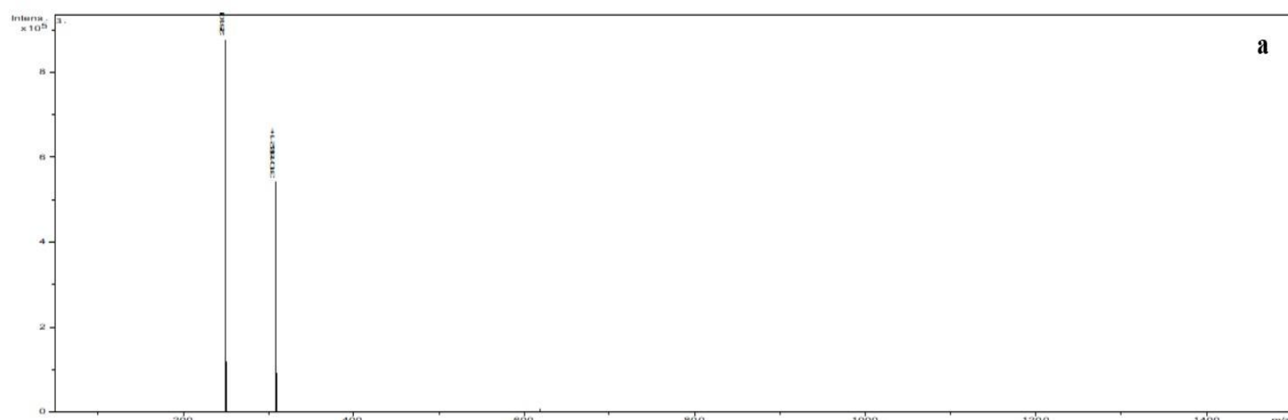
A - HPLC chromatogram of ultrasound assisted extracts at 180°C at 2 min (blue), 5 min (light blue), 10 min (green), 15 min (brown), 20 min (pink), and control 37°C (black)

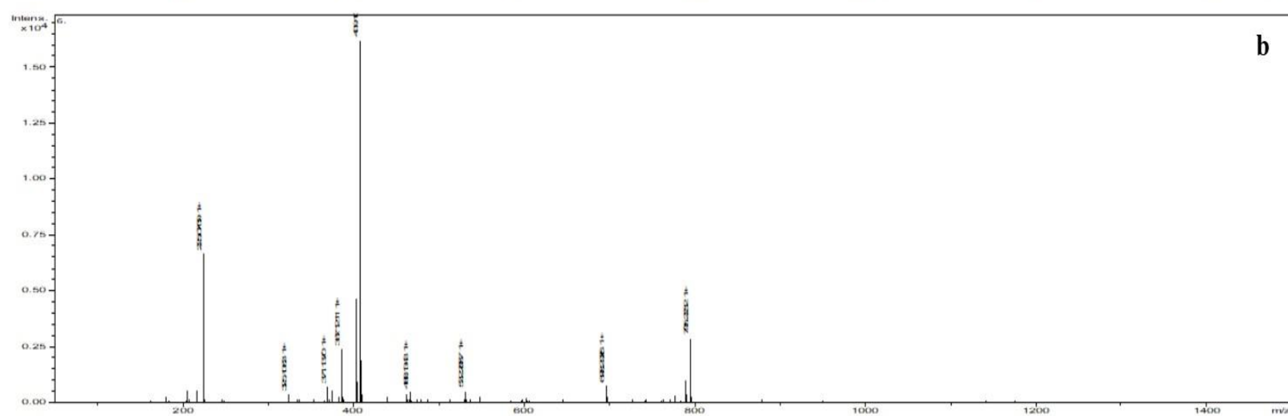


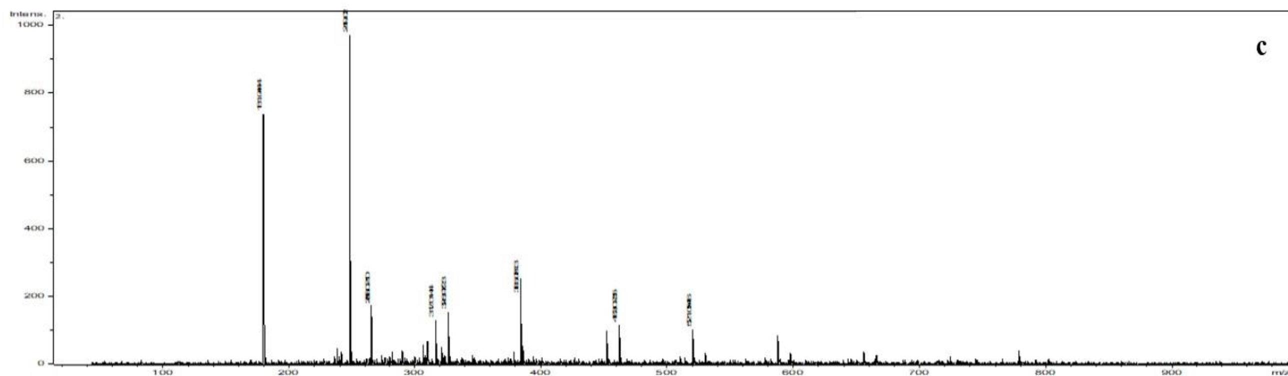
B - HPLC chromatogram of ultrasound assisted extracts at 180°C at 2 min (blue), 5 min (light blue), 10 min (green), 15 min (brown), 20 min (pink), and control 37°C (black)

Appendix D: LC-MS/MS fragmentation patterns for standard compounds

Appendix D1: Positive electrospray ionization for sinapine (a), sinapic acid (b), canolol (c)

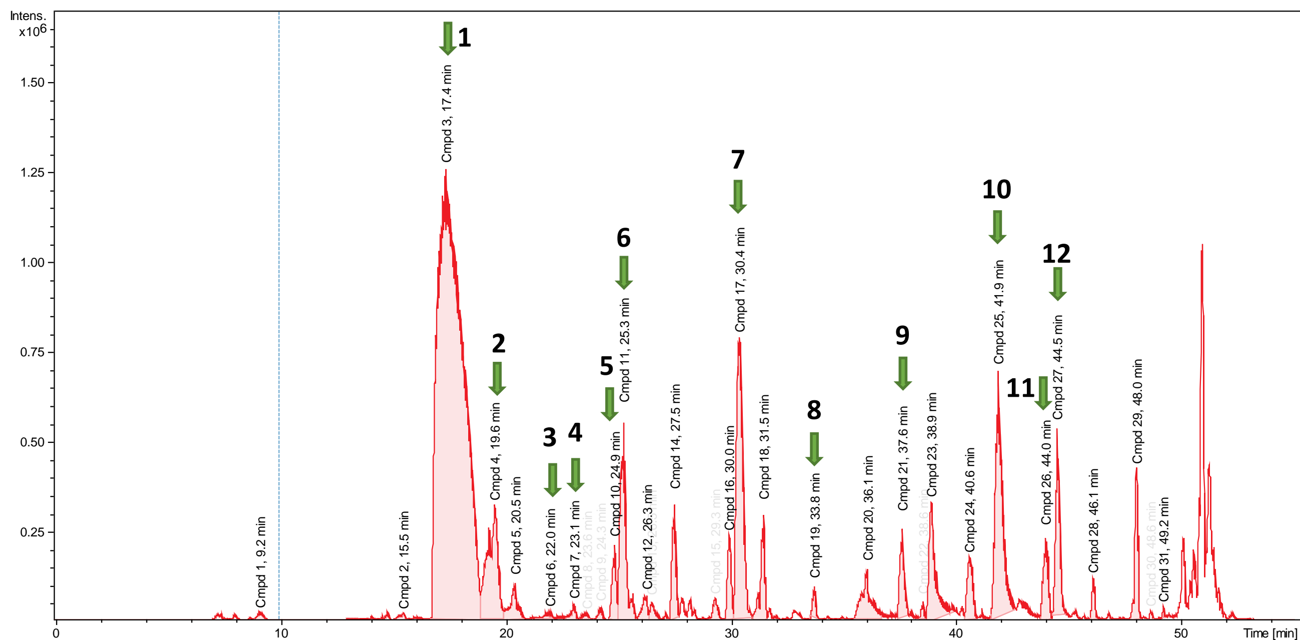






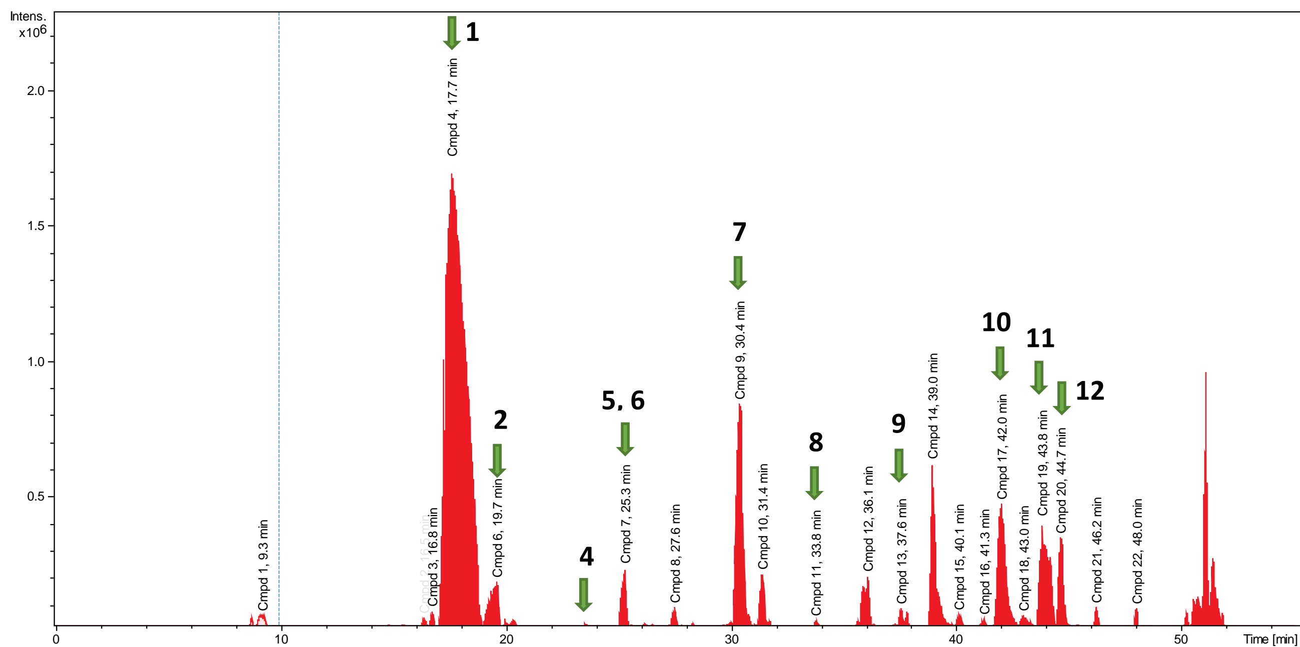
Appendix D2: LC-MS analysis of canola meal extracts

Appendix D2.1: Canola meal extracts treated at 160°C, 1.0 mm ASE



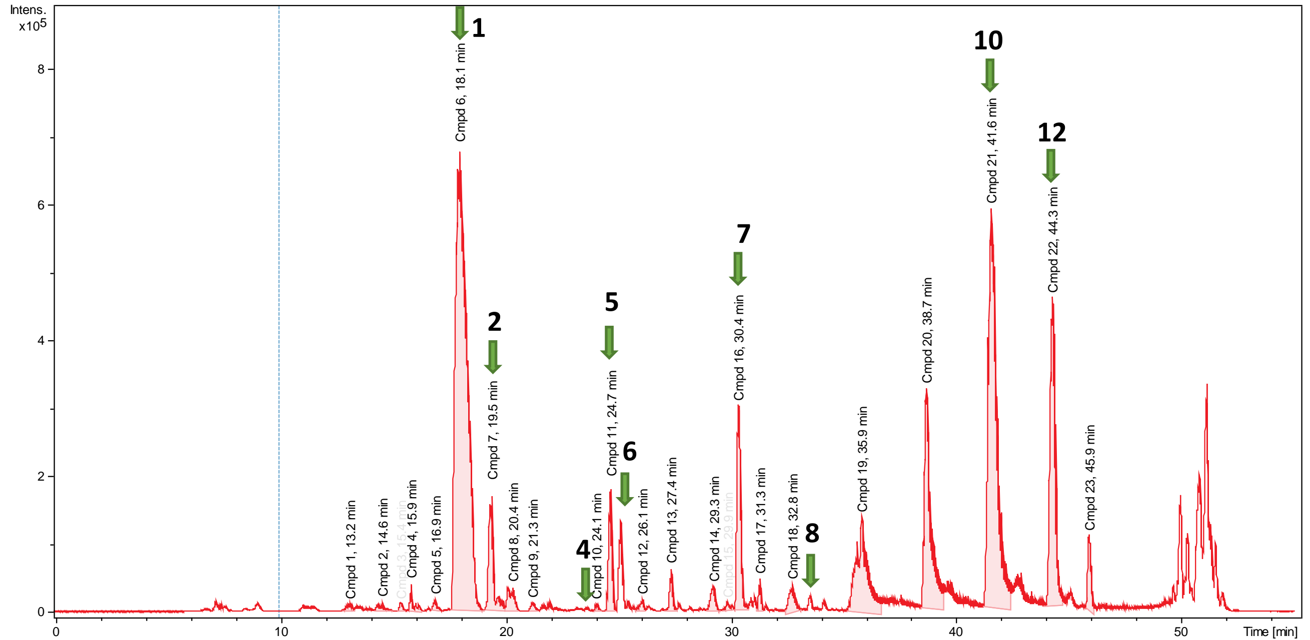
Compound #	RT (min)
1 - Sinapine	17.4
2 - Syringic Acid	19.6
3 - Sinapoyl Alcohol	22.0
4 - Sinapic Acid	23.1
5 - Kaempferol 3-O-(2”-O-Sinapoyl-ß-sophoroside)	24.9
6 - Thomasidioic Acid	25.3
7 - Kaempferol 3-O- ß-sophoroside	30.4
8 - Canolol	33.8
9 - Kaempferol 3-(2''-hydroxypropionylglucoside)-4'-glucoside	37.6
10 - Kaempferol 3-[2''-glucosyl-6''-acetyl-galactoside] 7-glucoside	41.9
11 - Methyl Sinapate	44.0
12 - Sinapic Acid Dimer	44.5

Appendix D2.2: Canola meal 70% (v/v) methanol extracts treated at 180°C, 1.0 mm ASE



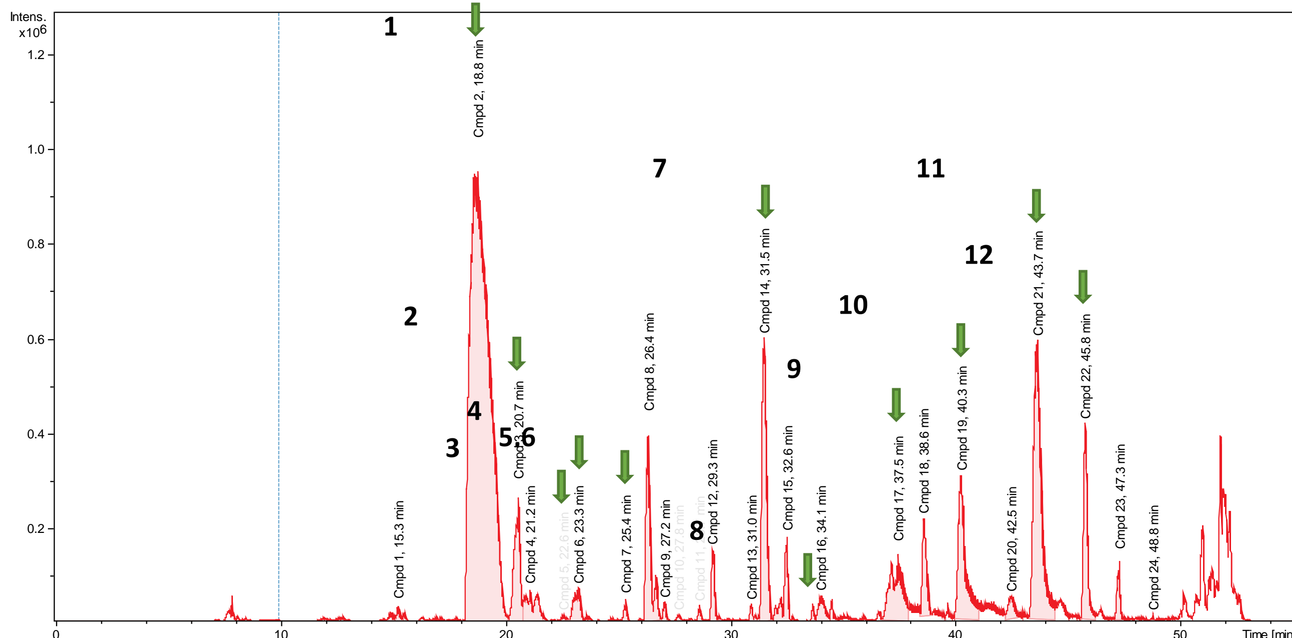
Compound #	RT (min)
1 - Sinapine	17.4
2 - Syringic Acid	19.6
4 - Sinapic Acid	23.1
5 - Kaempferol 3-O-(2”-O-Sinapoyl-ß-sophoroside)	24.9
6 - Thomasidioic Acid	25.3
7 - Kaempferol 3-O- ß-sophoroside	30.4
8 - Canolol	33.8
9 - Kaempferol 3-(2''-hydroxypropionylglucoside)-4'-glucoside	37.6
10 - Kaempferol 3-[2''-glucosyl-6''-acetyl-galactoside] 7-glucoside	41.9
11 - Methyl Sinapate	44.0
12 - Sinapic Acid Dimer	44.5

Appendix D2.3: Canola meal water extracts treated at 180°C, 0.5 mm ASE



Compound #	RT (min)
1 - Sinapine	17.4
2 - Syringic Acid	19.6
4 - Sinapic Acid	23.1
5 - Kaempferol 3-O-(2”-O-Sinapoyl-ß-sophoroside)	24.9
6 - Thomasidioic Acid	25.3
7 - Kaempferol 3-O- ß-sophoroside	30.4
8 - Canolol	33.8
10 - Kaempferol 3-[2''-glucosyl-6''-acetyl-galactoside] 7-glucoside	41.9
12 - Sinapic Acid Dimer	44.5

Appendix D2.4: Canola meal 70% (v/v) methanol extracts at room temperature, 0.5 mm ASE



Compound #	RT (min)
1 - Sinapine	17.4
2 - Syringic Acid	19.6
3 - Sinapoyl Alcohol	22.0
4 - Sinapic Acid	23.1
5 - Kaempferol 3-O-(2”-O-Sinapoyl-ß-sophoroside)	24.9
6 - Thomasidioic Acid	25.3
7 - Kaempferol 3-O- ß-sophoroside	30.4
8 - Canolol	33.8
9 - Kaempferol 3-(2''-hydroxypropionylglucoside)-4'-glucoside	37.6
10 - Kaempferol 3-[2''-glucosyl-6''-acetyl-galactoside] 7-glucoside	41.9
11 - Methyl Sinapate	44.0
12 - Sinapic Acid Dimer	44.5

Appendix E: Identification of major phenolic compounds present in canola meal extracts using LC-MS/MS analysis

Compound	RT (min)	Mol formula	monoisotopic neutral mass	theoretical calculated charged masses		[M-H]-	[M+H]$^+$	[M+Na]$^+$			
				1.0073 [M+H]$^+$	22.9892 [M+Na]$^+$						
Protocatechuic acid		C7H6O4	154.0261	155.0333	177.0153						
p-coumaric acid		C9H8O3	164.0468	165.0541	187.0360						
Syringaldehyde		C9H10O4	182.0574	183.0646	205.0466						
Syringic acid	19.6	C9H10O5	198.0523	199.0596	221.0415	-121956.92					
Sinapoyl alcohol	23.1	C11H14O4	210.0887	211.0959	233.0779						
Sinapic acid	22	C11H12O5	224.0679	225.0752	247.0571						
Methyl sinapate	44	C12H14O5	238.0836	239.0909	261.0728						
UC#7 (phenolic choline ester)		C15H26NO4+	284.1862	285.1935	307.1754						
Kaempferol	-	C15H10O6	286.0472	287.0545	309.0364						
quercetin		C15H10O7	302.0421	303.0494	325.0313						
Sinapine	17.4	C16H24NO5+	310.1649	311.1722	333.1541	1000000.00	49.0886	-6.5435	Frac 16 (-7) 333.1563		
1-O-feruloyl-β-D-glucose (Ferulic acid 4-O-hexoside)		C16H20O9	356.1102	357.1175	379.0994						
Syringate 4-O-hexoside		C15H20O10	360.1051	361.1124	383.0943						
1-o-Sinapoylglucose (Sinapate 4-O-glucoside)		C17H22O10	386.1207	387.1280	409.1100						
Thomosidiasic acid	25.3	C22H22O10	446.1213	447.1286	469.1105	1000000.00	3.5180	58.8731	Frac 26 (4) 447.1270		
SyC 4-O-hexoside		C20H32NO10+	446.2000	447.2073	469.1892						
kaempferol-3-O-D-glucoside		C21H19O11-	447.0927	448.1000	470.0820	-171.36	5.8291	-261.3225	Frac 19 (6) 448.0974		
UC#2 (choline ester)		C25H31N2O6+	455.2200	456.2273	478.2092	17724.79	3.4544	42.0757	Frac 20 (5) 456.2250	Frac 23 (-2) 456.2284	Frac 28 (3) 456.2257

Kaempferol 3-O- ß-sophoroside	29.3	$C_{27}H_{30}O_{16}$	610.1528	611.1601	633.1421	55.42	4.4375	2.3012	Frac 28 (4) 611.1574		
Kaempferol 3-(4",6"-diacetylglucoside)-7-rhamnoside		$C_{31}H_{34}O_{17}$	678.1796	679.1869	701.1688						
Kaempferol 3-(2"-hydroxypropionylglucoside)-4'-glucoside	37.6	$C_{30}H_{34}O_{18}$	682.1745	683.1818	705.1637	1000000.00	11.8417	-0.3758	Frac 45 (12) 683.1737		
SC(4-O-8′)S 4-O′-hexoside		$C_{33}H_{48}NO_{15}^{+}$	698.3018	699.3091	721.2911						
Kaempferol 3-sophorotrioside		$C_{33}H_{40}O_{21}$	772.2062	773.2135	795.1954	1000000.00	63.4805	105.9224	Frac 34 (63) 773.1644		
Kaempferol 3-[2"-glucosyl-6"-acetyl-galactoside] 7-glucoside	41.9	$C_{35}H_{42}O_{22}$	814.2162	815.2235	837.2054	1000000.00	-10.5493	-4.7240	Frac 34 (-13) 815.2345		
Kaempferol 3-O-(2”-O-Sinapoyl-ß-sophoroside)	26.5	$C_{38}H_{40}O_{20}$	816.2107	817.2180	839.2000	1000000.00	6.5111	-3.1387	Frac 25 (6) 817.2127		
Kaempferol-sinapoyl-trihexoside	26.5	$C_{44}H_{50}O_{25}$	978.2636	979.2708	1001.2528	1000000.00	4.9476	-0.0100	Frac 20 (-3) 1001.2556	Frac 21 (-2) 979.2728	Frac 38 (5) 979.2660