Practical Methods for Lignans Quantification

Simona Mihaela Nemes

Bioresource Engineering Department, McGill University,
Montreal, Canada
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Abstract

An optimized microwave-assisted extraction (MAE) method is proposed, in conjunction with high performance liquid chromatography (HPLC) analysis, for the general quantification of lignans in flaxseed materials and other plant foods. The method involves hydrolyzing 0.5 – 1.6 g samples with 50 ml of 0.5 M NaOH at 156 W (power level estimated using calorimetric calibration) for 3 min, using intermittent microwave power application (30 s on/off). The final temperature of the extracts is 67°C. The MAE method extracts the lignan from the plant matrix completely, accurately (97.5 % recovery), efficiently (yields 21.4 and 26.6 % higher than those obtained with reference methods), and precisely (coefficients of variation < 4.03 % for repeated determinations).

An enzymatic hydrolysis (EH) method, complementary to the MAE method, was developed for the general determination of lignan aglycones in plant samples. The EH method involves hydrolyzing microwave-assisted extracts containing 100 mg sample in 3 ml of sodium acetate buffer (0.01 M, pH 5), with crude solutions of β-glucuronidase using ≥ 40 U of enzyme/mg sample (depending on the hydrolysing capacity of various batches of enzyme) by incubation at 37°C for 48 h. The lignan glucosides are hydrolysed in proportion of 95.6 %. The EH method is recommended for building databases of lignan contents in foods, useful for nutritionists and medical researchers who seek to assess the effects of dietary lignan intake on human health.

Artificial neural network (ANN) and partial least squares (PLS) regression models, which are complementary to the MAE method, were calibrated for the general quantification of lignans in a variety of flaxseed materials. The lignan values predicted with the ANN and PLS models were in the range of ± 0.67 to 4.85 % of the reference lignan values. Using the ANN and PLS models requires measuring the UV-Vis light absorption of microwave-assisted flaxseed extracts at 289, 298, 343, and 765 nm, following the Folin-Ciocalteu's assay; the models are

useful to the flaxseed processing industry for rapidly and accurately determining the lignan contents of various flaxseed raw materials.

A non-automated, affordable and accurate solid phase extraction (SPE) method was developed for purifying microwave-assisted flaxseed extracts. The method requires the preparation of extracts prior to SPE by adjusting the pH of extracts in two stages, 1st to pH 3 with sulphuric acid for removing the water soluble proteins and carbohydrates by precipitation, and 2nd to pH 5 with sodium hydroxide for improving the retention of lignan by the packed SPE phase in order to reduce the lignan losses in the wash-water eluate. Microwave-assisted extracts from 0.6 and 1.5 g defatted flaxseed meal can be purified by SPE in order to recover in the 10, 20 and 30 % ethanol pooled eluates 71.2 % and 60.6 %, respectively, of the amount of lignan subjected to purification. SPE purified extracts can be used for further experiments, such as testing the antioxidant activity and the stability of the lignan extracts during various storage conditions.

Méthodes Pratiques pour la Quantification des Lignanes

Résumé

Une méthode optimisée d'extraction assistée par micro-ondes (EAMO), en conjonction avec l'analyse par chromatographie liquide de haute performance, est proposée pour la quantification de lignanes, de façon généralisée, dans des échantillons des graines de lin et des aliments d'origine végétale. La méthode nécessite l'hydrolyse des échantillons de 0.5 - 1.6 g avec 50 ml de NaOH 0.5 M en appliquant 156 W (niveau de puissance estimé par calibration calorimétrique) de façon intermittente (30 s marche/arrêt) pour 3 min. La température finale des extraits était de 67°C. La méthode EAMO extrait les lignanes des matrices végétales complètement, avec exactitude (récupération de 97.5 %), avec efficacité (rendements de 21.4 et 26.6 % plus hauts que ceux obtenus avec des méthodes conventionnelles), et avec précision (coefficients de variation pour analyses répétées < 4.03 %).

Une méthode d'hydrolyse enzymatique (HE), complémentaire pour la méthode EAMO, a été développée pour la quantification généralisée des lignanes aglycones dans des échantillons végétaux. La méthode HE nécessite l'hydrolyse des extraits, obtenus par EAMO, qui contient 100 mg d'échantillons dans 3 ml de solution tampon d'acétate de sodium (0.01 M, pH 5), avec des solutions d'enzyme β-glucuronidase en concentrations de ≥ 40 U d'enzyme/mg échantillon dépendant de la capacité d'hydrolyse des différents lots d'enzymes), par incubation a 37°C pour 48 h. Les lignanes glucosides sont hydrolysés en proportion de 95.6 %. La méthode HE est recommandée pour construire des bases des données des contenus en lignanes des aliments, qui sont utiles aux chercheurs en santé et nutrition qui cherchent à évaluer les effets des apports nutritionnels des lignanes sur la santé humaine.

Des modèles de réseaux de neurones artificiels (RNA) et de régression par les moindres carrés partiels (MCP), qui sont complémentaires pour la méthode EAMO, ont été calibrés pour la quantification généralisée des lignanes dans une variété d'échantillons de graines de lin. Les valeurs des lignanes estimées avec les modèles RNA et MCP ont été dans des écarts de ± 0.67 jusqu'à 4.85 % des valeurs de référence des lignanes. L'utilisation de modèles RNA et MCP nécessite d'effectuer des tests de Folin-Ciocalteu afin de mesurer l'absorption de la lumière UV-Vis des extraits a 289, 298, 343, et 765 nm. Ces modèles sont utiles aux industries de transformations des graines de lin pour quantifier avec rapidité et précision les niveaux de lignanes dans les différentes sources de matières premières à base de graines de lin.

Une méthode non-automatisée, abordable et précise d'extraction sur phase solide (EPS) a été développée afin de purifier des extraits de graines de lin produits par EAMO. La méthode nécessite la préparation des extraits avant la EPS par ajustement du pH à deux reprises; premièrement au pH 3 avec de l'acide sulfurique pour enlever, par précipitation, les protéines et les hydrates de carbone qui sont solubles dans l'eau; et, deuxièmement au pH 5 avec de l'hydroxyde de soude pour améliorer la rétention des lignanes en phase solide par l'entonnoir EPS afin de réduire les pertes de lignanes dans l'eau de lavage. Des extraits produits par EAMO à partir de 0.6 et 1.5 g de farine de graines de lin dégraissée peuvent être purifiés par EPS afin de récupérer 71.2 et 60.6 %, respectivement, de la quantité des lignanes utilisée pour la purification, dans les liquides d'élution des10, 20 et 30 % d'éthanol mis en commun. Des extraits purifiés par EPS peuvent être utilisés pour tester la capacité antioxydante et la stabilité des extraits des lignanes durant leur entreposage dans des conditions variées.

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Nomenclature

A	linear combination of predictor variables used in ANN analysis
$A@$	absorption of UV-Vis light at specified wavelength
AF	accuracy factor
ANN	artificial neural network
ANSECO	anhydro-secoisolariciresinol
ANOVA	analysis of variance
BF	bias factor
CCD	central composite design
cm	centimeter
CP	continuous power application
C_{pv}	specific heat of borosilicate glass vessel [J/(g°C)]
C_{pw}	specific heat of water [J/(g°C)]
CV	coefficient of variation [%]
CV-R^2	cross-validation coefficient of determination used in ANN analysis
d	difference between reference and predicted values
$\bar{d}$	bias
DF	degree of freedom
DFM	defatted flaxseed meal
D_p	penetration depth [cm]
DPPH	1,1-diphenyl-2-picrylhydrazyl
ED	enterodiol
EL	enterolactone
Et-OH	ethanol
eV	electron volt
FA	ferulic acid
FAE	ferulic acid equivalent
FC	Folin-Ciocalteu

FCR	Folin-Ciocalteu's reaction
FerAG	ferulic acid glucoside
FH	flax hull
FRAP	ferric reducing antioxidant power
g	gram
GA	gallic acid
GAE	gallic acid equivalent
GC	gas chromatography
GHz	gigahertz
h	hour
H	node used in ANN analysis
HCl	hydrochloric acid
H_2SO_4	sulphuric acid
HMGA	3-hydroxy-3-methyl-glutaric acid
HPLC	high performance liquid chromatography
Int. $	international dollar
IPA	intermittent power application
IR	infrared
k	number of responses
K	subsets of data used in cross validation in ANN analysis
kt	kilo tonne
L	liter
LDL	low density lipoprotein
LS	least squares
M	molar, mole
MAE	microwave-assisted extraction
MAE-AC	microwave-assisted extraction followed by acidic hydrolysis
MAP™	microwave-assisted process
Me-OH	methanol
min	minute

ml	millilitre
mm	millimeter
MPa	mega pascal
MS	mass spectrometry
M_v	mass of borosilicate glass vessel [g]
M_w	mass of water [g]
n	number of observation
N	normal
nm	nanometer
nM	nanomole
NaOH	sodium hydroxide
OFAT	one-factor-at-a-time
OFDM	oil-free dry meal
OLS	ordinary least squares
p	probability used for establishing the statistical significance
P	microwave power output [W]
P_a	actual microwave power output [W]
PCouAG	*p*-coumaric acid glucoside
PLS	partial least squares
PROC CORR	correlation procedure
PROC REG	regression procedure
PVDF	polyvinylidene difluoride
q	number of predictors
r	correlation coefficient
R^2	coefficient of determination
$R^2_{adj.}$	adjusted coefficient of determination
REML	residual maximum likelihood
RP	reversed phase
RPD	the ratio of the standard deviation of the reference data to standard error of performance
rpm	rotation per minute

RSM	response surface methodology
RMSD	root mean square difference
S	logistic function used in ANN analysis
SDG	secoisolariciresinol diglucoside
SD_x	reference data standard deviation
SECO	secoisolariciresinol
SEP	standard error of performance
SMG	secoisolariciresinol monoglucoside
SPE	solid phase extraction
RSE	relative standard error [%]
RT	ramp-to-temperature
s	second
STAR	Simultaneous Temperature Assisted Reactions
STDV	standard deviation
t	time [s]
tanδ	loss tangent
U	unit of enzyme
UV	ultraviolet
UV-Vis	ultraviolet-visible
VIP	variable importance for projection used in PLS analysis
W	watt
x	reference value
X	matrix of predictors used in PLS analysis
y	predicted value
Y	matrix of chemical indexes used in PLS analysis
wb	weight basis
ΔT	temperature difference [°C]
ε*	complex permittivity
ε’	dielectric constant
ε”	dielectric loss
ε''_t	total dielectric loss

ε_0	free space permittivity [F/m]
λ_0	free space microwave wavelength [cm]
σ	electric conductivity [S]
ω	angular frequency [rad/s]
μ^*	complex permeability
μm	micrometer

Chapter 1 - General Introduction

1.1. Scientific Background for Thesis Research

Flaxseed (*Linum usitatissimum* L), the richest food source of the lignan secoisolariciresinol diglucoside (SDG) (Peterson et al., 2010), has been the subject of intensive research since the late 1990s. The interests were varied, and included:

- fundamental studies for elucidating the composition and the structure of the complex lignan macromolecule (Ford et al., 2001; Johnsson et al., 2002; Kamal-Eldin et al., 2001; Kosińska et al., 2011a; Struijs et al., 2009; Struijs et al., 2007; Struijs et al., 2008),

- studies aiming at developing methods of lignan quantification and analysis (Eliasson et al., 2003; Johnsson et al., 2000; Nemes and Orsat, 2010, 2011b; Qiu et al., 1999),

- studies aiming at identifying the potential of flaxseed lignans to promote health and prevent diseases, and at understanding the underlying mechanisms (Adolphe et al., 2010; Bassett et al., 2009; Buck et al., 2010; Clark et al., 2000; Morisset et al., 2009; Peterson et al., 2010; Prasad, 1997, 1999; Prasad, 2000a, b, 2002; Prasad et al., 2000; Webb and McCullough, 2005),

- development and patenting of procedures for producing flaxseed lignan or lignan-rich flaxseed ingredients (Abe et al., 2005; Cui and Han, 2006; Hosseinian and Beta, 2009; Pihlava et al., 2004; Shukla et al., 2004; Westcott and Muir, 1998).

1.2. Problem Statement

The advancements in the science of flaxseed lignans (section **1.1**) have triggered the need for efficient and standardised methodologies of lignan determination in flaxseed (Adolphe et al., 2010; Muir, 2006a; Oomah and Hosseinian, 2008) and other food samples of plant origin (Schwartz et al., 2009), and for accurate methods of purification of crude extracts of flaxseed lignans (Nemes and Orsat, 2011c).

1.3. Thesis Hypothesis

It is hypothesised that efficient and accurate methods for the extraction, analysis and purification of flaxseed lignans can be developed by means of using advanced methodologies and techniques of extraction in conjunction with design of experiments, statistical analysis, and advanced chemometric procedures for the development of predictive models.

1.4. Thesis Rationale

The research presented in this thesis was designed to develop and propose solutions to the problems outlined in section **1.2**. Briefly, the solution to the problem of lack of accurate standardized methodologies for the extraction and analysis of lignans in flaxseed materials and in other plant sources is to use a previously optimized microwave-assisted extraction (MAE) for flaxseed lignans (Nemes and Orsat, 2010, 2011b), and to evaluate its accuracy, precision, repeatability and suitability for extracting lignans from a variety of samples. In addition, an enzymatic hydrolysis (EH) method was developed to be complementary to the MAE method for the general determination of lignan aglycones in plant samples. Also, artificial neural network (ANN) and partial least squares (PLS) regression models were calibrated to be complementary to the MAE method for the general determination of lignans in flaxseed samples. The

solution to the problem of lack of accurate methods of purification for crude flaxseed lignan extracts is to develop a solid phase extraction (SPE) method in order to maximize the recovery of purified lignan and model the recovery patterns to ensure the repeatability of results.

1.5. Thesis Objectives

- Identify the potential problems that might prevent the successful replication of the optimized MAE method in other laboratories, present solutions to these problems and give recommendations to ensure its successful replication; the results are presented in **Chapter 3** and **Chapter 4.**

- Design experiments for assessing the accuracy (recovery of lignan), precision (degree of reproducibility of results for different users over time), repeatability (degree of reproducibility of results for a single user within a day), efficiency of extraction (comparison with reference methods), and applicability for extracting lignans from samples other than flaxseed (e.g., chia seeds, sesame seeds, sunflower seeds, and almonds); the results of these experiments are presented in **Chapter 5**.

- Develop a complimentary SPE method for purifying lignans from crude microwave-assisted flaxseed extracts by using design of experiments and regression procedures; the results are presented in **Chapter 6**.

- Develop a complimentary EH method for converting the lignan glucosides from microwave-assisted extracts to lignan aglycones by using flaxseed as a reference plant material rich in lignan glucoside, crude and purified preparation of β-glucuronidase and cellulase enzymes, and designs of experiments; the results are presented in **Chapter 7**.

- Develop complimentary methods for the quantification of lignans in microwave-assisted flaxseed extracts that do not require the use of advanced chromatographic techniques, by using advanced chemometric methodologies such as ANN analysis and PLS regression; the results are presented in **Chapter 8.**

1.6. Contribution of the Authors to the Manuscript-Based Chapters

The Chapters 3 to 8 are manuscript-based, and were either presented in their edited form, or are soon to be published. Selected results from these chapters were also used to prepare conference presentations. The titles of manuscripts and conference presentations, and their respective authors are specified in the connecting statements to each chapter.

The contributions of the authors to the research presented in this thesis are as follows:

- Mrs. Simona Mihaela Nemes, in her quality of author of this thesis and first author of the manuscripts and the conference presentations enumerated in the connecting statements to each manuscript-based chapter, conducted the reviews of literature, the designs of experiments, the practical experimental work in the laboratory, the statistical analysis and interpretation of results, the writing of this thesis, and the preparation of manuscripts for publication.

- Dr. Valérie Orsat and Dr. G. S. Vijaya Raghavan, in their quality of PhD Thesis supervisors, provided their technical expertise, guided the author of this thesis throughout all the stages of planning and executing the experimental work, analysing and interpreting the results, writing and correcting this thesis, and preparing the manuscripts for publication.

Connecting Statement to Chapter 2

In **Chapter 1**, references were made to various scientific publications using flaxseed which triggered the interest for designing and conducting the research presented in this thesis. In **Chapter 2**, a review of literature is presented that highlights the economical aspects of the flax crop and presents the present state of knowledge related to the health benefits of flaxseed lignans, and the conventional methods of extraction and quantification.

Chapter 2 - Flaxseed a Key Food Source of Lignan Nutraceutical

2.1. Introduction

Flax is an economically important crop in the world, and especially in Canada which is the top producer of flaxseed, due to its numerous industrial uses. Flaxseed contains high levels of functional components such as ω-3 fatty acid, soluble fibre and lignan, which confer protection against cardiovascular diseases, diabetes and certain types of cancer. This chapter focuses specifically on the lignan component, its associated health benefits, and the conventional methods of extraction and analysis. The more advanced aspects pertaining to these topics, such as the microwave-assisted extraction of lignans, the purification of lignan extracts, the deglucosilation of lignans from microwave-assisted extracts by enzymatic hydrolysis, and the use of chemometric methods for the quantification of lignans are presented and discussed in **Chapter 3** to **Chapter 8** of this thesis.

2.1. Economical Importance of Flax Crop

Flax (also known as linseed, botanical name *Linum usitatissimum*, genus *Linum*, family Linaceae) is an annual herbaceous plant primarily grown for oil and fibre (Jhala and Hall, 2010). In 2010, the world production of flaxseed was 1,922.8 kilo tonnes (kt); the top six producers of flaxseed in the world were Canada with 423 kt and a value of 123.7 million international dollars (Int. $), followed by China (350 kt, 76.6 million Int. $), the U.S.A. (230 kt, 67.6 million Int. $), the Russian Federation (178 kt, 30.4 million Int. $), Ethiopia (150 kt, 44.4 million Int. $) and India (146 kt, 42.5 million Int. $) (FAOSTAT, 2012). In Canada, flaxseed is mainly grown in the western provinces of Manitoba, Saskatchewan and Alberta (Jhala and Hall, 2010; Puvirajah, 2012).

The uses of flax can be categorized in three main groups: (1) production of oil for edible purposes (as natural health product; for cooking; and as ingredient for bakery, transfat-free shortening and margarine products) and industrial applications (as curing agent; enters the composition of linoleum, oilcloth, painting, printer's ink and varnish products); (2) production of fiber for the textile industry; and (3) the use of the seed as raw material for producing value-added products (flax hull, lignan natural health products), as ingredient in the food industry (enters the composition of bakery, pasta and extruded breakfast cereal products), as functional food (to achieve protection against certain types of cancer, heart disease, hyperglycemia, stroke and thrombosis), and as animal feed in the form of flaxseed cake resulting from oil extraction (for buffalos, cattle, horses, poultry, cats and dogs) (Jhala and Hall, 2010; Oomah, 2001; Singh et al., 2011b).

2.2. Flaxseed as a Key Raw Material for the Functional Food and Nutraceutical Industries

Flaxseed has been identified as a key raw material for the functional food and nutraceutical industries due to its favorable composition (Oomah, 2001; Singh et al., 2011b). In 2011, the western Canadian flaxseed contained on an average 21.9 % protein (on a 8.5 % moisture basis), and 45.9 % oil (an a dry matter basis), 57.6 % of which represented α-linolenic acid (ω-3 fatty acid) (Puvirajah, 2012). Flaxseed also contains the lignan secoisolariciresinol diglucoside (SDG) in proportions of 0.74 – 1.9 % (on a whole grain basis) (Muir, 2006b; Nemes and Orsat, 2011a; Spence et al., 2003), and soluble dietary fibre (polysaccharidic mucilage) in proportions of 6 – 8 % (on a dry matter basis) (Ho et al., 2007; Westcott and Muir, 2003). SDG is concentrated in the seed coat or the hull of the grain, where it was found in concentrations of 3.1 to 4 % (Nemes and Orsat, 2011a; Oomah and Sitter, 2009; Singh et al., 2011b). Due to its composition, flaxseed offers health benefits such as protection against cardiovascular disease, anti-inflammatory effects, reduction of low density lipoprotein (LDL) cholesterol,

and prevention of certain types of cancer (Bassett et al., 2009; Oomah, 2001; Singh et al., 2011b). The nutraceuticals and functional food industries are interested in the development of proprietary (patent protected) extraction and purification processes for the commercial production of these specific functional flaxseed components (Hosseinian and Beta, 2009; Oomah, 2001).

The patented technologies for the production of flaxseed lignans use mostly defatted flaxseed meal or the whole grain as raw materials for extraction, as flaxseed is the richest lignan food source. The lignan is extracted with aliphatic alcohols in the form of a SDG-containing lignan macromolecule, which, in some cases is alkali hydrolysed in order to release the SDG in view of obtaining lignan products with higher SDG concentrations (Hosseinian and Beta, 2009). Patented technologies for the extraction of the complex lignan macromolecule involve: extraction with 70 % ethanol at room temperature for 8 h, followed by ultra filtration purification (Westcott and Paton, 2001), and extraction for 4 to 8 h with 50 – 80 % aqueous methanol, ethanol or iso-propanol at the boiling point of the solvents, followed by chromatographic purification and solvent evaporation or spray drying (Shukla et al., 2004). Hydrolysis with sodium hydroxide has been used for releasing the SDG from the complex lignan macromolecule (Abe et al., 2005; Muir and Westcott, 2000; Pihlava et al., 2004; Westcott and Muir, 1998). The lignan health products commercialized presently contain 20, 35 and 40 % SDG (Anonymous, 2003; Daniells, 2007; Douaud, 2007a, b). The worth of the flaxseed lignan health supplements market was appreciated to $50 million in 2005 (Halliday, 2005).

2.3. The Health Benefits of Dietary Flaxseed Lignan

The interest in producing flaxseed lignan nutraceutical ingredients at industrial scale has been motivated by its numerous conferred health benefits (Hosseinian and Beta, 2009; Oomah, 2001). The complex lignan macromolecule is of great interest for the human diet, as upon ingestion SDG is released by the acid in the

stomach, to be further converted by the enzymes secreted by the bacteria that colonize the intestines, to the more bioactive mammalian lignans enterodiol (ED) and enterolactone (EL) (Webb and McCullough, 2005). Although most studies focused on correlating the achieved health benefits with the levels of ED and EL metabolites in blood or urine, it was suggested that SDG, its aglycone secoisolariciresinol (SECO), and other related compounds such as 2, 3-dibenzyl-butane-1,4-diol might have contributed as well to the observed health benefits (Muir, 2006b). Prasad (2000a) showed that the antioxidant activity of SDG in human blood was increased 3.8 times by deglucosilation (formation of SECO), 3.4 times by conversion to EL and 3.9 times by conversion to ED. Both, ED and EL, exhibit weak estrogenic (simulating the fixation of estrogen on estrogen receptors) and anti-estrogenic (preventing the fixation of estrogen on estrogen receptors) activities depending on the various tissues and organs in the body, and on the ratio of concentrations of mammalian lignans to endogenous estrogen (Buck et al., 2010; Lamblin et al., 2008), and have been shown to protect against breast cancer in post-menopausal women and prostate cancers if produced in high concentrations in the colon (Adolphe et al., 2010; Touré and Xueming, 2010; Wang, 2002). Due to its antioxidant properties, SDG has the capacity to reduce the risk of type I diabetes by decreasing the oxidative stress (Prasad, 2000b; Prasad et al., 2000), and type II diabetes by inhibiting the expression of phosphoenolpyruvate carboxykinase, a rate-limiting enzyme for gluconeogenesis in liver (Prasad, 2002). High blood levels of EL have been linked to reduced body mass index and reduced total body fat in post-menopausal women (Morisset et al., 2009). The presence of ED and EL metabolites in the body has also been associated with significant reduction of the risk of developing cardiovascular diseases through lowering lipids concentrations in blood, blood pressure, oxidative stress and inflammation (Adolphe et al., 2010; Peterson et al., 2010). The SDG dose observed to provide protection against cardiovascular diseases was of 500 mg per day, taken daily for a period of eight weeks (Adolphe et al., 2010; Peterson et al., 2010).

2.4. Conventional Methods Used for Extracting Flaxseed Lignan

The extraction methods have to account for the fact that in flaxseed, SDG is part of a complex macromolecule, in which it is ester linked to 3-hydroxy-3-methyl-glutaric acid (Ford et al., 2001; Johnsson et al., 2002; Kamal-Eldin et al., 2001; Klosterman and Smith, 1954); the average number of SDG molecules in the lignan macromolecule is 3, but it can range from 1 to 7 (Peterson et al., 2010). The lignan macromolecule also includes *p*-coumaric acid glucoside, ferulic acid glucoside, herbacetin diglucoside (Johnsson et al., 2002; Struijs et al., 2009; Struijs et al., 2007; Struijs et al., 2008) and caffeic acid (Kosińska et al., 2011a).

The lignan macromolecule can be extracted from flaxseed with alcohols, and the SDG can be released from it by alkaline hydrolysis. Extraction with equal parts 1,4-dioxane and 95 % ethanol at room temperature for 48 h yielded 2 - 4 % lignan macromolecule (Klosterman and Smith, 1954). Extraction at room temperature for 1 - 4 h, with 65 – 75 % ethanol in a solvent to flaxseed meal ratio of 4 - 3: 1 v/w, followed by hydrolysis with 1 M solutions of sodium hydroxide or potassium hydroxide at room temperature for 4 - 24 h, then followed by solid phase extraction purification yielded extracts with SDG purity > 90 % (Westcott and Muir, 1998).

The extraction procedures published by Klosterman and Smith (1954) and Westcott and Muir (1998) had a great influence on the methods of SDG extraction from flaxseed at analytical scale. Extraction with 1,4-dioxane-ethanol followed by alkaline hydrolysis (Klosterman and Smith, 1954) has been applied for isolation and characterization of flaxseed lignan macromolecule constituents (Johnsson et al., 2002; Kamal-Eldin et al., 2001; Qiu et al., 1999), for developing high performance liquid chromatography (HPLC) methods (Eliasson et al., 2003; Johnsson et al., 2000), and for isolating SDG for research purposes (Frank et al., 2004). Extraction with aqueous ethanol or methanol followed by alkaline hydrolysis (Westcott and Muir, 1998) has been applied for quantifying SDG in

bread (Muir and Westcott, 2000) and flaxseed hulls (Madhusudhan et al., 2000), for isolating SDG for studying its health benefits (Clark et al., 2000; Prasad, 2000b; Prasad et al., 2000; Spence et al., 2003), for quantifying lignans in foods (Thompson et al., 2006), and for studying the structure of the lignan macromolecule (Struijs et al., 2007; Struijs et al., 2008).

The lignan macromolecule was also extracted from whole flaxseed using a pressurised solvent extraction method with subcritical water at 140 - 160°C and 5.2 MPa. The obtained yields were 14 to 20 % lower than those achieved with the direct alkaline hydrolysis method (Cacace and Mazza, 2006).

It is worth noting that alcoholic extraction followed by alkaline hydrolysis is not the most efficient procedure for the extraction of lignans in view of precise quantification purposes. Eliasson et al. (2003) demonstrated that even if the alcoholic extraction was carried out for 48 h, the lignan macromolecule was not completely extracted from the flaxseed matrix, and that such extraction procedures were outdone by a simple direct alkaline hydrolysis with 1 M NaOH for 1 h at room temperature. When, the direct alkaline hydrolysis was carried out in a microwave environment, additional benefits were achieved such as very short extraction duration (3 min only) and significantly higher extraction yields (6 to 26.6 % higher) (Nemes and Orsat, 2011a, b). The microwave-assisted extraction (MAE) method and the results obtained with it are presented and discussed in **Chapter 3** and **Chapter 5**.

Based on the results of the literature review presented above, it can be concluded that the MAE method using the direct alkaline hydrolysis approach was the fastest and the most efficient method of SDG extraction from flaxseed; as the MAE method achieved extraction yields higher than the direct alkaline hydrolysis extraction method (Nemes and Orsat, 2011a, b), which in turn achieved significantly higher extraction yields than other methods involving alcoholic

extraction followed by alkaline hydrolysis (Eliasson et al., 2003), and extraction with subcritical water (Cacace and Mazza, 2006).

2.5. Quantification of Secoisolariciresinol Diglucoside in Flaxseed Extracts

Chromatographic analysis of lignans is mostly done by HPLC (Oomah and Hosseinian, 2008). HPLC based methods are preferred to gas chromatography (GC) based methods, as the latter require extensive sample preparations through purification and chemical derivatization (Muir, 2006b). The chromatographic columns used in HPLC analyses are reversed phase (RP) silica-based C18. Acidic mobile phases are used in order to suppress the ionization of phenolic and/or carboxylic groups, which is not desired as it could reduce the retention times and therefore negatively affect the resolution and reproducibility of the chromatographic method (Nollet, 2000). A HPLC method for analysing lignans in flaxseed was developed by Johnsson et al. (2000) and was later applied by others (Eliasson et al., 2003; Frank et al., 2004; Johnsson et al., 2002; Nemes and Orsat, 2010, 2011b). This method uses the following gradient elution of solvent A (5% acetonitrile in 0.01 M phosphate buffer pH 2.8), and solvent B (acetonitrile); at 0 min – A is 100 % and B is 0 %, at 30 min – A is 70 % and B is 30 %, at 32 min – A is 30 % and B is 70 %. The separation is carried out on RP C18 columns (length 250 mm, internal diameter 4.6 mm, particle size 5μm). The flow rate is 1 ml/min, and the chromatograms are recorded at 280 nm with UV detectors. Before HPLC injection, the extracts are cleaned of water soluble proteins and carbohydrates by precipitation through acidification to pH 3 and alcohol addition, followed by the separation of solid and liquid phases through centrifugation and cleaning of the liquid phase by microfiltration ($\leq$ 45 μm) (Eliasson et al., 2003). In addition, the salts resulting from hydrolysis can be removed from the extracts by solid phase extraction (SPE) purification (Johnsson et al., 2000).

2.6. Problems Related to the Quantification of Secoisolariciresinol Diglucoside in Flaxseed

The precise quantification of SDG is required for studies that use dietary supplementation with flaxseed products in order to assess their effects on human health (Adolphe et al., 2010; Peterson et al., 2010). Although, a variety of methods have been reported in the literature, none has been recognized as a generally accepted standard method for SDG quantification (Oomah and Hosseinian, 2008). This has affected the results of all the research involving SDG quantification in the flaxseed products fed to animals or humans. The type of hydrolysis (enzymatic, acidic or alkaline) used for releasing the SDG from the complex lignan macromolecule has had a significant impact on the published SDG concentration ranges (0.1 – 0.4 % SDG if enzymatic hydrolysis was used; > 0.42 % if acidic or alkaline hydrolysis were used), and on the reported SDG levels associated with the observed health benefits (Muir, 2006b). Thus, it is challenging to reproduce the results of flaxseed supplementation studies that underestimated the SDG levels, conducted on animals and human subjects while using purified SDG extracts (Muir, 2006b). Although, the most efficient conventional method used for the quantification of SDG in flaxseed products was published in 2003 (Eliasson et al., 2003), this was not applied for analysing the flaxseed products used in supplementation studies conducted with animal and human subjects (Muir, 2006b). The above observations highlight the need for an efficient and easy to reproduce method of SDG analysis in order to avoid such problems with any future SDG health related studies. The MAE method presented in **Chapter 3** and **Chapter 5** is a potential solution to this problem.

2.7. Conclusions

Flaxseed is an abundant source of health beneficial compounds with high potential of value addition especially through the development of nutraceutical and functional food products. The patented technologies for the production of

flaxseed lignan nutraceutical involve conventional extraction methods with aqueous alcohols which are time consuming (Hosseinian and Beta, 2009; Oomah, 2001). The literature review indicated that substantial gains in terms of extraction yields and reduced extraction times can be obtained with direct alkaline hydrolysis methods in a microwave environment (Nemes and Orsat, 2011a, b) or at room temperature (Eliasson et al., 2003). However, in order to produce lignan rich nutraceutical products with such methods, the flaxseed extracts must by purified using expensive chromatographic processes (Nemes and Orsat, 2011c; Westcott and Muir, 1998). Future studies using flaxseed products as dietary supplements in order to assess their health effects in humans should consider accurate methods of SDG extraction, such as the MAE method developed by Nemes and Orsat (2010, 2011a, b).

Connecting Statement to Chapter 3

In **Chapter 2**, the economical importance of the flax crop, the composition of flaxseed, the health benefits of the flaxseed lignan and the conventional methods for the extraction and the analysis for the flaxseed lignan have been presented and discussed.

The conventional methods of extraction published are time consuming and do not extract the lignan from the flaxseed matrix completely. Thus, these methods are not recommended for the precise quantification of flaxseed lignan for analytical purposes; as a result, the need for an extraction method that is precise and accurate has been identified. The proposed solution to this problem is to use microwave-assisted extraction (MAE), a techniques that is fast, efficient and has been largely applied for the extraction of natural compounds over the past twenty five years.

In **Chapter 3**, the theoretical aspects pertaining to the MAE technique are presented and discussed. Recent literature reporting MAE methods for plant phenolic compounds, including flaxseed lignans, are critically reviewed and suggestions for future research in the field of MAE of plant compounds are made.

Part of the literature review presented in **Chapter 3** has been used to prepare two conference presentations, as follows.

- Nemes, S.M., Orsat, V., 2009. Microwave Extraction for the Production of Specialty Ingredients or Analytical Purposes, The Annual International Meeting of the American Society of Agricultural and Biological Engineers ASABE, Reno, Nevada, USA, June 21-24.

- Nemes, S.M., Orsat, V., 2009. Exploiting the Selectivity of Microwave Heating for the Extraction of Biologically Active Compounds from Plant

Matrices, Journée d'information scientifique et technique en génie agroalimentaire, CRDA de Saint-Hyacinthe, QC, Canada, March 25.

The MAE method for flaxseed lignan has been previously developed (the method development is not part of this thesis) by the author of this thesis and the manuscripts have been prepared and published under the supervision of Dr. Valerie Orsat.

- Nemes, S.M., Orsat, V., 2010. Screening the Experimental Domain for the Microwave-Assisted Extraction of Secoisolariciresinol Diglucoside from Flaxseed Prior to Optimization Procedures. Food and Bioprocess Technology 3, 300-307.

- Nemes, S.M., Orsat, V., 2011. Microwave-Assisted Extraction of Secoisolariciresinol Diglucoside - Method Development. Food and Bioprocess Technology 4, 1219-1227.

Chapter 3 is written in manuscript style and is soon to be submitted for publication as follows.

- Nemes, S.M., Orsat, V., Raghavan, G.S.V., 2012. Critical Review of Experimental Procedures Used for Developing Optimised Microwave-Assisted Methods for Extracting Phenolic Compounds from Plants.

Chapter 3 - Critical Review of Experimental Procedures Used for Developing Optimised Microwave-Assisted Methods for Extracting Phenolic Compounds from Plants

3.1. Abstract

This chapter presents the theory related to the microwave-assisted extraction (MAE) technique, and reviews recent experimental procedures used for developing MAE methods for extracting plant phenolic compounds. Only the methods developed by means of response surface methodology (RSM) for process optimization were considered truly optimized. RSM has been generally used in conjunction with the power-control approach and mono-mode type microwave systems. The microwave power densities (W/g of extraction mixture) of interest for applications in mono-mode systems were in the range of 1.99 – 3.12 W/g during the power-on cycles. Based on the results of the critical review of literature, the following power densities were recommended for the initial ramp-to-temperature stage of the temperature-control approach: 1.34, 3.35, and 5.36 W/g when using solvents with good, medium, and low microwave heating abilities, respectively. A critical insight into the MAE of plant phenolic compounds is provided, which will help researchers select the best methods among the published ones, and design and develop better optimized MAE methods while using less time and resources for obtaining the most informative results. Recommendations were made to assist researchers in the task of reproducing and scaling up published MAE methods.

Key words: mono-mode, multi-mode, microwave power level, microwave power density, method development, method optimization

3.2. Introduction

Microwave-assisted extraction (MAE) has emerged over the past 25 years as a technique suitable for extracting nutraceutical compounds from plant matrices. Microwaves instantaneously interact with the extraction mixtures causing thermal energy dissipation through dipolar polarization and ionic conduction. The main reasons for which researchers had chosen MAE over conventional extraction techniques were: reduced extraction times and solvents use, and improved extraction yields (Kaufmann and Christen, 2002; Wang and Weller, 2006). The factors commonly studied in MAE are: the solvent composition and volume, the extraction temperature, the microwave power level, and the duration of microwave extraction (Eskilsson and Björklund, 2000; Huie, 2002).

The designs of MAE experiments have to allow for assessing the two-factor (second order) interactions [third order and higher order interactions are considered inactive (Lundstedt et al., 1998)] and the quadratic effects of the studied factors, in order to predict the extraction yields among the studied factors' levels [e.g., central composite design (CCD)]. This is necessary as the microwave power factor is expected to interact significantly with at least one of the other studied factors, such as the concentration of solvent or the time of microwave power application. Further, the prediction power of the design is necessary as the optimum extraction conditions will not necessarily occur at the studied fixed factors' levels (Krishnaswamy et al., 2012; Leardi, 2009; Lundstedt et al., 1998; Nemes and Orsat, 2011b; Singh et al., 2011a).

The objectives of this chapter are to present the theoretical aspects of MAE pertaining to the extraction of natural compounds from plants; and to review research articles in the field of MAE of plant phenolic compounds, in order to present a critical discussion on several points of importance for MAE method development such as: (1) the use of statistical procedures for process optimization; (2) the possibilities of reproducing and/or translating the published MAE methods in other laboratories using the same or a different type of MAE

system; (3) the applicability of the methods for analytical and/or production purposes; and (4) the trends and recommendations for future research in the field of MAE of phyto-compounds. The discussion provided here will help researchers select the best MAE methods among the published ones, and use better experimental procedures for optimising MAE methods, while using less time and resources for obtaining the most informative results. The purpose of this work is not to present an exhaustive review of literature on the MAE of natural compounds; as such reviews have been previously published by others (Desai et al., 2010; Kaufmann and Christen, 2002; Routray and Orsat, 2011; Zhang et al., 2011).

3.3. Theoretical Aspects of Microwave-Assisted Extraction

MAE is a relatively new, non-conventional, technique that has been applied increasingly for the extraction of natural compounds from plant matrices over the past 25 years (Desai et al., 2010; Kaufmann and Christen, 2002; Routray and Orsat, 2011; Wang and Weller, 2006). In the late 1980s two publications drew attention on the benefits of using domestic microwave ovens for the extraction of analytes from seeds, food, feed and soil samples; namely, the benefits were: extraction yields higher than those obtained with Soxhlet or shake-flask methods, rapidity, and suitability for extracting thermo-labile compounds (Ganzler and Salgo, 1987; Ganzler et al., 1986). The ease of process optimization was another attribute that made MAE suitable for developing simple and reliable processes for obtaining high-quality plant extracts (Huie, 2002).

3.3.1. Types of Research-Dedicated Microwave-Assisted Extraction Systems

Research-dedicated microwave instruments with multi-mode and mono-mode cavities functioning at 2.45 GHz were first designed in the 1980 for acid-digestion and organic synthesis reactions. Multi-mode cavities are rectangular in shape and have larger volumes [e.g., 13, 43, 48 or 66 L (Kappe et al., 2009b)] within which

the microwaves are reflected by the walls and form multiple modes (standing waves) of propagation. The distribution of the microwave field inside multi-mode cavities is chaotic, and most systems have a mode stirrer that helps in achieving power delivery uniformity. When multi-mode systems are used for processing small loads that occupy less than 20 % of the cavity, non-uniform heating and hot- and cold-spots can occur (Orsat et al., 2005). The hot- and cold-spots are "pockets" with different levels of energy intensity caused by the distribution of the multiple standing waves inside the cavity (Kappe et al., 2009b; Lidström et al., 2001). The hot-spot areas have higher temperatures than their surroundings caused by greater interactions with the electric field (Will et al., 2004). Thus, it is difficult to reproduce the same conditions for small loads, whether processed in the same, or a different microwave system with multi-mode cavity (Lidström et al., 2001). Mono-mode systems have only one mode of propagation, and the cavities that can be rectangular or cylindrical in shape, are designed to allow for the maximum electric field strength to occur precisely within the extraction (reactor) vessel (Kappe et al., 2009b; Orsat et al., 2005). For this reason, mono-mode microwave systems appear to be more suitable for research purposes (Lidström et al., 2001; Orsat et al., 2005). Both mono- and multi-mode systems can be used for atmospheric (open-vessel) or high pressure (closed-vessel) chemical processes. Confusion has arisen in the literature regarding the type of microwave cavities; open-vessel MAE was referred to as mono-mode or focused MAE regardless of being carried out in mono- or multi-mode cavities (Kaufmann and Christen, 2002; Wang et al., 2008; Wang and Weller, 2006). The volumes of samples that can be processed with mono-mode systems are smaller, with 0.6 - 50 ml, and up to 125 ml for closed- and open-vessel conditions, respectively; whereas, several liters can be processed under both conditions with multi-mode cavities (Kappe et al., 2009b). It is worth noting that most research-dedicated microwave systems are designed for synthesis, thus the range of volumes that can be processed does not necessarily apply to MAE. For example, the articles reviewed in this paper (section **3.5**) used 20 - 50 ml solvent for MAE in multi-mode systems.

3.3.2. Microwave Heating

The increased yields of chemical processes achieved when using microwave heating as opposed to conventional heating are attributable to the differences between the two types of heating, and especially to the rapid volumetric nature of microwave heating (Kappe, 2004; Will et al., 2004). The latter occurs through direct energy transfer into the samples by microwave-matter interactions instead of thermal gradients as is the case for conventional heating (Orsat et al., 2005; Venkatesh and Raghavan, 2004; Will et al., 2004). The energy transfer from microwaves to materials takes place by emission and absorption of microwave photons, which have a level of energy that is several orders of magnitude smaller than the energies of molecular bonds (Kappe, 2004; Stuerga and Delmotte, 2002; Will et al., 2004) as can be seen from **Table 3.1**. Thus, the energy of the microwave photon is not high enough to initialize any constructive or destructive reaction steps, and supports the hypothesis that the benefits of using MAE over conventional extraction techniques are due to the fast and volumetric nature of the microwave heating.

Table 3.1 The energies of the microwave photon, and of the chemical bonds (Kappe, 2004; Stuerga and Delmotte, 2002; Will et al., 2004)

Microwave photon energy at 2.45 GHz (eV)	Hydrogen bonds (eV)	Covalent bonds (eV)	Ionic bonds (eV)
1×10^{-5}	0.04 - 0.44	5.0	7.6

The instantaneous and volumetric heating aspects are the result of the coupling of microwave energy with the polar molecules and the ionic species present in the materials, which is governed by two complex parameters: the permittivity ε^*, and the permeability μ^*; they describe materials' interactions with the electric and magnetic fields, respectively. The permeability aspect is rather negligible in agri-food materials as they contain only traces of magnetic materials such as iron,

nickel, and cobalt. Thus, it is considered that agri-food materials do not interact with the magnetic component of the electromagnetic field (Mudgett, 1989; Venkatesh and Raghavan, 2004). The complex permittivity (ε*) contains real and imaginary components according to the expression $\varepsilon^* = \varepsilon' - j\varepsilon''$; where, ε' is the dielectric constant – a measure of material's ability to be polarized by the electric component of the microwave field and store energy; and ε" is the dielectric loss – a measure of material's ability to dissipate (convert) microwave energy in the form of heat. The ratio of the dielectric loss to the dielectric constant is known as the loss tangent (also called the dissipation factor) $\tan\delta = \varepsilon''/\varepsilon'$, which can be used as an index for estimating the capacity of a dielectric material to heat in a microwave environment (Meda et al., 2005; Orsat et al., 2005).

When microwaves propagate through a material, the polar molecules and the ionic species in the material polarize; they try to align with the electromagnetic field oscillating at the speed of 2.45 billion times/s (at the frequency 2.45 GHz of interest for MAE). This movement is responsible for the fast volumetric heating through dipolar polarization and ionic conduction (Meda et al., 2005), and the enhanced reaction rates in microwave-assisted chemistry (Kappe, 2004; Will et al., 2004). Dipolar polarization occurs when microwaves interact with polar molecules that have either permanent or induced dipole moments, which are not free to align with the oscillating electric field instantaneously. In liquids and solids the instantaneous alignment of molecular dipoles is prohibited by frictions and collisions with the neighbouring molecules. This results in a phase lag between the orientation of the electric field and that of the dipoles, which causes energy to be dissipated in the form of heat (Lidström et al., 2001; Meda et al., 2005). This is accounted for by the dielectric loss (ε") in the total loss equation (**Equation 3.1**). Ionic conduction occurs when ions are free to move through the material. They oscillate under the influence of the electric component of the microwave field and collide with other species. This motion results in heating through ohmic losses. The contribution of ionic conduction to the total loss equation (**Equation 3.1**) is described by the term $\frac{\sigma}{\omega\varepsilon_0}$. Generally, the ionic

conduction increases with temperature (Buffler, 1993; Meda et al., 2005). The capacity to generate heat is greater for ionic conduction than for dipolar polarization (Lidström et al., 2001). The total energy loss occurring at microwave frequencies includes the dielectric loss and the conductive (ohmic) loss as shown in **Equation 3.1** (Meda et al., 2005; Will et al., 2004).

$$\varepsilon_t'' = \varepsilon'' + \frac{\sigma}{\omega \varepsilon_0} \qquad \textbf{Equation 3.1}$$

Where ε_t" = total loss

ε" = dielectric loss

σ = electric conductivity [S]

ω = angular frequency [rad/s]

ε_0 = free space permittivity [F/m]

Although microwave heating is volumetric in nature it should be kept in mind that the microwave energy will be attenuated depending on the dielectric loss of the extraction mixture. The depth of penetration (cm) of microwaves in the extraction mixtures can be calculated over the range of temperatures of interest for MAE using **Equation 3.2**, if the dielectric properties of the mixtures are known. The penetration depth is defined as the depth into a sample where the incident microwave power drops to 1/*e* (*e* = 2.718) or 36.8% of its initial value (Meda et al., 2005; Venkatesh and Raghavan, 2004). The limited penetration depth can cause non-uniform heating especially in larger volumes of extraction mixtures (such as in the scale-up of MAE) processed in multi-mode cavities. In such cases MAE should be performed under stirring conditions.

$$D_p = \frac{\lambda_0 \sqrt{\varepsilon'}}{2\pi \varepsilon''} \qquad \textbf{Equation 3.2}$$

Where D_p = microwave penetration depth into the sample [cm]

λ_0 = free space microwave wavelength for a given frequency [cm]

ε' = dielectric constant

ε'' = dielectric loss

The penetration depths at 2.45 GHz were calculated for water, methanol and ethanol: methanol (9:1, v/v) using the dielectric constant and dielectric loss values published by Liao et al. (2001). As it can be seen from **Figure 3.1,** the penetration depth of microwaves is greater in water than in alcohols.

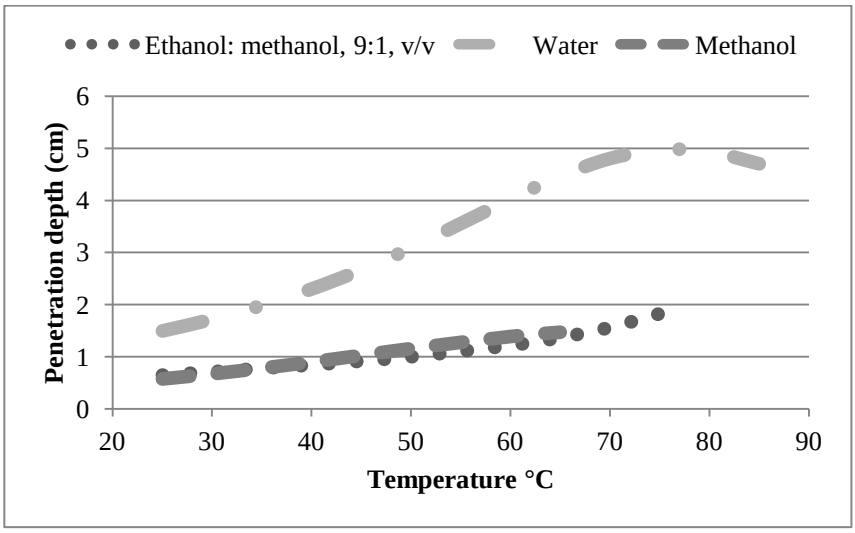

Figure 3.1 The penetration depth at 2.45 GHz for water, methanol and ethanol: methanol (9:1, v/v) based on the dielectric data from Liao et al. (2001)

3.3.3. Microwave-Assisted Extraction Selectivity Attributes

MAE, as opposed to conventional extraction techniques, benefits from selective extraction processes. Selective MAE can be achieved by localizing the heating into the sample rather than the solvent, or choosing organic solvents in order to extract particular compounds (Kubrakova and Toropchenova, 2008). The selectivity trait according to which microwave heating is localized in the samples is well exemplified by the microwave-assisted process (MAP™) which uses microwave-transparent solvents for MAE, and allows for releasing the extracted

compounds in a cooler solvent thus preventing their degradation (Paré et al., 1994). MAP™ was patented for the extraction of a variety of soluble components into a solvent that is relatively transparent to microwave, such as hexane (Paré and Bélanger, 1997). The greater the difference between the loss tangent values of samples and solvents, the greater the efficiency of MAP™. This process was applied to extract essential oils from fresh mint leaves using hexane (Paré and Bélanger, 1997; Paré et al., 1994). The water in the fresh leaves absorbed microwave energy, heated, and the increasing vapour pressure built into the glandular and vascular systems of the leaves caused them to burst. Thus, the essential oils were exposed and were dissolved readily by the microwave transparent solvent which acted as a coolant. It has been demonstrated that the quality of the essential oils obtained by MAP™ was improved as opposed to that of the oils obtained by Soxhlet extraction. It appeared that MAP™ extracted selectively the oils due to cells rupture, in a very short time, thus not allowing for the co-extraction of undesirable pigments (Paré and Bélanger, 1997; Paré et al., 1994). Briefly, the selectivity of MAP™ comes from the selective energy application right into the sample and not into the surrounding solvent (Paré and Bélanger, 1997; Paré et al., 1994). However, MAP™ using microwave-transparent solvents does not necessarily generalise to all kinds of plant matrices. For example, Dai et al. (2001) used dichloromethane, which is relatively microwave-transparent, and methanol, which has a good microwave heating ability, to extract azadirachtin-related limonoids from the leaf, the seed and the seed-shell of the neem tree according to the MAP™ principles. Although, both solvents were appropriate for extracting the azadirachtin-related limonoids, methanol lead to significantly higher MAE yields which were possibly due its greater microwave heating ability.

Another selectivity trait of MAE depends on choosing organic solvents in such a way as to selectively extract particular compounds (Kubrakova and Toropchenova, 2008). However, it is known that in general compounds with comparable polarities are co-extracted; thus, requiring the cleaning and

purification of extracts (Eskilsson and Björklund, 2000; Huie, 2002). Two examples of selective MAE of phenolic compounds, that were provided by the work of Krishnaswamy et al. (2012) and Singh et al. (2011a), will be presented later in this paper (section **3.5**). They showed that the MAE process parameters could be modulated in order to promote the extraction of specific compounds with high antioxidant capacities. It appears thus, that MAE is not necessarily selective due to obtaining microwave-assisted extracts that are cleaner (containing less co-extracted compounds) than the extracts obtained by conventional techniques, but it is selective rather due to the possibility of modulating the MAE process parameters in order to dissolve specific compounds.

The selectivity of MAE also depends on the type of microwave system used. MAE processes performed at high temperatures and pressures in closed-vessel systems tend to be more exhaustive, thus less selective than MAE processes performed at atmospheric pressures in open-vessel systems (Bélanger and Paré, 2006). This can be explained by the fact that the polarity of solvents, such as water, slightly decreases at temperatures greater than 100°C at few atmospheres. The pressure is a consequence of heating the extraction mixture in closed-vessels, and it depends on the volume and the boiling point of the solvents (Wang and Weller, 2006). Considering this aspect, closed-vessel MAE uses harsh conditions that lead to exhaustive extraction (Bélanger and Paré, 2006). Moreover, the slightly decreasing polarity of aqueous solvents under high temperatures and high pressure conditions leads to the co-extraction of polar, medium-polar, and non-polar components (Kubrakova and Toropchenova, 2008; Raynie, 2006). Bélanger and Paré (2006) stated that open-vessel systems are more suitable than closed-vessel systems for developing and refining selective microwave extraction processes, due to the gentler extraction conditions and the possibility of performing multi-step protocols that require hydrolysis and derivatization of compounds.

Another selectivity trait of MAE results from the possibility of modulating the microwave heating ability of solvents by mixing solvents with different dielectric properties (Wang and Weller, 2006) or by the addition of ionic liquids (Kappe, 2004; Kubrakova and Toropchenova, 2008). For example, when non-polar compounds have to be extracted with hexane, which is microwave transparent, and when heat must be induced into the extraction mixture, hexane can be mixed with small volumes of water or acetone (Wang and Weller, 2006). In some cases, mixing solvents with different dielectric properties promotes enhanced microwave heating rates. For example, it is known that aqueous alcohols are heated better by microwaves than either pure alcohol or water, as aqueous alcohols exhibit a synergistic behaviour under microwave environment. This synergistic behaviour is attributed to the stabilization of the hydrogen bonds in the water-alcohol mixtures (Decareau and Mudgett, 1985).

3.4. Review Methodology for the Microwave-Assisted Extraction of Plant Phenolic Compounds

In order to allow the comparison of the reviewed MAE methodologies in terms of the choice of power levels used for the extraction of specific compounds (especially thermo-labile ones), and their possible negative effects on the quality of the extracts, the microwave power densities (W/g, Watts applied per gram of extraction mixture during the power-on cycles) were calculated. With the exception of one MAE method, published by Beejmohun et al. (2007), all the other reviewed methods used intermittent microwave power applications. When the microwave power delivery was controlled by the user (generally, when using mono-mode systems), the intermittent delivery patterns are known; when the power delivery was controlled by the microwave system based on the temperature of the extraction mixtures (generally, when using multi-mode systems), the intermittent delivery patterns are not known. Hence, the W/g values calculated for the MAE methods that used unknown power delivery patterns will not be used for a straight forward comparison, but they will provide information on the possibility

of hot-spots occurrence in the extraction mixtures during the initial ramp-to-temperature stages, and the power-on cycles during the hold-at-temperature stages.

For the sake of simplification and generalization it was assumed that: (1) the dry samples, regardless of their mass, did not absorb microwave energy; thus, they were not used in the calculation of microwave power density; (2) for the samples containing water, their water content (g) was used in the calculations; and (3) in the case of polar solvents, most of the microwave energy is absorbed by the mass of the solvent (g, density × volume) used for extraction, if it has favourable dielectric properties (medium or high loss tangent values). The solvents' capacity to convert microwave energy into heat is the theoretical basis for the calculation of microwave power densities; and it is presented below.

The microwave heating capacity of solvents is generally classified as high if the loss tangent is greater than 0.5, medium if the loss tangent is in the range of 0.1 - 0.5, and low if the loss tangent is smaller than 0.1 (Kappe et al., 2009a). Examples of loss tangent values for most common solvents used in MAE can be obtained from Kappe et al. (2009a) and Liao et al. (2001). As can be seen from **Figure 3.2**, the loss tangent values of water, methanol, and ethanol: methanol (9:1, v/v) at 25°C were medium to high (0.150, 0.736, and 1.045, respectively). The loss tangent curves were generated using statistical models developed by Liao et al. (2001). Hexane and acetone are relatively microwave transparent; their respective loss tangent values at 20°C are 0.020 and 0.054 (Kappe et al., 2009a). The loss tangent values alone, cannot be used to predict the heating behaviour of extraction mixtures during MAE, as other attributes of the extraction mixture (the specific heat capacity, and the heat of vaporization) along with the depth of penetration of microwave energy into the mixture also have an important influence (Leadbeater, 2011). However, the loss tangent values can be used as indexes in order to compare different solvents in terms of their microwave heating abilities (Lidström et al., 2001; Mingos, 2005).

From **Figure 3.2**, it can be seen that the ethanol: methanol mixture and methanol have the ability to heat very efficiently in a microwave environment, thus, it could be assumed that by comparison with an equal volume of water, alcohols need less energy and time in order to increase their temperature by 1°C increments; in other words alcohol has a higher microwave heating rate than water. This observation coincides with the fact that the specific heat of alcohols is much smaller than that of water [≈ 2.4, 2.5 vs. 4.18 J/(g °C)].

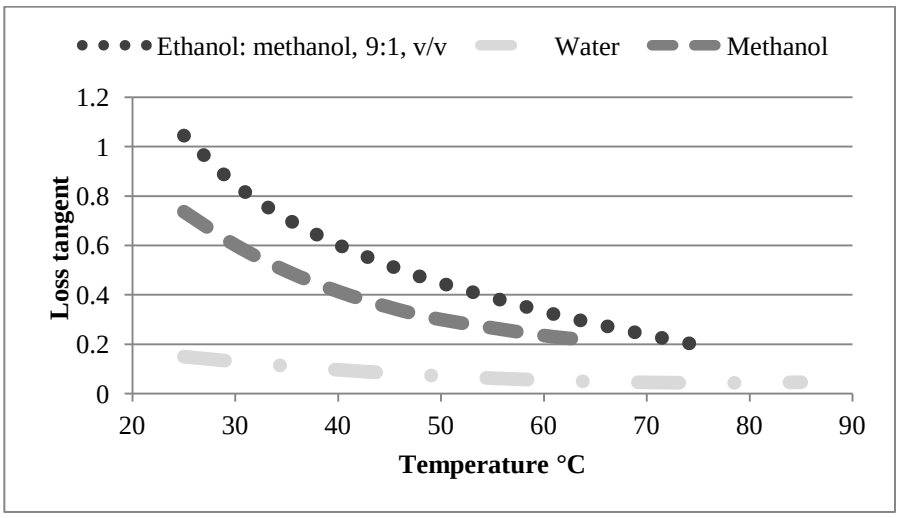

Figure 3.2. Loss tangent curves at 2.45 GHz for water, methanol and ethanol: methanol (9:1, v/v) based on the statistical models developed by Liao et al. (2001).

Based on these observations, it is assumed that the calculation of microwave power density (W/g) is useful for comparisons of MAE methods that use solvents with similar microwave heating abilities, as a way of identifying the most energy efficient methods.

3.5. Review of Developed Microwave-Assisted Extraction Methods for Plant Phenolic Compounds

3.5.1. Microwave-Assisted Extraction of Polyphenols from Chinese Herbs

Wang et al. (2008) developed MAE methods for the extraction of polyphenols from Chinese herbs, namely, resveratrol and emodin from *Rhizma polygoni cuspidati*, myricetin and quercetin from *Myrica rubra* tree leaves, and safflomin A from *Flos carthami.* Among these compounds, resveratrol, myricetin and safflomin A were thermo-labile. The effects of solvent volume (ml), duration (min) and temperature (°C) of extraction, and the level of vacuum (kPa, gauge pressure) on the extraction yields of the 5 polyphenolic compounds were studied using the one-factor-at-a-time (OFAT) approach without providing statistical analysis. The microwave instrument used was a multi-mode system (MAS I, by Sineo) functioning at 2.45 GHz with a nominal power of 1000 W, provided with a magnetic stirring device (the authors did not specify if magnetic stirring was used during the MAE), and an IR temperature sensor for automatic process control. The vacuum was created in the extraction vessel before starting the MAE by connecting a vacuum pump to the condenser placed on top of the extraction vessel.

The findings of the study indicated that vacuum MAE efficiently extracted the thermo-labile compounds resveratrol, myricetin and safflomin A as it achieved extraction yields higher by 7.4, 6.4 and 9.4 %, respectively than MAE at atmospheric pressure, and by 12.9, 29.5, and 7.9 %, respectively than reflux extraction. There were no noticeable differences in the extraction yields of emodin and quercetin among the three extraction methods. The best experimental conditions for extracting polyphenolic compounds were: 5 g sample/30 ml methanol, 15 min extraction at 50°C and 50 kPa gauge pressure, for *Rhizma polygoni cuspidati*; 5 g sample/30 ml 95 % ethanol, 20 min extraction at 70°C and 40 kPa gauge pressure, for *Myrica rubra* tree leaves; and 5g sample/50 ml

50 % ethanol supplemented with HCl (concentration not specified), 10 min extraction at 50°C and 60 kPa gauge pressure for *Flos carthami*.

Although these methodologies were not optimized, enough information was provided for using the findings of the study for designing more advanced optimization experiments in the field of MAE of polyphenols from Chinese herbs. Central composite design (CCD) with multiple response optimization using the desirability function is recommended in order to determine the MAE parameters suitable for the co-extraction (from the same plant matrix) of thermo-labile and thermo-stable polyphenols. These methods can be reproduced in microwave systems with accurate temperature control (fibre optic or IR sensors under mechanical or magnetic stirring or movement from rotating sample holders) for analytical purposes; however, they might need refining through optimization procedures before being translated to pilot scale applications. For future applications of these methods in multi-mode systems, it is recommended to use larger volumes of extraction mixtures in order to avoid hot-spots formation within the volume of the extraction mixture and prevent the degradation of the thermo-labile compounds. It is well known that when small loads, that occupy less than 20% of the cavity volume, are heated in multi-mode systems, non-uniform heating and hot-spots will occur in the load (Orsat et al., 2005). If one larger volume extraction (for scale-up experiments) is carried out, the efficient microwave heating of the extraction mixture is limited by the penetration depth, thus, stirring should be provided.

The work by Wang et al. (2008) used the temperature control approach to program the MAE system, meaning that a significant amount of microwave power was directed to the samples in order to bring the temperature to the desired levels (stage 1 – ramp-to-temperature), and then the power dropped or was automatically turned on and off in order to maintain the temperature (stage 2 – hold-at-temperature). This requires setting the ramp time, and the initial power level which were not specified by the authors. Due to the fact that the microwave power

level was not among the studied factors, it was assumed that the instrument was programmed to use the maximum power output in order to achieve the set temperature, which corresponds to: 22.4 W/g for *Flos carthami*, 34.8 W/g for *Rhizma polygoni cuspidati*, and 44.7 W/g for *Myrica rubra*. Considering that the MAE processes were temperature controlled and considerable microwave power densities were used to heat extraction mixtures with good microwave heating abilities, the microwave power was probably delivered briefly and intermittently. Due to this unknown microwave delivery pattern, the microwave power densities presented above are not applicable to MAE systems that need to use the power control approach.

3.5.2. Microwave-Assisted Extraction of Polyphenols from Potato

Singh et al. (2011a) developed optimized MAE methods for extracting phenolic antioxidant compounds from potato peel. The authors used response surface methodology for process optimization, in order to maximize the yields of antioxidants as a function of the following factors: the time of extraction (min), the concentration of methanol (%), and the microwave power level (W). The microwave power was applied intermittently; the system was programmed to turn the power on/off automatically at 30 s intervals. The MAE instrument used was a mono-mode, open-vessel (Star system 2, by CEM, 800 W nominal power, 2.45 GHz) with IR temperature sensors built-in under the two extraction vessels, operating under reflux conditions. This MAE instrument does not have built-in stirring devices; thus, temperature gradients may occur during the extraction which can impact dramatically the control of microwave power delivery based on temperature readings from the IR sensors. For this reason, the authors used the power control approach which allowed for a more accurate control of the process parameters mandatory for the optimization of MAE.

An interesting aspect of this work was the selective optimization of process parameters to extract (or degrade less) of certain antioxidant compounds in order

to produce extracts with high radical scavenging activity as measured using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. The optimized MAE methods were developed for 2 g dry potato peel (Russet Burbank variety) extracted with 40 ml solvent. The optimized MAE conditions were: 15 min extraction with 67 % methanol at 102 W of programmed power for extracting total phenolic compounds expressed as gallic acid equivalents (GAE); 15 min extraction with 100 % methanol at 63 W of programmed power for extracting ascorbic acid, caffeic acid and ferulic acid; and 5 min extraction with 100 % methanol at 63 W of programmed power for extracting chlorogenic acid and obtaining potato peel extracts with a high radical scavenging activity. The ultimate purpose of developing optimised MAE processes for the extraction of bioactive compounds is to ensure the preservation of their natural qualities in the end product. The work of Singh et al. (2011a) demonstrated that when dealing with thermo-labile or oxidizable compounds the MAE processes have to be optimized for obtaining a maximum of bioactive compounds with the highest quality.

Although, the final temperature of the extracts was not specified, all the information necessary for reproducing these methods was published. The microwave power densities during the power-on cycles were: 2.96 W/g for obtaining maximum amounts of phenolic antioxidants (in terms of GAE), and 1.99 W/g for obtaining extracts with high radical scavenging activity and maximum amounts of ascorbic, caffeic, ferulic, and chlorogenic acids. These methods are useful for analytical and/or pilot-scale applications, and can be easily reproduced in other laboratories when using mono-mode MAE systems; for translation to multi-mode systems, the total volume of the extracts has to be increased while keeping the solid to liquid ratio of 2 g/40 ml, and the microwave power densities specified above. Higher extraction volumes are required for MAE in multi-mode cavities (as discussed above in section **3.5.1**) in order to avoid hot spot formation that might result in potato peel extracts with reduced antioxidant capacity; stirring should be provided.

Another study that reported a developed MAE method for extracting polyphenols from various potato samples (diced peeled and un-peeled potatoes, and downstream waste from industrial potato processing) was reported by Wu et al. (2011). An orthogonal array of 16 factorial combinations of 4 factors was used for determining the best MAE conditions. The studied factors were: the concentration of ethanol (%), the sample to solvent ratio (g/ml), and the time (min) and temperature of extraction (°C). The MAE instrument used was a multi-mode system (MES 1000, by CEM; 950 W, 2.45 GHz) provided with fibre optic and IR temperature sensors and magnetic stirring. The system was programmed to use 300 W to reach the desired temperature in 1 min then to hold the temperature for 2 - 8 min, under magnetic stirring (120 rpm). The best conditions for MAE of total phenolics from potatoes samples were selected based on one-way analysis of variance (ANOVA) of the 4 studied factors; no factorial interactions were assessed. The selected MAE conditions that achieved the highest yield of total phenolics in terms of GAE were: 0.5 g dry powder of various potato samples, 20 ml of 60 % aqueous ethanol, and 2 min of extraction at 80°C, followed by 10 min cooling time outside the microwave. This MAE method achieved a 6.8 % higher total phenolic extraction yield than conventional reflux extraction for 30 min with methanol. The MAE extracts also appeared to have higher antioxidant activity than those obtained by reflux extraction; but in fact, they were simply more concentrated in total phenolics.

The individual components contributing to the total phenolics yields were predominantly the chlorogenic and caffeic acids. It is worth noting, that in comparison Singh et al. (2011a) found that the MAE conditions that yielded the highest amounts of total phenolics were different from those which yielded the highest amounts of chlorogenic and caffeic acids (65 vs. 100 % methanol, respectively; and 2.96 vs. 1.99 W/g, respectively). The experimental design used by Wu et al. (2011) did not allow for assessing such differences. However, the two methodologies agree on the fact that aqueous alcohols are preferred for the

total phenolics extraction from potato by MAE, and indicated that most of the antioxidant activity is due to the high concentrations of chlorogenic acid.

The microwave power densities used by Wu et al. (2011) were 17.5 W/g, much higher than those of 2.96 and 1.99 W/g used by Singh et al. (2011a). Both studies used intermittent microwave power delivery; following an unknown pattern affected by the temperature fluctuations in the extraction mixtures for the work of Wu et al. (2011); and, a user controlled pattern (30 sec on/off) for the work of Singh et al. (2011a). There is sufficient information provided in this study to allow for the replication of the developed MAE methodology in other laboratories using multi-mode microwave systems. However, the translation of microwave power density (17.5 W/g) for use in mono-mode systems will not be successful in the absence of stirring during the extraction, as the system would require accurate temperature measurements in order to deliver microwave power intermittently for maintaining the required 80°C. This method would benefit from optimization by means of CCD using the desirability function in order to modulate the MAE parameters for maximizing both the total phenolic content and the antioxidant capacity of the extracts.

3.5.3. Microwave-Assisted Extraction of Polyphenols from Mushrooms

Zhang et al. (2012) developed a MAE method for extracting six phenolic compounds (gallic acid, protocatechuic acid, catechin, syringic acid, myricetin, and quercetin) from dry *Agaricus blazei muril* mushrooms. The effects of the factors: ethanol concentration (%), sample to solvent ratio (g/ml), microwave power (W), temperature (°C), duration (min) and number of extraction cycles were studied using the one-factor-at-a-time (OFAT) approach without providing statistical analysis. The microwave instrument used was a multi-mode, closed-vessel system (MAS II, by Sineo; 1000 W, 2.45 GHz) provided with magnetic and mechanical stirring (the authors did not specify if the MAEs were performed under stirring), and temperature sensors inside and outside the extraction vessel.

The system was programmed to control the temperature of the extracts and the power level during the initial stage of ramp-to-temperature. The best MAE conditions for the extraction of the six phenolic compounds from dry mushrooms were: 1g dry mushroom powder, 30 ml of 60 % ethanol, 3 extraction cycles of 5 min each at 110°C, and 500 W of programmed power. The purpose of the extraction cycles was to re-extract the sample residues; each extraction cycle was followed by 30 min of cooling down below 35°C. A comparison of MAE with reflux, ultrasonic, and maceration extraction methods using 1 g sample in 30 ml of 60 % ethanol was carried out, though no statistical analysis was provided. The extraction yields obtained with MAE were higher than those obtained with: 3 cycles of 1 h of reflux extraction at 90°C, 3 cycles of 40 min of ultrasonic extraction at 25°C, and 3 cycles of 12 h of maceration at 25°C.

The microwave power density programmed during the initial ramp-to-temperature stage was 19.1 W/g, lower than that (44.7 W/g) used by Wang et al. (2008). It is worth noting that myricetin is a thermo-labile compound, and it was extracted at 70°C by vacuum MAE by Wang et al. (2008), thus the MAE at 110°C reported by Zhang et al. (2012) might not be suitable for the precise quantification of myricetin in mushrooms. This method would benefit from further experimentation using CCD and the desirability function for determining the MAE parameters that maximize the co-extraction of both thermo-labile and thermo-stable polyphenolic compounds. This experiment provides all the necessary information for reproducing the MAE method in other laboratories using similar multi-mode microwave systems. The drawback of this MAE method is the length of the total extraction procedure of 105 min due to the 3 MAE steps of 5 min at 110°C, each followed by 30 min cooling time.

3.5.4. Microwave-Assisted Extraction of Polyphenols from Grape Seeds

Krishnaswamy et al. (2012) reported an optimised MAE method for the extraction of antioxidant phenolic compounds from grape seeds. The MAE method was

developed by studying the effects of process parameters: ethanol concentration (%), duration of extraction (min) and microwave power (W), on the extraction yields of total phenols (expressed in terms of gallic acid, tannic acid, and catechin equivalents), and the antioxidant capacity of the extracts [using the 1,1-diphenyl-2-picrylhydrazil (DPPH), and the ferric reducing antioxidant power (FRAP) assays]. The MAE parameters were optimized my means of response surface methodologies for process optimization (screening and central composite designs) using the desirability function for multiple responses optimization.

The MAE instrument used was a mono-mode, open-vessel (Star system 2, by CEM, 800 W nominal power, 2.45 GHz) with IR temperature sensors built-in under the two extraction vessels, not provided with built-in stirring devices, operating under reflux conditions. The MAE system was programmed to turn the power on and off automatically at intervals of 30 s. The optimized MAE conditions that allowed for the extraction of maximum total phenolics yields with higher antioxidant capacity were: 0.5 g dry defatted grape seed powder, 50 ml of 32.6 % ethanol, and 6 min extraction at 121 W. The microwave power density during the power-on cycles was 2.6 W/g. It is worth noting that the conditions that lead to maximum total phenols used higher power levels and lower ethanol concentrations than those which yielded extracts with higher antioxidant power (160 - 170 W and 30 – 34 % ethanol, vs. 100 W and 39 % ethanol, respectively). Thus, a multiple response optimization using the desirability function was necessary to find the general MAE conditions that satisfied all the requirements in terms of high yields of phenolic compounds with high antioxidant quality. In this case the optimized MAE conditions satisfied these requirements in proportion of 94 % (based on the desirability function).

Although the final temperature of the extracts was not specified, this work provided all the necessary information for reproducing the optimized MAE method in other laboratories using mono-mode or multi-mode microwave systems. In order to avoid un-even extraction conditions in multi-mode systems, it

is recommended to increase the total volume of the extraction mixtures while keeping the sample to solvent ratio of 1 g sample/100 ml solvent and the microwave power density of 2.6 W/g. Based on the findings of Krishnaswamy et al. (2012) and Singh et al. (2011a), it can be concluded that un-even heating and hot-spots formation should be avoided particularly when extracting antioxidant phenolics which require accurate power delivery control in order to maintain the natural antioxidant capacity of the extracts. Care should be taken when adapting the MAE parameters developed for extracting thermo-labile compounds using mono-mode systems to multi-mode systems provided with magnetic stirring devices, as the electric field concentrates around the edges of the stirring bars (Robinson et al., 2010) providing hot surfaces that can cause compounds degradation. This MAE method is applicable for analytical purposes, and can be translated to pilot scale (under mechanical stirring or movement from rotors) for production purposes.

3.5.5. Microwave-Assisted Extraction of Lignans from Flaxseed

Zhang and Xu (2007) reported a MAE method for extracting a complex lignan macromolecule, containing the lignan secoisolariciresinol diglucoside (SDG), from defatted flax hull meal. The MAE method had to be followed by a conventional alkaline hydrolysis (2 h, 0.25 M NaOH, room temperature) in order to allow for the quantification of SDG by high performance liquid chromatography (HPLC). The extraction method was developed in two stages. In stage 1, the effects of the method of pre-soaking the sample in the extraction solvent (5 min sonication at 80 W, or without sonication at 25 and 60°C, for 60 and 15 min, respectively), of the concentration of ethanol (%), and of microwave power (W) on the yield of SDG were studied using the one-factor-at-a-time (OFAT) approach, without providing statistical analysis. In stage 2, the MAE method was refined by maximizing the extraction yield of SDG in terms of ethanol concentration (%), sample to solvent ratio (g/ml), and irradiation time (s) using a CCD.

The instrument used for MAE was a multi-mode microwave oven (probably manufactured for domestic use) functioning at 5 power levels (50, 130, 220, 315, and 390 W; 2.45 GHz), and not provided with temperature monitoring or stirring devices. The microwave power was applied intermittently (on/off) by the user in stages of 10 s in order to allow the extracts to cool between two consecutive irradiation steps. It is not clear how the power levels were modulated by the instrument. In domestic microwave ovens the power levels are defined by duty cycles [e.g., 10 s duty cycle (Kabza et al., 2000) or 30 s duty cycle (Diprose, 2001)] during which the microwave power is turned on and off. For example, if the system uses 10 s duty cycles, the magnetron delivers power for 5 s out of 10 in order to achieve an average power level of 50 %. It is necessary to know the duration of the duty cycle in order to be able to translate MAE parameters optimized using domestic microwave ovens to research-dedicated microwave systems, and to industrial applications as well. In addition, the final temperature of the extraction mixtures should be specified.

The optimized MAE method involved: pre-soaking (5 min sonication, 80 W) 1 g defatted flax hull meal in 22 ml of 41 % ethanol, followed by MAE for 3 min at 130 W (power level dependent on a duty cycle of unknown duration), with intermittent power application (10 s on/off). The extraction yields achieved with the MAE method were 35 and 14.5 % higher than those obtained with Soxhlet extraction (1 g/40 ml 75 % ethanol, 2.5 h, 95°C) and with stirring extraction (1 g/20 ml 50 % ethanol, 3 h, 60°C), respectively. The calculated microwave power density programmed during the power-on extraction steps was 6.47 W/g, but in reality it could be very different depending on the duration of the duty cycle (10 or 30 s). The final temperature of the extracts was not specified, and it is not known how the MAE instrument used in this work controlled the microwave power delivery. Thus, the MAE method reported by Zhang and Xu (2007) requires further experimentation in view of determining the optimum power density using a mono-mode system, as the volume of extraction mixture is too small for accurate power control when using multi-mode systems.

Beejmohun et al. (2007) reported a MAE method for extracting SDG from pressed flaxseed cake. The method was developed using factorial experimental combinations of: microwave power (W), duration of extraction (min) and concentration of NaOH (M) in 70 % methanol, without providing statistical analysis. The MAE instrument used was a mono-mode open-vessel system (Discover by CEM; 300 W nominal power, 2.45 GHz). The best MAE conditions for extracting SDG from pressed flaxseed cake were: 0.5 g sample, 20 ml 1 M NaOH in 70 % methanol, and 3 min of extraction at 50 W (continuous power delivery). This MAE method achieved extraction yields 20.5 % higher than a conventional extraction with 70 % methanol at 60°C for 3 h followed by hydrolysis at room temperature for 3 h with 1 M NaOH. The microwave power density was 2.93 W/g, while the final temperature of the extracts was not specified. This method could be replicated for analytical purposes using mono-mode MAE systems; however, larger extraction mixture volumes, and stirring are required when using multi-mode cavities in order to achieve uniform heating.

An optimized MAE method for extracting SDG from defatted flaxseed meal was developed by Nemes and Orsat (2010, 2011b) using response surface methodology for process optimization (screening designs and CCD), and a mono-mode open-vessel microwave instrument (Star System 2 by CEM; 800 W nominal power, 2.45 GHz). The factors studied were: the microwave power (W), the mode of application of microwave power [continuous or intermittent (30 s power on/off)], the concentration of aqueous NaOH (M), and the duration of extraction (min). The optimised parameters were: 1 g defatted flaxseed meal, 50 ml of 0.5 M NaOH, and 3 min of extraction at 135 W with intermittent power application (30 s on/off) (Nemes and Orsat, 2011b). The final temperature of the extraction mixtures was 67°C (Nemes and Orsat, 2011a). It is worth noting, that although the optimum power level was 135 W, the response surface modeling of the method development data published by Nemes and Orsat (2011b) indicated that statistically similar results can be obtained for microwave power levels ranging from 100 to 160 W (corresponding to a range of programmable power level

settings in the STAR System 2 instrument of 17 to 27 %, the optimum power level setting being 22 %). The conversion of programmable power level (%) into watts was done by calculating the corresponding value from the maximum microwave power output of 600 W (Dai, 2006; Nemes and Orsat, 2010) obtained by calibration in our laboratory (unpublished data) using the calorimetric calibration method published by Cheng et al. (2006). However, a new calorimetric calibration was developed for the STAR System 2 instrument (section **4.3.1**) by adapting the method published by Swain et al. (2006) to the specific needs of the STAR System 2 instrument (results presented in **Chapter 4**, section **4.4.1**). The optimum power level setting of 22 % translates into 156 W by using the calibration **Equation 4.2** [Actual power output (W) = 7.105 × Power level (%)] presented in section **4.4.1**. According to this new information, the optimum microwave power to be programmed for replicating the optimized MAE method developed by Nemes and Orsat (2010, 2011b) is 156 W, which corresponds to a power density of 3.12 W/g.

The optimised MAE achieved SDG extraction yields greater by 6 % than a well-known reference method (Nemes and Orsat, 2011b) for analysing lignans in flaxseed using conventional direct hydrolysis at room temperature with 1 M NaOH. This optimised MAE method was further evaluated through repeatability, recovery and efficiency testing (Nemes and Orsat, 2011a). The extraction of the SDG from the flaxseed matrix was complete; the repeatability was good as demonstrated by the MAE tests which were performed equally well by different users over time; and the recovery of SDG was of 97.5 % with coefficients of variation < 1%. The efficiency was excellent in comparison with two well-known methods for analysing lignans in foods; the MAE method extracted 21.4 and 26.6 % more SDG than a conventional sequential hydrolysis method (1 h at 60°C with 0.3 M NaOH in 70 % methanol, followed by 1 h at 60°C with 0.01 M HCl), and a two-step conventional extraction method followed by alkaline hydrolysis (2 extraction steps of 2 h at 60°C with 70 % methanol, followed by 3 h hydrolysis at room temperature with 0.3 M NaOH), respectively.

The MAE method was evaluated for the analytical extraction of lignans from various flaxseed samples (different cultivars and flax hull) and a variety of oil seeds (sesame, chia, sunflower and almonds) (Nemes and Orsat, 2011a), and can be used in conjunction with optimized enzymatic hydrolysis procedures for the general analysis of lignan aglycones in plant samples (the development of the enzymatic hydrolysis conditions is presented in **Chapter 7**). The applications of this optimized MAE method are varied and well established. For example, other than the analytical applications for the quantification of lignans in plant samples (Nemes and Orsat, 2011a), the method can be used to produce purified SDG from flaxseed (Nemes and Orsat, 2011c), and to analyse the SDG content of flaxseed cultivars and derived products in conjunction with the Folin-Ciocalteu's colorimetric method, and calibrated partial least squares (PLS) and artificial neural network (ANN) predictive models (Nemes et al., 2012). This method can be easily reproduced in other laboratories whether using a mono-mode or multi-mode microwave system (requires larger volumes of extraction mixture and stirring), or it can be translated to pilot scale for the production of SDG from flaxseed.

3.6. Assessment of Trends in Microwave-Assisted Extraction of Phenolic Compounds

In order to assess the trends in MAE of phenolic compounds, a set of key-point observations based on the reviewed literature are compiled in **Table 3.2**. Generally, it was observed that using MAE as opposed to conventional extraction methods did not necessarily reduce solvent usage. It was often stated in the literature that MAE has the advantage of using less solvent than conventional extraction methods. However, the solvent volumes used were small; in the range of 20 to 50 ml for sample sizes of 0.5 - 5 g.

Table 3.2. Comparison of MAE methods in terms of development strategies, efficiency, and possible applications of the end-product

Compound/ plant source	Method development strategy	Microwave system type, control strategy	Sample/solvent (g/ml)	[1]Microwave power density (W/g extract)	Comparison with other methods	Possible applications of developed MAE method	Reference
Resveratrol, emodin/ *Rhizma polygoni cuspidaty*	[2]OFAT, without statistical analysis	Multi-mode, temperature control	5 g/30 ml methanol	34.8 - RT	yes	Analytical purposes in multi-mode systems; optimization by [3]CCD using the desirability function	(Wang et al., 2008)
Myricetin, quercetin/ *Myrica rubra*			5 g/30 ml 95 % ethanol	44.7- RT	yes		
Safflomin A/ *Flos carthami*			5 g/50 ml 50 % ethanol	22.4- RT	yes		
Total phenolics/ potato peel	Response surface optimization (CCD)	Mono-mode, microwave power control	2 g/40 ml 67 % methanol	2.96 - IPA	no; results in the range of literature data	Analytical purposes, and translation to pilot scale	(Singh et al., 2011a)
Chlorogenic, caffeic, ferulic, and ascorbic acids/ potato peel			2 g/40 ml methanol	1.99 - IPA			
Total phenolics, chlorogenic and ferulic acids/ potato, and industrial downstream waste	OFAT, orthogonal array	Multi-mode, temperature control	0.5 g/20 ml 60 % ethanol	17.5 - RT	yes	Analytical purposes in multi-mode systems; optimization by [3]CCD using the desirability function	(Wu et al., 2011)

[1]The Microwave power densities (W/g) apply to the initial stage of ramp-to-temperature (RT) for the MAE methods that used the temperature control approach, and to the power-on cycles for the MAE methods that used intermittent power application (IPA) approach defined by the user. [2]OFAT stands for one-factor-at-a-time. [3]CCD stands for central composite design.

Compound/ plant source	Method development strategy	Microwave system type, control strategy	Sample/solvent (g/ml)	[1]Microwave power density (W/g extract)	Comparison with other methods	Possible applications of developed MAE method	Reference
Catechin, myricetin, quercetin, & gallic, protocatechuic, and syringic acids/ *Agaricus blazei muril*	[2]OFAT, without statistical analysis	Multi-mode, temperature & microwave control	1 g/30 ml 60 % ethanol	44.7 - RT	yes	Analytical purposes in multi-mode systems; optimization by [3]CCD using the desirability function	(Zhang et al., 2012)
Total phenolics with high antioxidant capacity/ grape seeds	Response surface optimization, screening design and CCD	Mono-mode, microwave power control	0.5 g/50 ml 32.6 % ethanol	2.6 - IPA	no; results in the range of literature data	Analytical purposes, and translation to pilot scale	(Krishnaswamy et al., 2012)
SDG/ flax hulls	OFAT, and response surface optimization (CCD)	Multi-mode, power control	1 g/22 ml 41 % ethanol	6.47 - IPA	yes	Analytical purposes in mono-mode systems	(Zhang and Xu, 2007)

[1]The Microwave power densities (W/g) apply to the initial stage of ramp-to-temperature (RT) for the MAE methods that used the temperature control approach, and to the power-on cycles for the MAE methods that used intermittent power application (IPA) approach defined by the user. [2]OFAT stands for one-factor-at-a-time. [3]CCD stands for central composite design.

Compound/ plant source	Method development strategy	Microwave system type, control strategy	Sample/solvent (g/ml)	[1]Microwave power density (W/g extract)	Comparison with other methods	Possible applications of developed MAE method	Reference
SDG/ pressed flaxseed cake	[2]OFAT, without statistical analysis	Mono-mode, microwave power control	0.5 g/20 ml 1 M NaOH in 70 % methanol	2.93 - CP	yes	Analytical purposes	(Beejmohun et al., 2007)
SDG/ flaxseed, oil seeds, and plant food in general	Response surface optimization, screening designs and [3]CCD	Mono-mode, microwave power control	1 g/ 50 ml 0.5 M NaOH	3.12 - IPA	yes	Analytical purposes, and translation to pilot scale	(Nemes and Orsat, 2010, 2011a, b)

[1]The Microwave power densities (W/g) denoted CP and IPC apply to a MAE method that used continuous power delivery, and to the power-on cycles of a MAE method that used intermittent power application approach defined by the user, respectively.
[2]OFAT stands for one-factor-at-a-time. [3]CCD stands for central composite design.

Reductions in the concentrations of solvents or reagents were observed with MAE, when the method development strategy used response surface optimization procedures, which allowed for assessing two-factor interactions and the quadratic effects of the solvent concentration factor (Krishnaswamy et al., 2012; Nemes and Orsat, 2010, 2011b; Singh et al., 2011a; Zhang and Xu, 2007). Such designs are recommended as opposed to factorial combinations (orthogonal arrays) which are carried out according the one-factor-at-a-time (OFAT) approach and are analysed using one-way ANOVA. OFAT designs do not allow for assessing the interactions among the studied factors and identifying the optimum extraction conditions (Leardi, 2009). The so called "optimum" conditions identified by OFAT and any other factorial designs that do not use regression analysis are in fact limited to, and localized at the factors' levels chosen by the researcher (Leardi, 2009).

In the case of MAE, for at least one of the studied factors, the optimum level would not coincide with the fixed experimental level (Krishnaswamy et al., 2012; Nemes and Orsat, 2011b; Singh et al., 2011a). Therefore, among the reviewed literature, only the MAE methods which were developed by means of response surface methodology using central composite designs were considered truly optimized. It is worth noting that using response surface methodologies (e.g., screening design and CCD) requires reduced experimental time and efforts, provides the most informative results, and allows for a true optimization of the MAE process parameters (Leardi, 2009; Lundstedt et al., 1998).

In general, when multi-mode microwave systems were used, the MAE methods were developed using OFAT designs, often without providing statistical analysis; and the control of the MAE systems was achieved through temperature control approach using higher microwave power densities (6.47 – 44.7 W/g) during the initial ramp-to-temperature stage. It appears that, the interest for developing mild yet efficient extraction methods was lacking when using multi-mode systems provided with stirring devices and accurate (or perceived as accurate) temperature

monitoring; an observation confirmed earlier by Bélanger and Paré (2006). Using the temperature control approach in such microwave systems seems to be error-proof; hence the lack of interest for using optimization procedures. However, as a result, the developed MAE methods cannot be translated to mono-mode systems that lack stirring devices without the re-optimization of the process parameters.

Most of the MAE methods developed using mono-mode systems and power control approach were optimized by means of CCD. These methods used lower microwave power densities (1.99 – 3.12 W/g) which are more appropriate for extracting phenolic compounds or any other bio-active compounds from plants. Two examples of selective MAE were observed for the extraction of total phenolics from potato peel (Singh et al., 2011a), and grape seeds (Krishnaswamy et al., 2012). In these two cases, the MAE conditions that achieved the highest yields of total phenolics did not coincide with those that allowed for producing extracts with high antioxidant capacity. In order to modulate the MAE parameters to influence the composition of the extracts towards extracting more (or degrading less) chlorogenic acid from potato peel (Singh et al., 2011a), and catechin compounds from grape seeds (Krishnaswamy et al., 2012), which exhibited high antioxidant activities, the microwave power and the polarity of the extraction solvents had to be reduced. The MAE methods optimised for mono-mode microwave systems can be easily applied in multi-mode systems and translated to pilot scale for the production of phenolic extracts.

In general, the superiority of MAE was proven by comparison with conventional extraction methods, and the gains due to using microwave energy for extraction were dramatically reduced extraction times (2 - 15 min) and increased extraction yields.

3.7. Recommendations for Future Microwave-Assisted Extraction Research

To ensure that the published MAE methods can be replicated in other laboratories, it is mandatory to describe the type of microwave instrument used, and how the

power delivery and temperature control were achieved. It was observed that confusion has arisen in the literature regarding the type of microwave cavity; the atmospheric pressure MAE in multi-mode cavity was mistakenly reported as focused (mono-mode) MAE (Kaufmann and Christen, 2002; Wang et al., 2008; Wang and Weller, 2006). When the temperature control-approach was used in multi-mode MAE systems, the initial power levels and/or the duration of ramp-to-temperature stages were often not reported. According to Kappe et al. (2009b) any publication reporting microwave-assisted chemistry methods that use the temperature control approach should specify how the ramp-to-temperature stage was programmed. The duration of the ramp-to-temperature stage increases with increasing volumes of extraction mixtures. It is important to select an appropriate initial power level in order to avoid overshoots of temperature, which could lead to the degradation of thermo-labile compounds from the beginning of the MAE. Some microwave systems designed for synthesis allow for programming the initial power levels depending on the solvents' capacities to absorb microwave energy. For example, for medium and low microwave energy absorbing solvents, the recommended power levels are 2.5 and 4 times greater than that recommended for good microwave energy absorbing solvents, respectively (Kappe et al., 2009c). For the MAE of bio-active compounds, the power densities reported in **Table 3.2** for the methods developed using the intermittent power application (IPA) approach in mono-mode systems can be used as a guideline. All the extraction solvents used in these cases had good microwave heating capacities. The microwave power density averaged over the total extraction time (includes the power off time) for the methods that used IPA is 1.34 W/g. Thus, 1.34, 3.35 and 5.36 W/g [3.35 and 5.36 were obtained by multiplying 1.34 with 2.5 and 4, respectively, according to the recommendations given by Kappe et al. (2009c)] could be used to program the initial power level in multi-mode systems using the temperature-control approach when the extraction solvent has good, medium, and low microwave heating abilities, respectively.

The accurate temperature monitoring during MAE depends on: (1) using appropriate extraction mixture volumes within the range specified by the manufacturer of the microwave system; (2) efficient magnetic or mechanical stirring; (3) the type of temperature sensor used; and (4) the calibration of the IR sensor (Kappe et al., 2009b; Robinson et al., 2010). The mono-mode systems are recommended for smaller scale applications (up to 150 ml) and the multimode-systems for larger scale applications (up to several liters) (Kappe et al., 2009b). The volumes of the extraction mixtures presented in **Table 3.2** are in the ranges of 20 - 50 ml for both mono- and multi-mode microwave systems. It should be kept in mind that the multi-mode microwave systems are designed for carrying multiple chemical reactions using smaller volumes in parallel (many replicates), or one larger volume chemical reaction (Kappe et al., 2009b). The temperature monitoring in a microwave environment is problematic. The IR sensors measure the radiation, from a distance, on the surface of the extraction vessel (bottom or side of the vessel depending on the system) and converts it to temperature, which will generally be lower than the inside temperature even with appropriate stirring. It is worth noting that the IR sensors use one-point calibration, often done at 100°C while boiling water and measuring the temperature with a fiber optic, which means that the IR sensor should only be used to monitor temperatures close to 100°C. The use of magnetic stir bars and fiber optics in the extraction vessel will cause significant concentrations of the electric field density around these objects and can cause localised heating (Robinson et al., 2010). Thus, using magnetic stirring in an attempt to achieve homogeneous heating might result in hot-spots around the edges of the magnetic stir bar which can lead to the degradation of the thermo-labile compounds, and reduce the quality of natural plant extracts. Choosing the appropriate volume of extraction mixture for the type of cavity is important not only for obtaining a homogeneous microwave heating but also for an efficient absorption of the microwave energy by the mixture. If too small volumes are heated in mono-mode cavities, most of the microwave energy will be reflected and will lead to big disparities between the programmed power output and the absorbed microwave energy (Robinson et al., 2010). If too small

volumes are heated in multi-mode cavities, inefficient heating and hot- and cold-spots can result due to the uneven distribution of the multiple modes of propagation of the microwaves inside the cavity (Lidström et al., 2001; Orsat et al., 2005), and adversely affect the extraction yield and the quality of the extracts. In both cases, the reported microwave power levels lose their meaning and the translation of MAE methods from one system to another will not be successful. The translation of reported power levels is further complicated by the fact that the nominal power output declared by the manufacturers can differ significantly from the actual power outputs (Cheng et al., 2006) which in turn vary significantly as a function of the calibration method used as discussed in section **3.5.5**. Based on these observations, in order to translate power densities from one system to another, the researchers could start by carrying out initial tests using the values reported by the developers of the methods, while accurately monitoring the temperature during the MAE (for methods developed using the temperature control approach) or measuring the final temperature of the extraction mixture (for methods developed using the power control approach). If the achieved conditions differ from the target ones, the researchers should consider varying the power density (e.g., 5 % at a time) until the optimum MAE conditions reported by the developer of the method are reached.

For modifying the scale of the extraction process, the volume of the extraction mixture should be increased while keeping the power density in W/g for the applications that use the power control approach, and also increasing the duration of the ramp-to-temperature stage for the applications that use the temperature control. It should be kept in mind that non-uniform heating and hot- and cold-spots can occur when using multi-mode systems for MAE regardless of the volume of the extraction mixture. The multiple modes of propagation of microwaves inside a multi-mode cavity form multiple hot- and cold-spots, which are “pockets” with different electric field strength intensities (Kappe et al., 2009b; Lidström et al., 2001; Orsat et al., 2005). The depth of penetration of microwaves in extraction mixtures with good and medium microwave heating abilities is an

additional factor to consider when carrying out large scale MAEs. Thus, stirring should be always provided when carrying out MAE in multi-mode cavities by means of movement from rotors or mechanical stirring (using microwave-transparent stirring devices) when extracting thermo-labile compounds, or by means of magnetic stirring when extracting thermo-stable compounds.

3.8. Conclusions

In this chapter, the mechanisms that govern the MAE of natural compounds from plant matrices were described; a critical review of MAE of plant phenolic compounds was presented; and recommendations for future research in the field were provided. This work is useful for replicating and further improving existing MAE methods, and for developing new optimized MAE methods for analytical applications that require accurate quantification of bio-active compounds (nutraceuticals) in plants. Recommendations were made for helping researchers translate small scale MAE methods to pilot scale for productions purposes. This work was considered necessary for advancing the field of MAE of natural compounds as currently many of the published methods are difficult to reproduce due to the less desirable method-development approaches used, or to missing information. This work is different from other published literature reviews of concern for the MAE of natural compounds due to the following aspects: (1) the methodology of review used the power density concept for comparing different methods and for understanding the microwave power density requirements for the MAE of nutraceuticals; (2) the interpretation of results accounted for the type of experimental designs and statistical analyses used in order to identify the strengths and the weaknesses of the reviewed methods; and (3) the results of the review in conjunction with the MAE theory were used to make recommendations for future research in the field of MAE of plant sourced chemicals.

Connecting Statement to Chapter 4

In **Chapter 3**, the theoretical aspects related to the microwave-assisted extraction (MAE) technique have been presented and recently published MAE methods for plant phenolic compounds have been critically reviewed. The control of the extraction temperature and of the microwave power level is problematic during MAE processes. The accurate temperature monitoring during the MAE process is subject to physical limitations within the extraction system. Thus, often, the successful replication of MAE methods published in the literature depends on the correct application of the microwave power energy. The successful replication of published microwave power levels depends on (1) respecting the ranges for the reaction volumes recommended by the manufacturers of MAE systems, (2) on calibrating the actual microwave power output as this differs from the nominal microwave power declared by manufacturers, and (3) on the calibration methodology used.

In order to help understand the factors that influence the correct microwave power applications during MAE processes, the calibration of the microwave power output in the MAE system used in this thesis has been carried out. A calibration reference method is adapted for the specific needs of the MAE instrument (Star System 2) used in this thesis. The results are presented in **Chapter 4**.

Chapter 4 is written in manuscript style and is soon to be submitted for publication as follows.

- Nemes, S.M., Orsat, V., Raghavan, G.S.V., 2012. Calibration of Microwave Power Output in a Research-Dedicated Microwave-Assisted Extraction System

Chapter 4 - Calibration of Microwave Power Output in a Research-Dedicated Microwave-Assisted Extraction System

4.1. Abstract

The successful control of microwave power during microwave-assisted extraction (MAE) processes depends on using calibration and understanding how the instrument controls the incident power level. In this work, a MAE system with one magnetron, and two mono-mode cavities placed along one waveguide was used for calibrating the actual microwave power outputs achieved in the two cavities when these were used individually and simultaneously. The power outputs were calibrated by heating loads of 200 ml distilled water using the calorimetric calibration method. The results revealed that the maximum actual power output was 11.2 % lower than the nominal microwave power claimed by the manufacturer of the instrument. The distribution of the microwave field was achieved in a similar fashion when the two cavities were used individually, but its intensity was 11.7 % lower in the second cavity when the two cavities were used simultaneously. The two cavities cannot be used for carrying simultaneous replications due to the significantly lower microwave power output achieved in the second cavity. The results and discussions presented in this chapter are useful to researchers as they address several aspects critical for the correct application of desired microwave power levels during MAE processes.

Key words: microwave power output, calorimetric calibration, power level, calibration equation, mono-mode cavity

4.2. Introduction

Microwave-assisted extraction (MAE) was identified as an attractive replacement technique for the conventional Soxhlet extraction in the late 1980s as the former

was faster, more efficient and prevented the degradation of thermo-labile compounds (Ganzler and Salgo, 1987). At the beginning of the 1990s, research-dedicated microwave systems designed for digesting environmental samples in closed vessels were used for extraction processes. The experimental parameters studied for developing MAE processes in closed vessel systems were: the solvent composition and volume, the extraction time and temperature, and the matrix characteristics. Among these factors, the extraction temperature was the parameter the most studied because solvents could be heated above their atmospheric boiling points in closed vessels and could enhance the efficiency and the speed of extraction for environmental samples (Eskilsson and Björklund, 2000). Later, Nóbrega et al. (2002) and Bélanger and Paré (2006) recommended the use of open vessel mono-mode systems for developing gentler and more selective MAE processes that prevent the degradation of thermo-labile compounds as opposed to extracting the matrix exhaustively at high temperatures and pressures in closed vessel multi-mode systems. The experimental factors studied for developing MAE methods when using mono-mode microwave systems were: the characteristics of the sample, the solvent volume, the microwave power level, and the time of extraction (Nóbrega et al., 2002). More recently, Desai et al. (2010), Routray and Orsat (2011), and the results presented in **Chapter 3** confirmed the importance of controlling the microwave power level during the development and the optimization of MAE processes for natural compounds (nutraceuticals).

The shift from temperature control in multi-mode systems to microwave power control in mono-mode systems could be due to the type of technology used for temperature measurements in these systems (**Chapter 3**). Kappe et al. (2009b) described the most known commercially available mono- and multi-mode microwave systems designed for chemistry applications. While in general, multi-mode systems allow for direct temperature measurements in the reaction vessels by means of immersed temperature probes (fibre optic, or gas balloon thermometer), many mono-mode systems use remote IR sensors for measuring the temperature on the outer surface of the reaction vessels. It is known that the

temperature within the reaction mixture is not accurately reflected by the temperature of the outer surface of the reaction vessel (Herrero et al., 2008) even if efficient stirring is used (Robinson et al., 2010). This is due to the selective and volumetric nature of the microwave heating which leads to inversed temperature gradients (temperatures higher within the load inside the vessel than on the outer surface of the vessel), to decreasing temperature gradients (temperatures decreasing within a volume of reaction mixture from the top to the middle and to the bottom of the vessel in mono-mode systems not using stirring), and to the limited depth of penetration of microwaves into the volume of reaction mixtures (Herrero et al., 2008; Kappe et al., 2009a, b). Temperature differences of 15 to 40°C were recorded with fiber optic between the bottom and the middle of the vessels when heating solvents with good microwave heating abilities in mono-mode systems without stirring. However, the temperature gradients could be minimized (< 6°C) when using effective magnetic stirring (Herrero et al., 2008).

Although temperature is known to be a major driving factor in chemical processes, it cannot always be controlled accurately during the MAE processes due to physical limitations within the extraction systems. Given, that microwave heating is the result of the interactions (dipolar polarization and ionic conduction) occurring between microwave energy and materials over time (Meda et al., 2005; Orsat et al., 2005; Venkatesh and Raghavan, 2004) the microwave power, the volume and composition of the extraction mixtures could be easily used as independent factors to be controlled during experimental optimization of MAE. There are two approaches used for controlling the heating of reaction mixtures during microwave-assisted chemical processes, the temperature control approach and the power control approach (Collins and Leadbeater, 2007). The temperature control approach has two stages, first it requires setting of the power level, the desired temperature and the duration of the initial stage of ramp-to-temperature (Kappe et al., 2009b), and then setting the duration of the maintain-at-temperature stage during which the power drops or is delivered intermittently by the system to prevent overheating (Collins and Leadbeater, 2007). The power control approach

involves setting the power level (calculated as percentage from the nominal power of the system) and the duration of power delivery in order to allow the temperature of the reaction mixture to rise continually (Collins and Leadbeater, 2007) until the desired threshold is met (Nemes and Orsat, 2011a) or to allow the reaction to proceed under reflux conditions at the boiling point of the solvents (Krishnaswamy et al., 2012; Singh et al., 2011a).

With the ongoing interest for developing and/or using optimized MAE methods for efficiently extracting natural compounds from a variety of samples, it is useful to raise the awareness of the users on MAE systems and the implications of appropriately setting the microwave power level. Costa et al. (2001) proved the necessity of calibrating the actual microwave power output in a system that used a single magnetron to deliver microwave power to six mono-mode cavities placed along a single wave guide (Star Systems 6, 2.45 MHz, 950 W nominal power; made by CEM, Mathews, NC, USA) as this varied by as much as 8.5 and 42 % among the 6 cavities when they were used individually and simultaneously, respectively. Cheng et al. (2006) showed that the actual microwave power output in a multi-mode system was 29 % lower than the nominal power declared by the manufacturer. Swain et al. (2006) demonstrated that the microwave power output in domestic microwave ovens diminished by 7 to 20 % during the first 5 min of continuous use, and by an average 17 % after 30 min of continuous use.

The objective of this work is to calibrate the actual microwave power output in a research-dedicated microwave system (Star System 2, CEM) that has two mono-mode cavities placed along the same wave guide. For this purpose, a calibration reference method was adapted to the specific needs of the Star System 2 instrument, in order to obtain accurate calibration data. The microwave power outputs were calibrated using regression analysis for the two cavities when used individually and simultaneously. The results presented here are useful for understanding the functioning of this type of microwave system in order to

correctly set the power levels during MAE method development studies, and for successfully reproducing optimized MAE methods reported in the literature.

4.3. Materials and Methods

4.3.1. Calculation of Actual Microwave Power Output of the Microwave-Assisted Extraction System

The actual microwave power outputs (*P*, **Equation 4.1**) in the two mono-mode cavities of the MAE apparatus (Star System 2, CEM, Mathews, NC, USA; frequency 2.45 GHz, nominal power 800 W) were calculated using a calorimetric method (Swain et al., 2006). The temperature differences (ΔT, **Equation 4.1**) from room temperature (22 - 23°C) were recorded after heating 200 g distilled water in borosilicate glass vessels (volume 250 ml) for 45 s. The heating experiments were carried out in triplicates at 100, 70, 40, and 10 % of the nominal power claimed by the manufacturer while using the two cavities individually; and at 100, 80, 60, and 40 % while using the two cavities simultaneously. When using the two cavities simultaneously, the incident power level is divided between the two cavities; thus, it was hypothesised that power levels < 40 % (e. g., 10 or 20 %) would be too low for obtaining accurate ΔT data. The temperature was measured before and after heating with a type K thermocouple (accuracy ± 0.5°C, Fisher Scientific) after briefly and vigorously stirring the water with a glass rod.

$$P = \frac{(M_w \times C_{pw} + M_v \times C_{pv}) \times \Delta T}{t} \qquad \textbf{Equation 4.1}$$

Where, P = the actual microwave power output [W]

M_w = the mass of water [g]

M_v = the mass of the borosilicate glass vessel [g]

C_{pw} = the specific heat of water [4.184 J/(g °C)]

C_{pv} = the specific heat of borosilicate glass [0.75 J/(g °C)]

ΔT = the temperature difference [°C]

t = the time of heating [s]

There are three potential sources of error when using the **Equation 4.1** for estimating the microwave power output: the changes in the heat capacity of water, the heat loss from the load of water to the surroundings (vessel, and the air surrounding the vessel), and the changes in the dielectric properties of water (Kingston and Jassie, 1986). The maximum temperatures recorded with this experiment were below 60°C [estimated heat capacity variation from 23 to 60°C < 0.13 % (Kingston and Jassie, 1986)], thus the errors due to changes in the heat capacity are considered negligible. The errors due to heat loss to the surroundings were minimized by accounting for the mass of the container that was in contact with the water [as opposed to not accounting for the mass of the container (Cheng et al., 2006; Costa et al., 2001; Kingston and Jassie, 1986)], and by minimizing the heating time [45 s in this experiment as opposed to 1 min used by Costa et al. (2001) and 2 min used by Kingston and Jassie (1986)]. In order to calculate the mass of the vessel that was in direct contact with the water, and thus heated by conduction, the volume of the vessel (measured by displacing water at 25°C in a 1 L graduated cylinder by fitting the menisci of the water inside and outside the vessel at the 1 L graduation) was multiplied with the density of borosilicate glass (2.23 g/cm^3). The changes in the dielectric properties of water with increasing temperatures are thought to affect the translation of the incident microwave power into observed (actual) power by decreasing the latter (Kingston and Jassie, 1986), but the significance of this decrease is not known as it cannot be quantified with **Equation 4.1**. The values of the loss tangent for water decrease from 0.150 at 25°C to 0.055 at 60°C, which corresponds to a 63.5 % reduction in the microwave heating ability, but in the same time the depth of penetration of microwaves in water increases from 1.49 cm at 25°C to 4.03 cm at 60°C (section **3.3.2**), which corresponds to a 170 % increase. Considering the complexity of heating dielectric materials in a microwave field, the results obtained with **Equation 4.1** are not perfectly reflecting the true microwave power output. However, this calorimetric method is widely used by manufacturers (Cheng et al., 2006; Swain et al., 2006) and accepted by researchers due to its simplicity and practicality.

4.3.2. Statistical Analysis

Regression models were fitted to the data obtained with **Equation 4.1** for each of the 2 cavities when used individually and simultaneously using the JMP 8.0.2 software (SAS Institute Inc., Cary, NC, USA); the tests were considered significant at $p < 0.05$.

4.4. Results and Discussion

The microwave system (STAR System 2; STAR is an acronym for Simultaneous Temperature Accelerated Reactions) used in this work was designed for digestion chemical processes. The description of the system can be found on the web site of the manufacturer (CEM at www.cem.com). The system comprises a magnetron that delivers power to two mono-mode cavities placed along the same waveguide. One IR sensor is placed underneath each of the two cavities and is used to send temperature feedback to the system. The system achieves temperature control in each cavity (independent of each other) by opening or closing the waveguide slots (Wu et al., 2002). The slots (apertures) are placed between the waveguide and each of the cavities and allow for controlling the delivery of microwave power to the cavities in an intermittent fashion (open slot/close slot) based on the temperature feedback from the IR sensors without varying the output of the magnetron (Moses and Cousins, 1997). When the microwave power is on, the slots are open and allow the passage of microwaves from the waveguide into the cavities. When the temperatures specified in the programmed methods are reached, the slots close automatically thus blocking the passage of microwaves from the waveguide to the cavities. The microwave power can be programmed between 1 and 100 % (in 1 % increments) calculated from the nominal microwave power output of 800 W. This microwave system does not have built in stirring devices.

As discussed in sections **4.2** and **3.7** , the feedback based on temperature readings from IR sensors in the absence of stirring does not allow for accurately controlling

the process kinetics during MAE of phyto-compounds, due to important volumetric and inverted temperature gradients. Therefore, the power control approach has been often used in such situations during the development and optimization of MAE methods (Desai et al., 2010; Krishnaswamy et al., 2012; Nemes and Orsat, 2010, 2011b; Nóbrega et al., 2002; Routray and Orsat, 2011; Singh et al., 2011a). In order to correctly program the desired power levels in any given microwave system it is first necessary to calibrate the actual microwave power output.

4.4.1. Calibration of the Actual Microwave Power Output in the Two Cavities Used Individually

All the calibration experiments were carried out with fresh loads of 200 ml of distilled water equilibrated at room temperature in the vessels. The microwave system was programmed to use 1 s ramp time in order to start delivering the microwave power (100, 70, 40, and 10%) into the cavities, and a programmed temperature impossible to attain while heating water for 45 s (e.g., 150°C) such as to prevent the closing of the slots due to temperature feedback from the IR sensors.

Initial regression models were fitted for the two cavities using the actual microwave power outputs calculated in triplicates at 100, 70, 40 and 10 % of the nominal power. The results are depicted in **Figure 4.1**; both models were highly significant ($p < 0.0001$) and had non-significant lack of fit tests ($p > 0.05$) and $R^2 > 0.99$. However, these models were considered unsatisfactory due to the significance of the quadratic effects of the power level factor ($p = 0.0116$ for cavity 1, and $p < 0.0001$ for cavity 2). The recorded ΔT values ranged from 3 to 34.5°C for cavity 1, and from 3.1 to 32.5°C for cavity 2. It is hypothesised that significant errors were induced in the calculation of power outputs at 100 % power levels due to higher temperatures (> 50°C, discussion follows) achieved in the loads of water (55.3 - 58.6°C for cavity 1, and 55.1 - 55.6°C for cavity 2),

which might have caused the upper body of the vessel, which was not in contact with the water, to heat at a certain extent by conduction. These errors translated into significant quadratic regression terms for both cavities, as the heat loss from the water to the upper body of the vessels caused the observed actual power outputs to be lower (hence, the downward curvature of the regression lines) than the incident power at 100 % power levels.

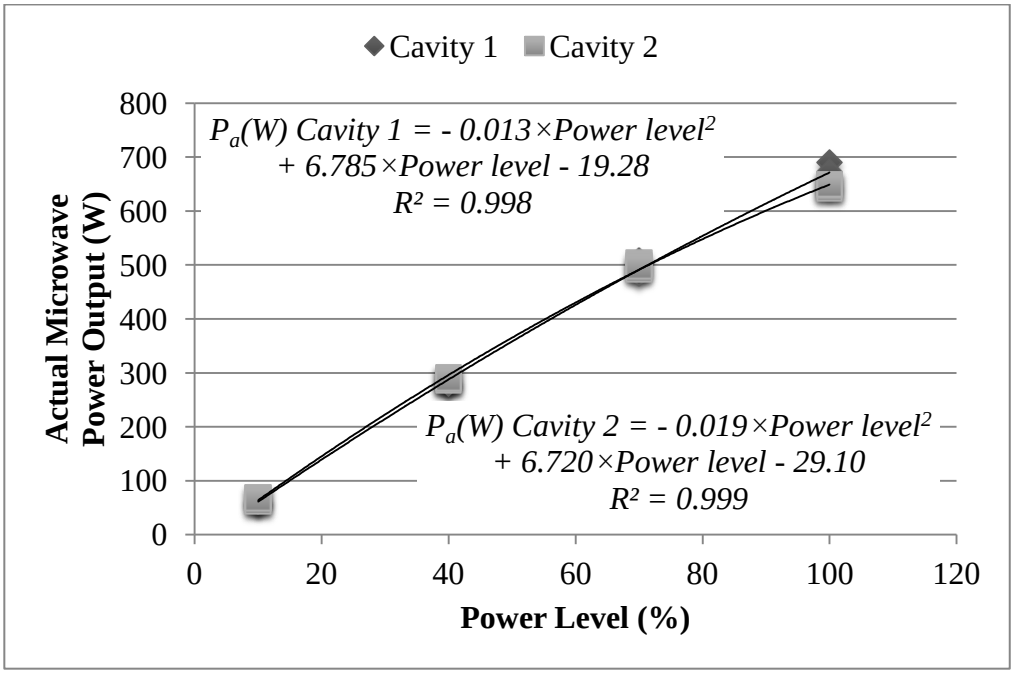

Figure 4.1. Initial quadratic models fitted for the two cavities when used individually

Further, a simple linear regression model was fitted to the actual power output data as a function of cavity (1 and 2) and power level (70, 40 and 10%). The results are graphically represented in **Figure 4.2**. The model was significant ($p < 0.0001$) and had R^2 and $R^2_{adj.} = 0.999$. The lack of fit test was not significant ($p = 0.293$). The actual power outputs were similar in the two cavities when used individually (cavity factor not significant, $p = 0.105$), and the distribution of the microwave field was achieved in similar ways in both cavities (cavity × power level interaction not significant, $p = 0.616$). These results indicated that the actual

power output data can be accurately described by simple linear regression models over the range of 10 to 70 % power levels. It appears that the errors due to heat losses by conduction from the water loads to the upper body of the vessels are acceptable when the final temperature of the water is < 50°C.

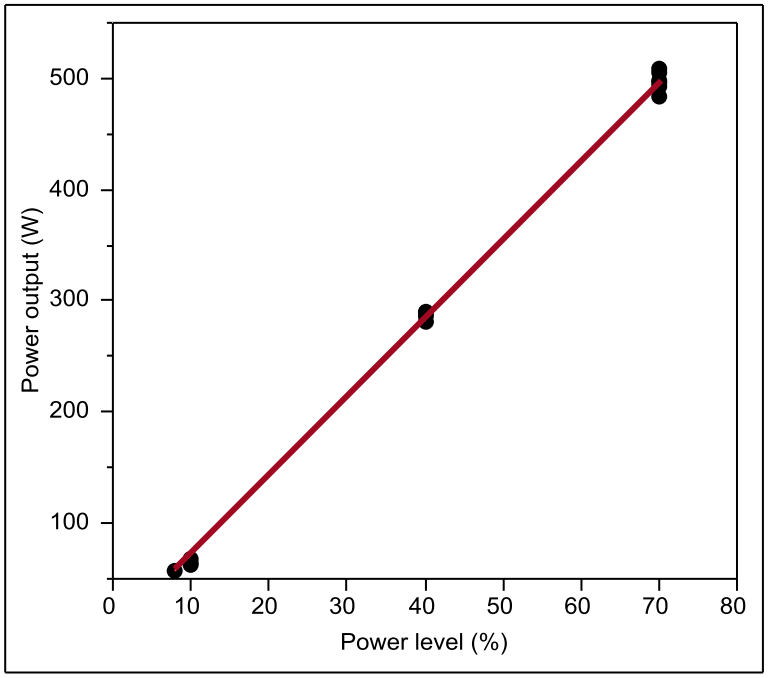

Figure 4.2 General regression line fitted for the two cavities when used individually

One general regression model (**Equation 4.2**) was established for predicting the actual power outputs (P_a) in the two cavities when used individually over the range of 10 to 70 % power levels. The parameter estimate (7.105) was highly significant ($p < 0.0001$) and had a standard error of 0.033.

$$P_a(W) = 7.105 \times Power\ level\ (\%) \qquad \textbf{Equation 4.2}$$

The maximum actual power output was calculated by extrapolation using **Equation 4.2**, and was found to be 710.5 W, which represents 88.8 % of the

nominal microwave power output claimed by the manufacturer of the microwave system.

4.4.2. Calibration of the Actual Microwave Power Output in the Two Cavities Used Simultaneously

The heating experiments using the two cavities simultaneously were carried out in triplicates as described above (section **4.4.1**) at 100, 80, 60 and 40 % of the nominal microwave power. An initial regression model was fitted for the actual power outputs as a function of cavity (1 and 2) and power levels (100, 80, 60 and 40 %) in order to find out if the intensities of the microwave field across the various power levels differed significantly between the two cavities when used simultaneously. The ΔT values ranged from 8.3 to 19.9°C for cavity 1, and from 7.3 to 17.5°C for cavity 2. As it can be seen from **Figure 4.3** significantly higher microwave power outputs were achieved in cavity 1 ($p < 0.0001$, $R^2 = 0.975$, $R^2_{adj.} = 0.971$, the lack of fit test was not significant $p = 0.475$). Thus, this requires calibrating individual regression lines for the two cavities when used simultaneously. Based on these results it can be concluded that the two cavities cannot be used for carrying out simultaneous replications of the same method while using the power control approach. These results are in agreement with the findings of Costa et al. (2001) who reported that the intensity of the microwave field varied by as much as 42 % among six mono-mode cavities placed on the same wave guide in a Star System 6 (made by CEM).

The regression model for predicting the actual power output in cavity 1 is shown in **Equation 4.3**. The model was highly significant ($p < 0.0001$) and had $R^2 = 0.974$, and $R^2_{adj.} = 0.971$. The lack of fit test was not significant ($p = 0.505$) indicating that the linear model fitted well the actual microwave power outputs in cavity 1. The intercept was significant ($p = 0.0226$) and had a standard error of 14.0. The regression coefficient for the power level term was highly significant ($p < 0.0001$) and had a standard error of 0.18.

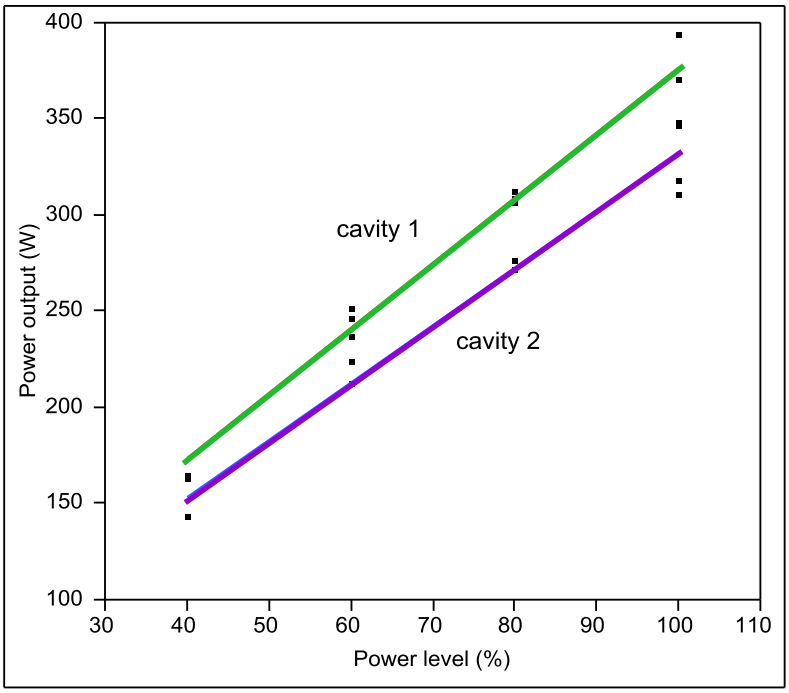

Figure 4.3 Regression lines fitted for the two cavities when used simultaneously

$$P_a(W) = 38.429 + 3.389 \times Power\ level\ (\%)$$

Equation 4.3

The regression model for predicting the actual microwave power output in cavity 2 is shown in **Equation 4.4.** The model was highly significant (p < 0.0001) and had R^2 = 0.973, and $R^2_{adj.}$ = 0.971. The lack of fit test was not significant (p = 0.364) indicating that the linear model fitted well the actual microwave power outputs in cavity 2. The intercept was significant (p = 0.0223) and had a standard error of 12.4. The regression coefficient for the power level term was highly significant (p < 0.0001) and had a standard error of 0.16.

$$P_a(W) = 34.258 + 2.979 \times Power\ level\ (\%)$$

Equation 4.4

The maximum actual power level outputs achieved in cavities 1 and 2 when used simultaneously were 376.3 W and 332.2 W (11.7 % lower than in cavity 1), respectively. The total maximum microwave power delivered simultaneously was 708.5 W, which corresponds to 88.6 % of the nominal microwave power output, and it is close to the 710.5 W extrapolated using **Equation 4.2**.

4.4.3. Considerations for Correctly Setting the Microwave Power Level During Microwave-Assisted Extraction Studies

The microwave system used in this study controls the heating of samples in the two mono-mode cavities by opening and closing the waveguide slots (placed between the waveguide and the two cavities) based on feedback from the IR temperature sensors placed underneath the reaction vessels. The role of the slots is to allow the passage of microwaves from the waveguide to the cavities until the desired temperature is reached, upon which the slots are closed automatically. The opening of and the closing of the slots do not control the microwave power output of the magnetron (Moses and Cousins, 1997). The system is not provided with built-in stirring devices, which can cause important temperature gradients to occur within the volume of the reaction mixtures. The surface temperatures read by the IR sensors at the bottom of the reaction vessels in mono-mode cavities without stirring are lower than those achieved within the reaction mixtures in the lower part of the vessel, and much lower than those reached at the middle and close to the surface of the reaction mixtures (Herrero et al., 2008; Robinson et al., 2010). It appears thus, that using the temperature control approach in such microwave systems while carrying out studies of MAE of phyto-compounds (especially of thermo-labile compounds) is less appropriate than using the power control approach. It is hypothesised that the degradation of thermo-labile compounds close to the surface of the extraction mixture can be avoided if using the power control approach in conjunction with optimization studies.

In order to correctly program the microwave power levels (which are generally calculated as percentage from the nominal power) during development, optimization, and replication studies of MAE of phyto-compounds, one must consider calibrating the actual microwave power output of the system. The results of the present study and that reported by Cheng et al. (2006) showed that the actual power output can vary significantly (lower by 11.2 and 29 %, respectively) from the nominal power output reported by the manufacturers of microwave systems. Calibration equations, such as those presented in sections **4.4.1** and **4.4.2**, are necessary in order to predict the actual power outputs as a function of programmable microwave power levels accepted by the microwave systems. It is recommended to accept only linear regression equations, since quadratic equations are the result of biased observed microwave power outputs. The bias is most likely due to enhanced heat loss by conduction from the water load to the vessel when the water temperature is > 50°C as result of increased thermal conductivity of the borosilicate glass. It is recommended to include the mass of the container (when this is filled with water) or the mass of the fraction of the container body which is in contact with the water (when the container is partially filled) into the calculation, and to work with final water temperatures < 50°C in order to reduce this type of bias. Based on the results presented above, linear regression equations are obtained for ΔT values in the range of 3 to 25.4°C. The maximum actual power output can be calculated by extrapolation rather than by inclusion of data obtained with loads of water heated at temperatures ≥ 55°C (ΔT ≥ 26°C). Every calibration test should be carried out with fresh loads of water equilibrated at room temperature in the vessel, in order to start every heating test with the same dielectric properties.

In order to correctly program the desired power levels, in addition to using microwave output calibration equations, the researcher has to verify how the programmed power levels function in systems that use one magnetron to supply microwave power to multiple mono-mode cavities placed on the same waveguide. For example, the control panel of the microwave system used in this work allows

for setting different power levels in the two cavities. Few tests were carried out by setting different power levels for the two cavities when these were used independently and simultaneously to heat loads of 200 ml distilled water for 45 s. One test was carried out by programming 10 and 60 % power levels in cavities 1 and 2 respectively, but the load of water was heated in cavity 2. Two tests were carried out by programming 80 and 10 % power levels in cavities 1 and 2 respectively, but the load of water was heated in cavity 2. The results of these three tests showed that when used individually, the power level programmed for cavity 1 controlled the actual power output in cavity 2, regardless of the power level programmed for cavity 2. Two tests were carried out by programming 40 and 60 % power levels for cavities 1 and 2, respectively, and two loads of water were heated simultaneously in the two cavities. The results showed that when the two cavities were used simultaneously the highest programmed power level controlled the heating in both cavities. It can be concluded that the fact that different power levels could be programmed for the two cavities, while the system had only one magnetron, might mislead researchers into thinking that the system could achieve different power levels while operating the two cavities simultaneously. The meaning of the power level settings in this system can be summarized as follows: (1) when the two cavities are used independently of each other, the power level set for cavity 1 controls the microwave power output to both cavities; (2) when the two cavities are used simultaneously, the highest programmed power level controls the power output to both cavities.

In spite of the problems that could arise while programming and controlling the power levels, this type of system was successfully used for developing and optimizing MAE methods for extracting nutraceuticals from plant sources (Krishnaswamy et al., 2012; Nemes and Orsat, 2010, 2011a, b; Singh et al., 2011a). Knowledge on correct microwave power output calibration and power level settings must go beyond the information provided by the manufacturer in the user's manuals in order to ensure successful replications of developed MAE methods by others. Considering the potential problems that can arise when using

the power control approach, any replication of MAE methods reported in the literature should consider varying the power level (e.g., 5 % at a time) until the temperature specified by the developer of the method is reached.

4.5. Conclusions

This chapter highlights the necessity of controlling the microwave power when carrying out MAE processes in mono-mode cavity microwave systems that are not provided with stirring devices. The correct setting of the microwave power levels in MAE systems depends on the calibration of actual microwave power outputs, and on the way the power settings programmed in the control panel of the instrument affect the microwave power delivery. The calibration regression equations provided here apply specifically to the microwave system that was used for experimentation. However, the interpretation of results, the considerations and the recommendations pertaining to the correct control of microwave power can be generalized to any MAE system. This chapter contributes to the field of MAE by acknowledging the problems that can arise during MAE processes due to the nature of microwave heating and the physical limitations of microwave systems, and presents solutions to these problems.

Connecting Statement to Chapter 5

In **Chapter 4**, the calibration of the actual microwave power output in the microwave-assisted extraction (MAE) system used in this thesis has been presented, and the ways that the system achieves power and temperature control during MAE processes have been explained. Together with the MAE theory and the recommendations presented in **Chapter 3**, **Chapter 4** provides the basis for carrying out MAE processes successfully.

In **Chapter 5**, a MAE method previously developed and optimized for extracting the flaxseed lignan is evaluated in terms of accuracy, precision, repeatability and applicability for the analytical quantification of lignans in a variety of flaxseed cultivars, flax hull, and oil seeds. The purpose of **Chapter 5** is to demonstrate that the optimized MAE method can be used for the general analysis of lignans in plants. Thus, the recommendations given in **Chapter 3** and **Chapter 4**, along with the method description provided in **Chapter 5** will assist researchers in successfully replicating the MAE method.

Chapter 5 is written in manuscript style and was published in edited form.

- Nemes, S.M., Orsat, V., 2011. Evaluation of a Microwave-Assisted Extraction Method for Lignan Quantification in Flaxseed Cultivars and Selected Oil Seeds. Food Analytical Methods 5, 551-563.

Some of the results presented in **Chapter 5** have been disseminated through a conference presentation.

- Nemes, S.M., Orsat, V., Raghavan, G.S.V., 2010. Microwave-Assisted Technology for Quantitative Analysis of Lignans in Foods, The XVIIth World Congress of the International Commission of Agricultural

Engineering CIGR, hosted by the Canadian Society for Bioengineering (CSBE/SCGAB), Québec City, Canada, June 13-17.

Chapter 5 - Evaluation of a Microwave-Assisted Extraction Method for Lignan Quantification in Flaxseed Cultivars and Selected Oil Seeds

5.1. Abstract

An optimized microwave-assisted extraction (MAE) method was evaluated through repeatability, recovery, and efficiency testing. The repeatability tests, performed by three users over time, were not significantly different. The recovery of lignan throughout the extraction, preparation and analysis steps is 97.5 % with a coefficient of variation < 1 %. The MAE method is efficient for extracting lignans from the plant matrix, and it achieves significantly higher extraction yields than two established reference methods. The applicability of the MAE method was demonstrated by extracting lignans from a variety of plant samples. The secoisolariciresinol diglucoside (SDG) content of seven flaxseed cultivars grown in Saint-Mathieu-de-Beloeil, QC, in 2009 ranged from 14.6 - 18.9 mg SDG/g of seed. Flax hulls produced in Winchester, ON in 2010 were very rich in lignan; their SDG content was 40.0 mg/g of flax hull. Sesame seeds contained 0.18 - 1.89 mg SECO (aglycone of SDG)/g of seed, with significant differences among black, white and brown sesame seed. Chia seeds contained 0.99 - 1.29 mg SECO/g of seed, with significant differences among batches of seeds. Sunflower seeds had 0.046 mg SECO/g of sample and almonds had 0.029 mg SDG/g of sample. The optimized and evaluated MAE method is recommended for the general analytical quantification of lignans in plant samples.

Key words: MAE, secoisolariciresinol, SDG, sesame, chia, sunflower, almonds

5.2. Introduction

Lignans are a major class of phytoestrogens that are found in decreasing order of concentration in oilseeds, nuts, soy products and cereals (Thompson et al., 2006). They are concentrated in the bran layer of cereals and the seed coat of oil seeds. Although plant foods in general may contain up to 2 mg lignan/100 g of food product, flaxseed and sesame seeds have more than 300 mg lignan/100 g of seeds (Peterson et al., 2010). Flaxseed contains the lignan SDG which is naturally occurring as part of a complex macromolecule. An important body of research has contributed to the elucidation of the composition and the structure of the flaxseed lignan macromolecule in the past ten years (Ford et al., 2001; Kamal-Eldin et al., 2001; Struijs et al., 2009; 2007; 2008). The flaxseed lignan macromolecule includes, besides SDG, 3-hydroxy-3-methyl-glutaric acid (HMGA), p-coumaric acid glucoside, ferulic acid glucoside, and the flavonoid herbacetin diglucoside. A sketch of the lignan macromolecule (**Figure 5.1**) was proposed by Peterson et al. (2010) upon reviewing the latest studies on the composition and the structure of the flaxseed lignan. The backbone of the macromolecule is made of units of SDG ester-linked to HMGA; the number of backbone units (represented by the letter n in **Figure 5.1**) can vary between 1 and 7 with an average of 3.

In order to correlate the health effects conferred by SDG consumption in humans, the quantity of SDG present in flaxseed products used in epidemiological and lignan supplementation studies has to be known, whether these products are SDG extracts, whole or defatted flaxseed meal (Adolphe et al., 2010; Peterson et al., 2010). Adolphe et al. (2010) summarized the health effects of SDG consumption upon reviewing only those animal and human studies that reported the dosage of SDG in the flaxseed products used. It appears that SDG benefits human health upon bioconversion in the digestive tract to the more potent mammalian lignans: enterodiol and enterolactone.

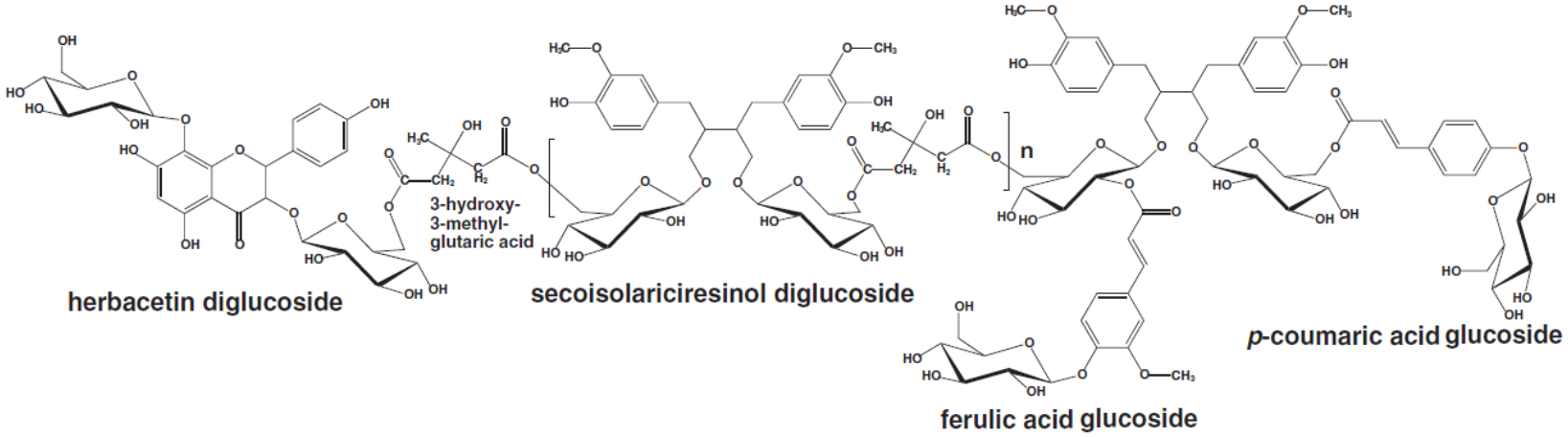

Figure 5.1. The structure of the flaxseed lignan macromolecule according to Peterson et al. (2010).

The mammalian lignans have antioxidant (Kitts et al., 1999; Prasad, 2000a) and weak estrogenic or anti-estrogenic effects [depending on the various tissues and organs in the body, and on the ratio of the concentrations of mammalian lignans to the endogenous estrogen (Buck et al., 2010; Lamblin et al., 2008)] thus providing protection against cardiovascular diseases (Adolphe et al., 2010; Peterson et al., 2010), the metabolic syndrome and certain types of cancer (Adolphe et al., 2010; Touré and Xueming, 2010; Wang, 2002).

The demand for known lignan contents in foods resulted in the publication of a variety of analytical methods for lignan analysis, and a number of databases reporting lignan contents in foods. A recent inventory of published phytoestrogen databases (Schwartz et al., 2009) has identified the need for a generally applicable sample preparation methodology. The inconsistencies in the lignan values in various databases were attributed to the use of different methods of extraction. Moreover, the limitations of acidic and enzymatic hydrolyses such as artefact formation and/or incomplete extraction from the matrix, led to the underestimation of results. Two main trends in sample preparation methodologies that were used for the construction of some of the databases were identified; these involved lignan extraction by methanolysis or alcoholic extraction followed by alkaline hydrolysis, both followed by enzymatic hydrolysis (**Table 5.5.1**).

Smeds et al. (2007) found that the yield of lignan extraction can be increased if an additional mild hydrolysis step is introduced between the alkaline and enzymatic hydrolysis steps. Schwartz et al. (2009) identified this sequential three-step hydrolysis methodology as trend-setting in lignan analysis, and as an indicator that further method development is required for analytical lignan extraction. Although the additional mild hydrolysis step was not used later by Smeds et al. (2009) for lignan analysis in cereals, it is worth further investigation.

Table 5.5.1. Trends in extraction methodologies used for lignan quantification in foods and construction of databases, and the values of lignan content in flaxseed achieved with these methodologies.

Methods description	Specifications	Analysed samples	*Results (mg lignan/g fresh weight flaxseed)	References
Extraction with 0.3M NaOH in 70% Me-OH at 60°C for 1 h. Extract acidification to pH 5-6 with acetic acid. Centrifugation and drying of pooled supernatants under N_2 stream.	Enzymatic hydrolysis of dried extracts.	Dutch food	3.01 aglycone (5.71 SDG equivalent)	(Milder et al., 2005)
		Food	3.35 aglycone (6.35 SDG equivalent)	(Penalvo et al., 2005)
		Japanese food	-	(Penalvo et al., 2008)
	Mild acidic hydrolysis of dried extracts with 0.01M HCl in 70% Me-OH at 60°C for 1 h, followed by enzymatic hydrolysis.	Cereals, oil seeds and nuts	1.69 aglycone (3.20 SDG equivalent)	(Smeds et al., 2007)
	Drying step eliminated. Enzymatic hydrolysis of acidified liquid extract.	Cereals	-	(Smeds et al., 2009)
Extraction with 70% Me-OH at 60-70°C for 2 hours twice. Evaporation of extracts under vacuum at 60 °C, followed by hydrolysis with 1M NaOH for 3 h, and neutralization with acetic acid.	Purification of neutralized extract by SPE followed by enzymatic hydrolysis and final purification by SPE.	Canadian food	3.79 aglycone (7.18 SDG equivalent)	(Thompson et al., 2006)
		Dietary supplements	-	(Thompson et al., 2007)

* The aglycone values represent the sum of SECO, pinoresinol, lariciresinol and matairesinol.

Although significant advancements have been done in the fields of extraction and analysis of lignans in the past ten years, the efficient extraction of lignans from plant matrices remains a challenge. It is known that lignan contents in flaxseed vary with the cultivated variety (Eliasson et al., 2003; Johnsson et al., 2000), but also with the location and the year of growth (Westcott and Muir, 2003). These are natural challenges that complicate the task of lignan quantification in foods, as it would be impractical and tedious to analyse various cultivated varieties of oil seeds and cereal grains harvested in different years at various locations. However, the methods of extraction and analysis of lignans are challenges that the researcher has a choice over.

The composition of flaxseed extracts vary with the extraction method used. If alcoholic extraction followed by alkaline hydrolysis, or direct alkaline hydrolysis are used, the reported lignan is SDG (Eliasson et al., 2003; Johnsson et al., 2000; Nemes and Orsat, 2010, 2011b). If the flaxseed alcoholic or alkaline extracts are further hydrolysed by acidic or enzymatic hydrolysis a variety of lignan aglycones are reported besides SECO. For example, when enzymatic hydrolysis has been used matairesinol, lariciresinol and pinoresinol have been reported by many as can be seen from **Table 5.5.1**; in addition to these, isolariciresinol and demethoxy-secoisolariciresinol have been reported by Sicilia et al. (2003). Enzymatic hydrolysis appears to be a better choice than acidic hydrolysis as the latter causes the transformation of lariciresinol into isolariciresinol, and the dehydration (loss of a water molecule) of SECO and demethoxy-secoisolariciresinol (Sicilia et al., 2003). The formation of lignan artefacts during the extraction increases the cost and the difficulty of the chromatographic analysis as more standard lignan compounds are required. As opposed to enzymatic and acidic hydrolysis, the alkaline hydrolysis is more efficient and robust (no artefact formation), and the chromatographic analysis of flaxseed lignans is straight-forward as only SDG is present (Oomah and Hosseinian, 2008).

The fact that SDG was the only lignan found in the flaxseed lignan macromolecule (Peterson et al., 2010) corroborated with earlier findings according to which SDG was as well the only lignan found by HPLC with mass spectrometry and nuclear magnetic resonance spectroscopy analysis of flaxseed extracts obtained by alcoholic extraction followed by alkaline hydrolysis (Ford et al., 2001). Further proof was provided by Eliasson et al. (2003) who used reversed phase HPLC with SDG, syringaresinol, pinoresinol and matairesinol as lignan standards for identification but found only SDG in flaxseed extracts obtained by direct alkaline hydrolysis.

The most efficient methods for lignan extraction from flaxseed use direct alkaline hydrolysis with dilute NaOH (Eliasson et al., 2003; Nemes and Orsat, 2011b). Alkaline hydrolysis has the role of breaking the ester bonds between SDG and HMGA thus releasing the SDG from the complex lignan macromolecule. The lignan macromolecule has been extracted often with aqueous alcohol or with alcoholic mixtures (Eliasson et al., 2003; Johnsson et al., 2000; 2002; Kamal-Eldin et al., 2001; Thompson et al., 2006). However, the SDG content of the macromolecule can only be analysed upon hydrolysis. The alcoholic extraction step is time consuming; some authors have used 4 h (Thompson et al., 2006) and others as much as 16 h (Johnsson et al., 2000) or even up to 48 h (Eliasson et al., 2003). The duration of alcoholic extraction is crucial for a quantitative recovery of the lignan macromolecule; the longer the extraction time the higher the yield of the lignan macromolecule, with a high yield being obtained after 48 h of alcoholic extraction. Moreover, a direct alkaline hydrolysis method was developed (1 h hydrolysis at room temperature with 1M NaOH) that gave higher results than 48 h alcoholic extraction followed by alkaline hydrolysis (Eliasson et al., 2003). A new MAE method that used a direct hydrolysis approach was developed by Nemes and Orsat (2010, 2011b). The MAE method was used for SDG extraction from defatted flaxseed meal and achieved a 6 % increase in the extraction yield as opposed to the conventional direct hydrolysis method developed by Eliasson et al. (2003), with additional benefits such as: reduction in extraction time by 95 %,

reduction in the NaOH concentration by half, and internal standard (o-coumaric acid) recovery of 97 %. MAE methods published by others showed improvements in the extraction yields of SDG from flax hull (Zhang and Xu, 2007) and flaxseed cake (Beejmohun et al., 2007) as opposed to conventional methods such as stirring extraction and Soxhlet extraction.

This chapter proposes to bring further proof in terms of efficiency and repeatability for our previously developed optimized MAE method, and as well to demonstrate its applicability for fast and efficient analytical quantification of lignans in flaxseed cultivars and other oil seeds samples. The first objective of this article is to evaluate our previously developed MAE method by: (1) assessing its repeatability when performed by different users over time, (2) assessing its recovery of standard SDG compound, and (3) assessing its efficiency by comparison with known trend-setting methods, and investigating the possibility of further increasing the extraction yield by using a sequential hydrolysis approach. The second objective is to use the evaluated MAE method for analysis of SDG in different flaxseed cultivars, flaxseed hulls and other oil seeds.

5.3. Materials and Methods

5.3.1. Chemicals

The reference lignan standards of HPLC grade: secoisolariciresinol diglucoside (SDG, molecular weight 686.7, purity 97.6 %) and anhydro-secoisolariciresinol (ANSECO, molecular weight 344.4, purity > 99 %) were purchased from Chromadex (Santa Ana, CA, USA); secoisolariciresinol (SECO, molecular weight 362.4, purity ≥ 95 %) was purchased from Sigma-Aldrich (Oakville, ON, Canada). The solvents: acetonitrile, methanol and hexane of HPLC grade were obtained from Fisher Scientific (Ottawa, ON, Canada). The reagents: sodium hydroxide ≥ 98 %, sulphuric acid 95 - 98 % ACS, phosphoric acid ≥ 85 %, and di-potassium hydrogen phosphate 98 % were purchased from Sigma-Aldrich (Oakville, ON, Canada).

5.3.2. Samples

The flaxseed (*Linum usitatissimum* L.*)* used for the MAE method efficiency assessment and validation was purchased in 2009 from a local grocery store (Montreal, QC, Canada); the country of provenance was Guatemala as specified on the package. The flaxseed cultivars (CDC Bethune, McBeth, Prairie Blue, Flanders, 09LS01, CRGL 8.1, and CRGL 8.2) analysed for SDG content were provided by Mr. Yves Dion from Centre de Recherche sur les Grains Inc. (CEROM). All the cultivars or advanced breeding lines were grown in Saint-Mathieu-de-Beloeil, (QC, Canada) in 2009. The Omega-3 Flax Hull, produced in May 2010, was provided by Dr. Nam Fong Han from Natunola Health Inc. (Winchester, ON, Canada). Other samples used for lignan analysis were purchased from local grocery stores in 2008 and 2010 as follows: white and black sesame (*Sesamum indicum* L.) seeds, and black chia (*Salvia hispanica* L.*)* seeds were purchased in 2008; white and black chia seeds, brown sesame seeds, sunflower seeds and almonds were purchased in 2010. All the samples were divided in batches of 100-300 g packed in plastic bags (the flaxhull was vacuum packed) and stored at -18°C until use.

5.3.3. Microwave-Assisted Extraction Apparatus

The MAE experiments were carried out in a mono-mode (focused) microwave apparatus (Star System 2, CEM, Mathews, USA; nominal power declared by the manufacturer 800 W, microwave frequency 2.45 GHz) which delivers a maximum power of 710.5 W (section **4.4.1**), as calibrated with the calorimetric method developed specifically for the Star System 2 instrument (**Chapter 4**, section **4.3.1**). The temperature was monitored by a built-in IR temperature sensor placed at the bottom of the extraction vessel. The extraction vessels (250 ml) were made of borosilicate glass and were fitted with a Graham-type reflux condenser.

5.3.4. High Performance Liquid Chromatography Analysis of Lignans

All extracts were analysed in triplicates (coefficient of variation < 5 %) with an Agilent 1100 series HPLC. The Chemstation software for LC systems [Rev. B.01.03 (204), Agilent Technologies] was used for instrument control and chromatographic data analysis. The chromatograms were recorded at 280 nm using a variable wavelength detector. The separation of lignans (SDG, SECO, ANSECO) was carried out at 25°C on a reversed-phase C18 column (Discovery; 5 µm, 25 cm × 4.6 mm; Sigma-Aldrich) fitted with a guard cartridge (Supelguard; 5 µm, 2 cm × 4 mm; Sigma-Aldrich) using a slightly modified version of the gradient elution method developed by Johnsson et al. (2000). The two solvents were: A – 0.01 M phosphate buffer with a pH of 2.8 containing 5 % acetonitrile, and B – acetonitrile. Originally, the solvent A decreased from 100 to 70 %, at a rate of 1%/min over a period of 30 min. In this work the rate of decrease of solvent A was maintained at 1%/min but it was extended over a period of 50 min. This extended gradient method allowed the quantification of the three lignans SDG, SECO and ANSECO together; their respective elution times were 19.5, 26.5 and 38.5 min. Standard curves were built using six levels of lignan concentrations ranging from 5 to 200 µg lignan/ml methanol; in all cases the coefficients of determination (R^2) were > 0.999. The following equations were used for quantification: SDG = peak height/0.4494, SECO = peak height/1.054, and ANSECO = peak height/0.8065.

5.3.5. Sample Preparation

5.3.5.1. Microwave-Assisted Extraction

Experiments were carried out in order to assess the repeatability and the efficiency of the optimized MAE method, and for the quantification of lignans in various flaxseed cultivars, flaxseed hulls, sesame seeds, chia seeds, almonds and sunflower seeds. Some experiments were carried out using defatted flaxseed meal (DFM) that was obtained by extracting flaxseed meal twice with hexane (sample

to liquid ratio 1:6, g/ml) for 1 h under magnetic stirring at room temperature (Nemes and Orsat, 2010). The MAE experiments were carried out following our previously published methodology (Nemes and Orsat, 2011b), which was updated in section **3.5.5**. Briefly, samples of 1g of defatted or non-defatted flaxseed meal were hydrolysed with 50 ml of 0.5 M NaOH at 22 % power level (156 W as calculated with the calibration in **Equation 4.2** = 7.105 × Power level (%), presented in section **4.4.1**); the microwave power was applied intermittently (30 s on/off) for 3 min. A type K thermocouple (accuracy ± 0.5°C, Fisher Scientific) was used to measure the final temperature of the extracts after brief stirring. The temperature of the extracts rose from room temperature (22 – 23°C) to about 67°C over the 3 min span. [Note: Statistically similar results can be obtained if using programmed power levels in the range of 17 to 27 % (section **3.5.5**)]. The extracts contain water soluble carbohydrates and proteins which must be removed before HPLC analysis. In order to do so, the extracts were acidified to pH 3 by addition of 5.55 ml of 5 N H_2SO_4 while measuring the pH with an Accumet 25 instrument (Fisher Scientific), then methanol was added to the acidified extracts in proportion of 2:1 (v/v, ml/ml) and the extracts were kept for 15 min under magnetic stirring (3000 rpm). The solid and liquid phases were separated by centrifugation (10 min at 3000 rpm) using a Spinette centrifuge (International Equipment Company, Needham Heights, MA, USA). If DFM was used for MAE, an aliquot of the clear liquid phase was passed through 0.22 µm Whatman Puradisc (13 mm) nylon syringe filters into 2 ml vials then analysed by HPLC. If non-defatted flaxseed meal samples were used for MAE, the liquid phase was transferred into a round bottom flask and the extract was stripped of methanol and concentrated to 3 - 5 ml by vacuum rotary evaporation (30 min, 65°C, 195 rpm) using a Buchi Rotavapor 205 equipped with a B490 heating bath. The aqueous concentrated extracts were transferred into 15 ml centrifuge tubes and hexane was added in proportion of 2:1 (v/v, ml/ml). The tubes were shaken to facilitate the defatting, and then centrifuged for 10 min at 3000 rpm to speed up the separation of the organic phase, which was then removed with a pipette. The concentration of the extracts was adjusted with methanol to bring the theoretical sample to liquid ratio to a

maximum of 10:1 (mg/ml), then the extracts were filtered through 0.22 µm membrane filters and analysed by HPLC.

5.3.5.2. Cumulative Effects of Microwave-Assisted Extraction, Sample Preparation and High Performance Liquid Chromatography Analysis on the Recovery of Secoisolariciresinol Diglucoside Standard

The methodologies described above for MAE, sample preparation and HPLC analysis were applied in order to assess the recovery of SDG standard; 120 µg SDG dissolved into 300 µl methanol were used per MAE test. The percentage of SDG recovered was calculated from the amount of SDG used per test upon HPLC analysis.

In order to assess the efficiency of the optimized MAE method, two reference methods were carried out for comparison purposes.

5.3.5.3. Reference Method A

A sequential extraction involving alkaline hydrolysis followed by mild acidic hydrolysis was performed as described by Smeds et al. (2007). Briefly, samples of 0.5 g DFM were hydrolysed with 24 ml of 0.3 M NaOH in 70 % methanol for 1 h at 60°C, then the pH was adjusted to 5 - 6 with 750 µl glacial acetic acid; the extracts were centrifuged and the liquid phase was dried by vacuum rotary evaporation (65°C, 195 rpm). Note: this type of alkaline hydrolysis was also used by Milder et al. (2005; 2004) and Penalvo et al. (2005; 2008). The dry extracts were dissolved in 24 ml of 0.01 M HCl in 70 % methanol and then hydrolysed for 1 h at 60°C. Methanol was added to the extracts to bring the theoretical concentration to maximum 10 mg sample per ml, then the extracts were filtered through 0.22 µm membrane filters and analysed by HPLC.

5.3.5.4. Reference Method B

Alcoholic extraction followed by alkaline hydrolysis was carried out following the procedures described by Thompson et al. (2006). Briefly, 0.5 g DFM were extracted twice with 25 ml of 70 % methanol at 60°C for 2 h, then the extracts were centrifuged and the liquid phase was dried by vacuum rotary evaporation (65°C, 195 rpm). The dry extracts were dissolved in 30 ml of 1 M NaOH and hydrolysed at room temperature for 3 h, and then the pH was brought to 7 by addition of 1.7 ml glacial acetic acid. The concentration of extracts was adjusted with methanol to correspond to a maximum of 10 mg DFM/ml, then the extracts were filtered through 0.22 µm membrane filters and analysed by HPLC.

5.3.5.5. Sequential Microwave-Assisted Extraction

A sequential MAE experiment was carried out in order to test the hypothesis that alkaline hydrolysis (MAE) followed by mild acidic hydrolysis (MAE - AC) might increase the total yield of lignan. This hypothesis was considered based on the findings of Smeds et al. (2007). The MAE - AC experiment was designed keeping in mind that the acidity of the solvent and the extraction temperature might be directly related to lignan artefact formation. Sicilia et al. (2003) found that both enzymatic hydrolysis (in sodium acetate buffer, pH 5, 37°C) and acidic hydrolysis (in 1 M HCl, 95°C) lead to artefact formation, but the harsh acidic hydrolysis produced more lignan artefacts. Furthermore, it is known that for stability, lignan extracts must be kept at pH 2 – 4 (Nollet, 2000), preferably 3 (Eliasson et al., 2003; Johnsson et al., 2000) in order to prevent the ionization of hydroxylic groups which would decrease lignans' retention times thus resulting in HPLC quantification losses. Therefore, the MAE - AC was carried out at pH 3 in order to preserve the integrity of extracted lignans and to avoid quantification losses. The sequential MAE was carried out as follows: samples of 1 g DFM were extracted by MAE then the pH was adjusted to 3 with 5.55 ml of 5 N H_2SO_4, further the extracts were subjected to MAE - AC which investigated the time (3, 9 min) and

temperature (60, 90°C) of hydrolysis according to the experimental design presented below. The temperature of extracts after MAE was about 67°C, therefore before running MAE - AC at 60°C the extracts needed to be cooled down to the required temperature in a cold water bath. For practical reasons the microwave power was kept at 156 W (programmed power level 22 %) for the MAE-AC as well. Given that the MAE - AC extracts already have a pH of 3 the ensuing preparation steps in view of HPLC analysis were done as described above for the MAE experiments.

5.3.6. Experimental Design and Statistical Analyses

One-way ANOVA and Tukey-Kramer HSD tests for pair-wise comparison of means were carried out for the repeatability and efficiency assessments of MAE, the comparison of lignan contents in various flaxseed cultivars, and the comparison of lignan contents among different batches of sesame and chia seeds. The number of replications for the pair-wise comparison tests ranged from 2 to 5 per sample. The sequential MAE was designed as a two-factor two-level full factorial screening design with two replications. The coded and actual levels for the factors studied were as follows: time (-1) = 3 min, and (+1) = 9 min; temperature (-1) = 60°C, and (+1) = 90°C. The design was analysed using response surface procedures. The significance of tests was established at p values < 0.05 in all cases. The statistical analyses were carried out with SAS 9.2 TS2M2 or JMP 8 (SAS Institute Inc., Cary, NC, USA).

5.4. Results and Discussion

5.4.1. Microwave-Assisted Extraction Repeatability

Three users tested the repeatability of the optimized MAE method by performing three extractions each over three weeks (one MAE/user/week) using the setup in our laboratory. The non-defatted flaxseed meal (flaxseed grown in Guatemala) used was from the same batch. The data obtained are presented in **Table 5.5.2**.

The MAE tests were performed equally well by the three users judging from the results of the pair-wise comparison test, by which the three means did not differ from each other significantly (p value = 0.551). The highest coefficient of variation was 4.03 %. In the literature, coefficients of variation ranging from 1 to 7 % were reported for two well known lignan extraction methods. The direct hydrolysis method reported by Eliasson et al. (2003) was tested by two analysts over three days; the coefficients of variation for SDG were ≤ 7 %. The complex lignan extraction method reported by Thompson et al. (2007) was tested for variation within a day and between different days; the obtained coefficients of variation ranged from 1 to 5.2 % and 1.4 to 6.3 %, respectively. Our results fall within the published range of acceptable coefficients of variation. Our optimized MAE method has excellent repeatability demonstrated not only over time (low coefficients of variation per user over three weeks) but also between users (non-significant one-way ANOVA and pair-wise comparison test).

Table 5.5.2. The repeatability of the optimized MAE method tested by three users over three weeks

Individual performing the MAE test	Mean value of SDG (mg /g fresh weight flaxseed)	Standard deviation	[1]Coefficient of variation (%)	[2]Pair-wise comparison, Tukey-Kramer HSD test
1	10.4	0.37	3.56	A
2	10.7	0.43	4.03	A
3	10.3	0.24	2.34	A

[1]The coefficient of variation was calculated as percentage from the mean (standard deviation×100/mean).

[2]The same letter (A) under the pair-wise comparison test heading indicates that there is no significant difference (p value = 0.551) between the three mean values of SDG.

5.4.2. Recovery of Secoisolariciresinol Diglucoside Standard

Four MAE tests were carried out in order to assess (1) the potential of MAE to decompose SDG to SECO or ANSECO, and (2) the magnitude of the cumulative

effects of MAE, additional preparations for non-defatted samples, and HPLC analysis on the recovery of SDG standard. There were no SECO or ANSECO detected in the MAE extracts meaning that the method did not produce lignan aglycone and/or artefacts. The mean value for the recovery of SDG was 97.5 % with a standard deviation of 0.61 and a coefficient of variation < 1 %. We have previously reported 97 % recovery of o-coumaric acid used as internal standard for MAE in the presence of flaxseed samples (Nemes and Orsat, 2011b). Lignans recovery results accounting for the cumulative effects of the extraction, preparation and analysis procedures were also reported by Thompson et al. (2007); the percentage of recovered lignans ranged from 73.8 to 92.3. Our optimized MAE method has better SDG standard recovery which could be explained in part by the much simpler procedure (less manipulation steps) but also by its robustness (no lignan degradation). Although the cumulative losses were very small (2.55 %), a multiplication correction factor was computed (100/97.5=1.026) in order to be able to account for the losses occurring during analytical MAE, sample preparation and analysis of SDG in oil seeds. Thus, original lignan values can be presented side by side with the corrected values.

5.4.3. Microwave-Assisted Extraction Efficiency

The efficiency of the optimized MAE method was assessed by comparison with two well known published methods (referred to in this chapter as reference method A and B). The samples extracted with the three methods were from the same batch of DFM (flaxseed grown in Guatemala). The results are presented in **Table 5.5.3**. The SDG extraction yield obtained with MAE was the highest and it was significantly different (p value < 0.0001) from the extraction yields obtained with the two reference methods. The SDG recoveries of the reference methods A and B relative to the MAE method were 78.6 and 73.4 %, respectively. As opposed to the reference method B, method A has an improved initial extraction step. Although both methods use 70 % methanol, for method A this is supplemented with 0.3 M NaOH. However, this concentration of NaOH is not

high enough to ensure a complete SDG extraction from the matrix. Eliasson et al. (2003) have demonstrated that alcoholic extraction followed by alkaline hydrolysis is superseded by a simple direct alkaline hydrolysis with 1 M NaOH for 1 h at room temperature. In addition, we have previously proven that the optimized MAE using 0.5 M NaOH achieved an increase of 6 % in the SDG extraction yield as opposed to the direct hydrolysis method (Nemes and Orsat, 2011b).

Table 5.5.3. Assessment of MAE efficiency by comparison with two reference methods

Extraction method	Mean value of SDG (mg/g DFM)	Standard deviation	No. of replicates	*Pair-wise comparison, Tukey-Kramer HSD test
MAE	22.9	0.26	4	A
Reference method A	18.0	0.43	3	B
Reference method B	16.8	0.03	3	C

*The different letters under the pair-wise comparison test heading indicate that there are significant differences (p value < 0.0001) between the mean values of SDG obtained with the three methods of extraction.

5.4.4. Sequential Microwave-Assisted Extraction

This experiment was carried out in order to verify if prolonged MAE extraction after acidification of extracts to pH 3 with H_2SO_4 improves the lignan extraction yield from flaxseed samples. This hypothesis was inspired by the findings of Smeds et al. (2007) which indicated that introducing an additional acidic hydrolysis step between the alkaline and enzymatic hydrolyses steps increased the lignan extraction yield. The assumption is that the increase in the lignan extraction yield was due to the additional acidic hydrolysis step rather than to the subsequent enzymatic hydrolysis. It is known that the enzymatic hydrolysis does not break the lignan macromolecule efficiently, its main benefit being the production of lignan aglycones from already released lignan glucosides (Oomah and

Hosseinian, 2008). The DFM used for this experiment was from the same batch that was used for the MAE efficiency tests (flaxseed grown in Guatemala). The sequential MAE did not produce SECO or ANSECO. The effects of the MAE-AC factors time (3, 9 min), temperature (60, 90°C) and their interaction did not have significant effects on the SDG extraction yield; their respective *p* values were 0.162, 0.333, and 0.130. The predicted maximum value for SDG was 22.9 mg/DFM (standard error 0.22) for 3 min MAE-AC at 60°C. This result is well depicted in the response surface plot (**Figure 5.2**). The maximum SDG value of 22.9 mg/g DFM obtained with MAE-AC is equal to the MAE reference SDG value shown in **Table 5.5.3**. This indicates that the additional MAE-AC step is not necessary as it is not increasing the SDG extraction yield. Therefore, it can be concluded that the MAE of SDG from flaxseed is complete as further increase of SDG is not possible, and robust as SDG is not transformed into SECO or ANSECO.

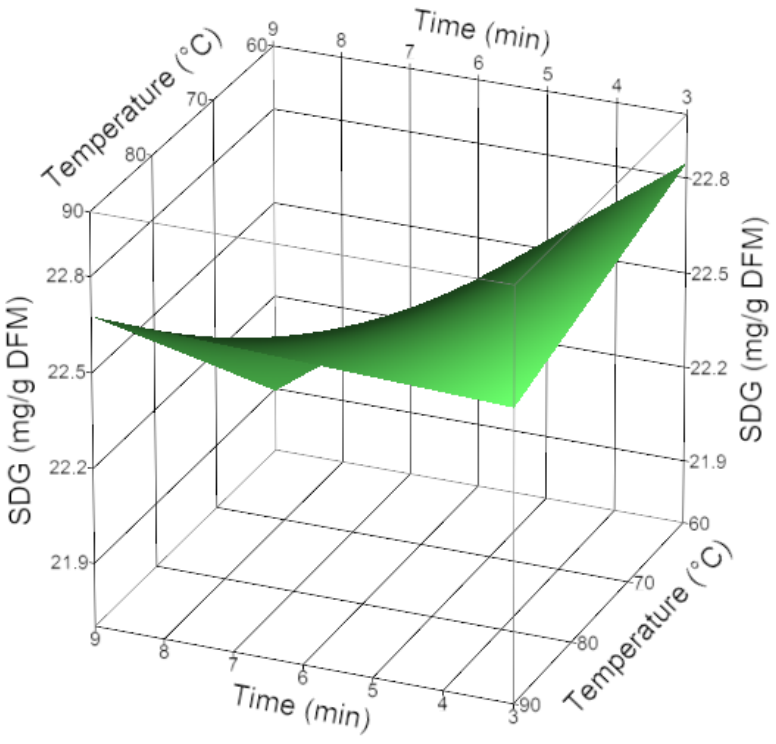

Figure 5.2. Response surface plot for SDG during MAE-AC

The complete proof of repeatability and efficiency, provided above for the optimized MAE method, along with its simplicity and rapidity make MAE the method of choice for analytical quantification of SDG in flaxseed and other plant samples. In order to demonstrate its utility and the range of samples that can be analysed with it, the optimized MAE method was further applied for analytical quantification of lignans in various flaxseed cultivars, flax hull, sesame seeds, chia seeds, sunflower seeds and almonds.

5.4.5. Quantification of Secoisolariciresinol Diglucoside in Flaxseed Cultivars

The optimized and evaluated MAE method was used for the quantification of SDG in flaxseed cultivars grown in the Québec province in 2009. The results of the pair-wise comparison tests, presented in **Table 5.5.4**, are the mean of three replicates and are expressed as mg SDG/g of fresh weight flaxseed meal. In order to account for the cumulative losses of SDG that occurred during MAE, sample preparation and HPLC analysis, the corrected SDG values are also presented in **Table 5.5.4**. The corrected value was calculated by multiplying the mean SDG values by 1.026 (correction factor computed based on the recovery of SDG standard, presented in section **5.4.2**). The letters assigned based on the pair-wise comparison test categorize the Québec flaxseed cultivars into three groups A, B and C which vary from each other significantly (p value < 0.0001). The group A has the highest SDG value (18.9 mg/g flaxseed meal) and includes only the cultivar 09LS01. A model of HPLC chromatogram for MAE extracts of the 09LS01 flax cultivar is shown in **Figure 5.3**. The group B is for the medium SDG values (16.5 – 17.3) and includes four cultivars: CDC Bethune, Flanders, CRGL 8.1 and 8.2. The group C is for the low SDG values (14.6 – 14.9) and includes the cultivars McBeth and Prairie Blue. For comparison purposes, the flaxseed sample (unknown cultivar grown in Guatemala) bought from a local grocery store in 2009 was also included in the table in order to highlight that all the flaxseed cultivars grown in Québec analysed by MAE contained significantly more SDG/g of fresh weight flaxseed meal.

Table 5.5.4. Quantification of SDG in flaxseed cultivars grown in the Québec province by MAE

Flaxseed cultivars	Mean value of SDG (mg/g fresh weight flaxseed meal)	Standard deviation	[1]Coefficient of variation (%)	[2]Pair-wise comparison, Tukey-Kramer HSD test	[3]Corrected SDG value (mg/g fresh weight flaxseed meal)
09LS01	18.4	0.49	2.66	A	18.9
CRGL 8.1	16.8	0.28	1.67	B	17.3
CDC Bethune	16.3	0.25	1.53	B	16.7
Flanders	16.2	0.07	0.43	B	16.6
CRGL 8.2	16.1	0.16	0.99	B	16.5
McBeth	14.5	0.22	1.52	C	14.9
Prairie Blue	14.2	0.23	1.62	C	14.6
[4]Unknown cultivar	10.4	0.33	3.17	D	10.7

[1] The coefficient of variation was calculated as percentage from the mean (standard deviation×100/mean).

[2]The different letters under the pair-wise comparison test heading indicate that there are significant differences (p value < 0.0001) between the mean values of SDG obtained for the various flaxseed cultivars.

[3]The mean value of SDG was multiplied with the correction factor of 1.026 in order to obtain the corrected SDG value.

[4]The flaxseed bought from a local grocery store was an unknown cultivar grown in Guatemala.

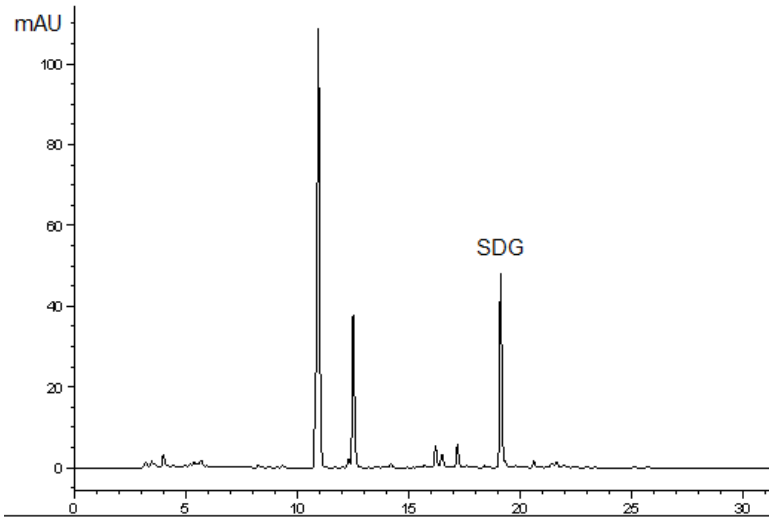

Figure 5.3. A model of HPLC chromatogram for MAE extract of 09LS01 flaxseed cultivar.

This chromatogram is also representative for MAE extracts of flax hull, defatted and non-defatted flaxseed meal, and it is similar with a previously published HPLC chromatogram for MAE of DFM (Nemes and Orsat, 2010).

Previous reports for SDG content in flaxseed cultivars, presented on an oil-free dry meal (OFDM) basis, include: 11.9 - 25.9 mg SDG/g OFDM in Swedish flaxseed cultivars (Eliasson et al., 2003), 11.7 - 24.1 mg SDG/g OFDM in Swedish and Danish cultivars (Johnsson et al., 2000), and 12.9 - 14.3 mg SDG/g OFDM in American cultivars (Madhusudhan et al., 2000). Westcott et al. (2002) reported SDG concentrations ranging from 11.1 to 17.6 mg/g defatted meal for flax cultivars grown at various locations in Canada between 1995 and 1998. Other published SDG concentration ranges include 1 - 10 µM (0.686 - 6.867 mg) SDG/g flaxseed, and 1.2 - 1.7 % SDG (12 – 17 mg SDG/g meal) in commercial flax meal obtained from Canadian cultivars (Daun et al., 2003). The latter SDG concentration range is similar with that published by Westcott et al. (2002) for defatted meal for Canadian cultivars. One publication reported SDG contents of three Canadian flaxseed cultivars expressed on a fresh weight flaxseed basis as follows: cultivar AC Linora had 9 mg SDG/g of seed, cultivar Flanders had 7.4 mg SDG/g of seed, and cultivar Linola 989 had 15 mg SDG/g of seed (Spence

et al., 2003). Thompson et al. (1997) analysed 10 flaxseed cultivars grown in Manitoba in 1989 by in vitro fermentation with human fecal inoculum and found 0.96 - 3.15 µM lignan/g seed, and great variations between the cultivars. However, the coefficients of variation calculated from the published data were 11.7 - 45.8 %, which are much greater than the current acceptable coefficients of variation for lignan analysis methods (max 7 %) as specified in the section **5.4.1** on MAE repeatability. The coefficients of variation recorded for the MAE of flaxseed cultivars shown in **Table 5.5.4** range from 0.43 to 3.17 %.

Lignan values expressed on a fresh weight basis are required for database construction (Milder et al., 2005; Thompson et al., 2006) as they give valuable information in terms of lignan concentration per edible serving and can be used in epidemiological and supplementation studies in order to find the relations between SDG content and health benefits (Adolphe et al., 2010; Peterson et al., 2010). The SDG content of flaxseed cultivars obtained with MAE cannot be compared with those expressed on an oil-free dry basis for the Danish and Swedish cultivars or with those expressed on a defatted meal basis from Canadian cultivars. Based on the results obtained with MAE for the extraction of defatted and non-defatted meal from the same flaxseed material (**Table 5.5.4**, 10.43 mg SDG/g non-defatted meal; and **Table 5.5.3**, 22.9 mg SDG/g DFM) it can be concluded that the SDG concentrations in defatted flaxseed materials should be at least 200 % higher than in non-defatted meals. Even if the results were expressed on a fresh weight flaxseed basis the comparison would be unjust towards flaxseed cultivars analysed with methods less efficient than the optimized MAE method. Moreover, the ranges for SDG concentration in flaxseed or flaxseed meal depend on the year of growth, the cultivated variety, the location of growth (Eliasson et al., 2003; Johnsson et al., 2000; Westcott and Muir, 2003; Westcott et al., 2002), and the methodology of extraction and analysis (Daun et al., 2003; Oomah and Hosseinian, 2008).

Here we have demonstrated that the flaxseed cultivars grown in the Québec province can be classified into three lignan cultivars groups (high, medium and low) based on their SDG content. Such a classification could be very useful for narrowing down varieties of flaxseed that are likely to produce similar amounts of lignan when grown in similar conditions. High lignan flaxseed varieties are desired for flax-dehulling processes in order to obtain high lignan flax hull. In addition, high lignan flaxseed cultivars are attractive for incorporation in health food products and dietary supplements.

The optimized MAE method is recommended for future applications such as analysis of SDG in flaxseed cultivars in view of database construction and also for adding lignan values to the Canadian quality data tables for flaxseed. The quality data for the Canadian flaxseed published annually by the Canadian Grain Commission include the oil, protein, and free fatty acids contents, the fatty acids composition, and the iodine values (Puvirajah, 2011). The flaxseed cultivars registered for production in Canada must have quality attributes values that are not statistically significantly lower than those of the check flaxseed cultivar. The check cultivar is determined every year by the Flax Evaluation Committee. The current check cultivar, which has been used for the past few years, is Flanders (Anonymous, 2008, 2011). Although the lignan content is not one of the quality attributes being monitored yet by the Canadian Grain Commission, if this would be the case, the statistical analysis presented in **Table 5.5.4** indicates that the cultivars McBeth and Prairie Blue do not have the required minimum lignan content. Setting a minimum standard for lignan content for flaxseed cultivar registration would contribute to an improved nutraceutical quality of newer flaxseed cultivars.

5.4.6. Quantification of Lignans in Other Samples

The flax hull contained on average 39.0 mg SDG/g of fresh weight sample (standard deviation 0.1, SDG value corrected for losses 40.0 mg/g), which was

2.12 to 3.73 times higher than the SDG contents of the flaxseed cultivars presented in **Table 5.5.4**. Oomah and Sitter (2009) reported 31.2 mg SDG/g defatted flax hull, as analysed with a direct hydrolysis method. The authors investigated 6 methods of oil extraction from the hulls prior to SDG extraction, and found that oil extraction with acetone and ethanol resulted in defatted hulls with significantly lower SDG content as opposed to oil extraction by cold press, hexane, petroleum ether and supercritical CO_2. Although, Oomah and Sitter (2009) analysed defatted flax hull and we analysed fresh (non-defatted) hull, some sources of variation, that account for the marked difference between the two results, can be identified. Both studies analysed flax hull from the same producer. However, the material was produced in different years (the present study used flax hull produced in 2010), and apparently, different cultivars were used as raw material for the dehulling process.

Flax hull has a naturally high concentration of SDG that has potential for future epidemiological or supplementation studies. For example, only 12.5 g of flax hull (calculated based on the MAE analysis of flax hull) would be required in order to provide the 500 mg SDG, which according to Adolphe et al. (2010) taken on a daily basis would reduce the risk of cardio-vascular diseases.

In order to demonstrate the applicability of the MAE method for lignan analysis in plant samples other than flaxseed, MAE was carried out for sesame seeds, chia seeds, sunflower seeds and almonds. The SECO content of sesame and chia seeds seemed to fall in the same range of values; therefore they are presented together in **Table 5.5.5**. SECO values corrected for losses are presented as well, as it is assumed that SECO has a recovery similar to SDG in the absence of artefacts formation during MAE and additional sample preparation. The differences in SECO concentration in sesame seeds were significant and large enough in order to be attributed to different seed colours (varieties) rather than different samples. Black sesame had the highest SECO concentration 1.898 mg/g of fresh weight sample, which is about 2.2 and 10.2 times higher than that found in white and

brown sesame seeds, respectively. However, the SECO concentration does not correlate with the intensity of the seed colour since brown sesame had the lowest concentration, rather than medium, as opposed to the white and black seeds. A model of HPLC chromatogram for MAE extract of black sesame is shown in **Figure 5.4**.

Table 5.5.5 Quantification of SECO in sesame and chia seeds by MAE

Sample information	Mean value SECO (mg/g fresh weight meal)	Standard deviation	[1]Pair-wise comparison, Tukey-Kramer HSD test	[2]Corrected SECO value (mg/g fresh weight meal)
White sesame	0.849	0.002	C	0.871
Brown sesame	0.181	0.008	D	0.186
Black sesame	1.850	0.003	A	1.898
White chia	1.257	0.135	B	1.289
Black chia sample 1	0.974	0.058	C	0.999
Black chia sample 2	1.026	0.070	BC	1.053

[1]The different letters under the pair-wise comparison test heading indicate that there are significant differences (p value < 0.0001) between the mean values of SECO obtained for the tested samples.

[2]The mean value of SECO was multiplied with the correction factor of 1.026 in order to obtain the corrected SECO value.

Lignan aglycone values (sum of SECO, pinoresinol, matairesinol and lariciresinol) for sesame seeds (seed colour is assumed to be white as it is the common one sold in grocery stores) reported in the literature by Thompson et al. (2006) and Milder et al. (2005) were 0.079 and 0.393 mg lignan aglycone per gram of fresh seeds, respectively. It is not known if SECO is naturally conjugated to carbohydrates or other compounds in sesame seeds. If sesame contains oil-soluble conjugated SECO this would be lost during sample defatting with hexane.

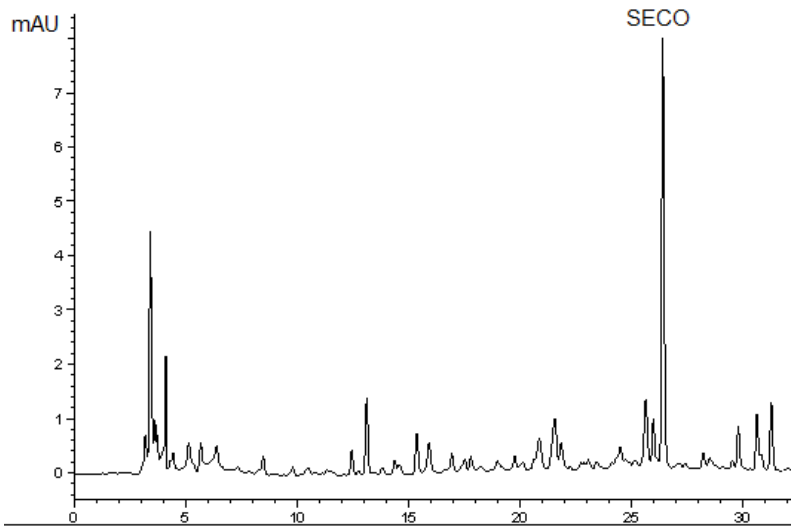

Figure 5.4. A model of HPLC chromatogram for MAE extract of black sesame seeds

This chromatogram is also representative for MAE extracts of white and brown sesame seeds.

Therefore, only non-defatted sesame meal samples were extracted with MAE, which could explain in part the much higher results obtained with MAE. In the literature, lignans other than SECO were reported both in sesame oil and in defatted sesame samples. The oil soluble lignans sesamolin and sesamin were found in concentrations up to 2.97 and 7.12 mg/g of fresh weight sesame samples, respectively by Moazzami and Kamal-Eldin (2006). The polar sesaminol diglucoside and triglucoside were found in alcoholic extracts (non-hydrolysed) of defatted sesame samples in concentrations up to 4.93 and 15.6 mg/g defatted sample, respectively, by Moazzami et al. (2006). Unlike for SECO, there was no significant difference found in the content of oil-soluble lignans and lignan glucosides between white and black sesame seeds. The fact that the SECO yields for white sesame seeds obtained with MAE were much higher than those reported in the literature (sum of lignan aglycones) can also be attributed to the high efficiency of MAE for releasing lignans from plant matrices as opposed to other lignan extraction methods.

The only significant difference in SECO content for chia seeds was recorded between white chia and black chia sample 1. Here, the variations in SECO content seem to be attributable to different samples rather than different colours (varieties), as both white and black chia sample 1 are not significantly different from black chia sample 2. The two black chia samples and white sesame have similar SECO content. A model of HPLC chromatogram for MAE extract of black chia is shown in **Figure 5.5**.

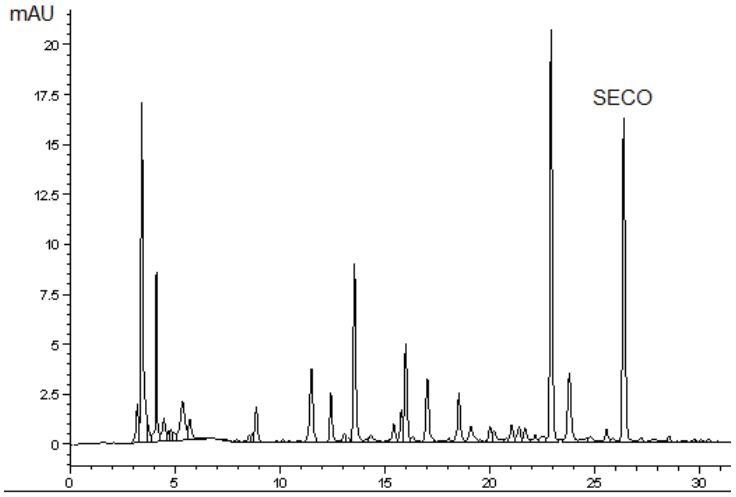

Figure 5.5. A model of HPLC chromatogram for MAE extracts of black chia seeds

This chromatogram is also representative for MAE extracts of white chia seeds.

To the best of our knowledge, this is the first report of SECO in chia seeds. However, SECO was previously reported by Powell and Plattner (1976) and Plattner and Powell (1978) in *Salvia plebeia* R. brown seeds. Both chia – *Salvia hispanica* and *Salvia plebeia* belong to the genus Salvia L. As a common trait they are both mucilaginous seeds (Dweck, 2000), which is also shared by flaxseeds. In *Salvia plebeia*, SECO is found in the form of a SECO diester and a SECO branched-fatty diester. The first diester was extracted with a mixture of pentane and hexane, and yielded SECO, 12-methyltetradecanoic and ferulic acids upon alkaline hydrolysis (Powell and Plattner, 1976). The second diester was

extracted with hexane and yielded SECO and 12-methyltetradecanoic acid upon alkaline hydrolysis (Plattner and Powell, 1978). Only non-defatted chia samples were extracted with MAE. If SECO is ester-linked to fatty and/or cinnamic acids in chia as it is in *Salvia plebeia*, then all SECO found in chia would be released by MAE.

Samples of non-defatted sunflower seeds and almonds were also extracted with MAE. Sunflower seeds contained 0.045 mg SECO/g of fresh weight sample with a standard deviation of 0.005; after multiplication with the correction factor for losses (1.026) the SECO value is 0.046 mg/g of fresh weight sample. Almonds contained 0.028 mg SDG/g of fresh weight sample with a standard deviation of 0.001; the SDG value corrected for losses, and its equivalent SECO value are 0.029 and 0.015 mg/g of fresh weight sample, respectively. Others have reported lignan contents for sunflower seeds and almonds (sum of SECO, matairesinol, pinoresinol and lariciresinol) as follows: 0.009 mg lignan/g of fresh weight sunflower (Milder et al., 2005), 0.002 mg lignan/g of fresh weight sunflower and 0.001 mg lignan/g of fresh weight almonds (Thompson et al., 2006). The lignan content found in both sunflower seeds and almonds with MAE are much higher than the values published in the literature. Models of HPLC chromatograms for MAE extracts of sunflower seeds and almonds are shown in **Figure 5.6**, and **Figure 5.7** respectively.

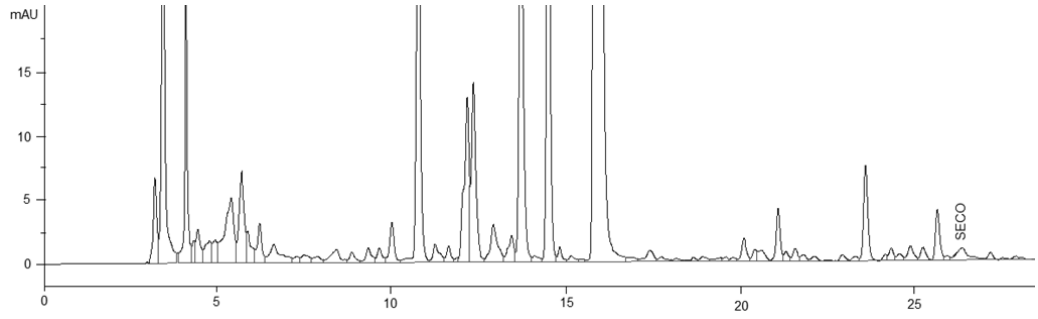

Figure 5.6. A model of HPLC chromatogram for MAE extract of sun flower seeds

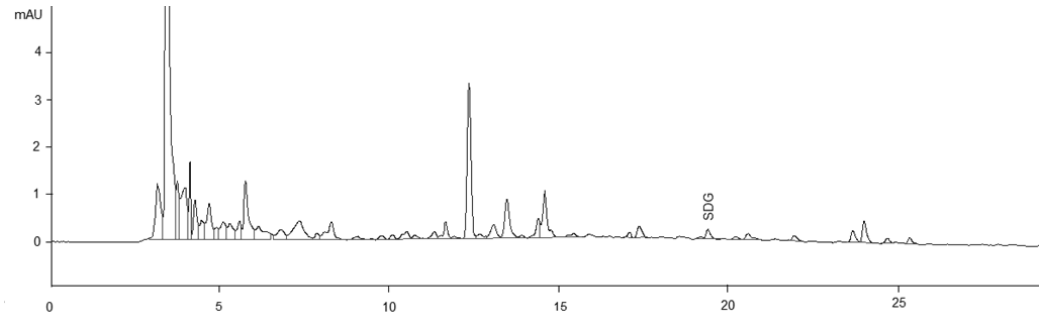

Figure 5.7. A model of HPLC chromatogram for MAE extract from almonds

The lignan yields obtained with MAE for samples other than flaxseed are much higher than the range of values published in the literature, thus further demonstrating the efficiency of the MAE method and its potential of becoming a generally applicable method for analytical lignan quantification in plants. In most plant sources lignans occur conjugated to a variety of carbohydrates; thus in order for the optimized MAE to be generally applicable, it has to be followed by an optimized enzymatic hydrolysis method.

5.5. Conclusions

The novelty of this work resides in the complete proof of efficiency, repeatability and reliability of an optimized MAE method for analytical quantification of lignans in plant materials. In addition, the applicability of the MAE method was demonstrated for a variety of plant samples such as: flaxseed cultivars, flaxseed hulls, sesame seeds, chia seeds, sunflower seeds and almonds. Therefore, it can be concluded that the MAE method was evaluated for the general analytical quantification of lignans in theses samples. For extending its applicability to the general analytical quantification of lignans in plants, the MAE method has to be followed by an optimized enzymatic hydrolysis. The optimized MAE method is recommended for applications such as: the classification of flaxseed cultivars based on their lignan content, for construction of lignan databases for plant foods, and for adding lignan values to the quality data tables for flaxseed.

Connecting Statement to Chapter 6

In **Chapter 5**, it has been demonstrated that the optimized microwave-assisted extraction (MAE) method for flaxseed lignan was precise, accurate, repeatable, and was significantly faster and more efficient than two conventional extraction reference methods. In addition, the applicability of the MAE method for analysing lignans in plant matrices other than flaxseed has been demonstrated. In order to use the MAE method for the general quantification of lignans in plants, it has to be followed by an efficient enzymatic hydrolysis method.

In view of developing an efficient enzymatic hydrolysis method it is first required to develop a solid phase extraction (SPE) purification method for removing the hydrolysis chemicals from the microwave-assisted extracts. The SPE method will be then used to assess the effects of prior purification of microwave-assisted extracts on the yields of the enzymatic hydrolysis.

In **Chapter 6**, the development of the SPE method and the modeling of the SPE yields are presented.

Chapter 6 is written in manuscript style and was published in edited form.

- Nemes, S.M., Orsat, V., 2011. Modeling the Recovery Patterns from Solid Phase Extraction Purification of Secoisolariciresinol Diglucoside, *P*-Coumaric Acid Glucoside, and Ferulic Acid Glucoside from Microwave-Assisted Flaxseed Extracts. Food and Bioproducts Processing 90, 453-465.

Some of the results presented in **Chapter 6** have been disseminated through conference presentations.

- Nemes, S.M., Orsat, V., Raghavan, G.S.V., 2011. Solid Phase Extraction Purification of Microwave-Assisted Flaxseed Extracts, Northeast Agricultural and Biological Engineering Conference (NABEC), South Burlington, VT, USA, July 24-27.

- Nemes, S.M., Orsat, V., Raghavan, G.S.V., 2010. Microwave-Assisted Technology for Quantitative Analysis of Lignans in Foods, The XVIIth World Congress of the International Commission of Agricultural Engineering CIGR, hosted by the Canadian Society for Bioengineering (CSBE/SCGAB), Québec City, Canada, June 13-17

Chapter 6 - Modeling the Recovery Patterns from Solid Phase Extraction Purification of Secoisolariciresinol Diglucoside, *P*-Coumaric Acid Glucoside, and Ferulic Acid Glucoside from Microwave-Assisted Flaxseed Extracts

6.1. Abstract

Flaxseed extracts obtained by microwave-assisted extraction (MAE) were cleaned of leftover salts from hydrolysis by solid phase extraction (SPE). The SPE set up was affordable, non-automated and vacuum-driven. The recovery patterns of secoisolariciresinol diglucoside (SDG), *p*-coumaric acid glucoside (PCouAG), and ferulic acid glucoside (FerAG) were modeled in two stages using regression procedures. At stage one, the recovery patterns were predicted as a function of SPE eluent concentration in ethanol (10 to 50 %). At stage two, the accuracy of the predictions was increased by enlarging the SPE eluent regressor's range (0 to 100 % ethanol in water) and arranging the solvent system into three practical elution groups. The groups 1, 2 and 3 reflected the major loss, the major recovery and the minor loss of SDG, respectively. Second degree polynomial regression models were fitted for accurately predicting the recoveries of compounds. Microwave-assisted extracts obtained from 0.6 and 1.5 g defatted flaxseed meal were purified; the total SDG recoveries from the SPE funnel were 97.8 and 99.8 %; and the SDG amounts obtained were 8.54 and 20 mg, respectively. The high performance liquid chromatography (HPLC) analysis of eluates pooled into practical groups allows for significant reductions in HPLC analysis time and solvent consumption which could have a positive impact on future purification studies. The results of this study allow for designing simplified, efficient and economical pilot scale studies for the purification of SDG from flaxseed extracts.

Key words: prediction accuracy, SDG, SPE, purification losses, lignan, MAE.

6.2. Introduction

Lignans are phytoestrogens found in great amounts in flaxseed and its derivatives (e.g., defatted flaxseed meal and flax hulls). Flaxseed lignans are bio-converted in the human digestive tract to the more potent mammalian lignans, enterodiol and enterolactone, which have beneficial effects for the human health such as reduction of the risk of developing cardiovascular diseases, the metabolic syndrome or certain types of cancer (Adolphe et al., 2010). The flaxseed lignan is secoisolariciresinol diglucoside (SDG); its concentration varies with the cultivated variety. SDG concentrations ranging from 1 to 1.9 % on a fresh weight basis (wb) were reported by Nemes and Orsat (2011a). Flaxseed derivatives such as defatted flaxseed meal or flax hulls have higher SDG concentrations; they were found to contain about 2.3 and 4 % SDG (wb) (Nemes and Orsat, 2011a). Flax hulls can be obtained from dry flaxseed by abrasive dehulling and separation of hulls from embryos by sieving or aspiration. Cui and Han (2006) found that the SDG content of flax hulls free of oil and mucilage was 2 to 10 times greater than that of the seed material used for dehulling. Weisenborn et al. (2003) found an inverse linear relationship between the SDG and the oil contents of the hull, the intact seeds and the embryos fractions resulted from a dehulling process. The authors reported that the hulls contained 46 times more SDG than the embryos. Therefore, defatted flaxseed meal and flax hulls are preferred to whole flaxseed meal as raw materials for SDG extraction, concentration and purification.

In order to improve the yield of producing purified SDG from flaxseed, efficient extraction methods have to be used in addition to selecting raw materials concentrated in SDG. A recent review of patented technologies for the extraction and the purification of SDG from flaxseed revealed that SDG is obtained mostly from defatted and whole flaxseed meal by alcoholic extraction followed by alkaline hydrolysis and sequential purification by liquid/liquid extraction, and/or anion exchange chromatography followed by reversed phase C18 chromatography (Hosseinian and Beta, 2009). In the literature, a variety of methods have been reported for the extraction of SDG for the purpose of purification by SPE. Two

categories of extraction methods can be distinguished: one that involves extraction with aqueous alcohols or mixtures of alcohols followed by alkaline hydrolysis (Johnsson et al., 2000; Muir and Westcott, 2000; Struijs et al., 2007; Thompson et al., 2006), and another that involves direct alkaline hydrolysis in aqueous or alcoholic solutions (Frank et al., 2004; Hyvarinen et al., 2006; Strandås et al., 2008). It has been demonstrated that the alcoholic extraction does not recover the lignan from the plant matrix completely thus limiting the extraction yield from the beginning. A direct alkaline hydrolysis approach is recommended as it is more efficient than using initial alcoholic extraction (Eliasson et al., 2003; Nemes and Orsat, 2011a). A conventional direct alkaline hydrolysis method was developed by Eliasson et al. (2003), and it requires 1 h of hydrolysis at room temperature with 1 M NaOH. A more efficient microwave-assisted extraction (MAE) method using a direct alkaline hydrolysis approach was recently developed by Nemes and Orsat (2010, 2011b) and was updated in section **3.5.5**; the MAE requires 3 min of extraction at 156 W (applied intermittently 30 s on/off) with 0.5 M NaOH. The MAE method was shown to extract the SDG from the flaxseed matrix completely; the coefficients of variation for three users over time were 2.3 – 4 % indicating excellent repeatability; and the total losses of SDG throughout the extraction and the HPLC analysis were only 2.5 % (Nemes and Orsat, 2011a). Therefore, it appears that MAE is the most efficient alternative to the methods presented above for the extraction of SDG from flaxseed.

Initial purification of SDG crude extracts was usually done by vacuum-driven SPE on C18 resins; the purpose was the fractionation of extracts in: (1) wash-fractions containing low molecular weight polar compounds, acids and leftover salts from hydrolysis, and (2) SDG-rich fractions that also contained *p*-coumaric acid glucoside (PCouAG) and ferulic acid glucoside (FerAG). The extracts were prepared for SPE by adjusting the pH to 3 (Johnsson et al., 2000; Strandås et al., 2008) or to 6.5 - 7 (Muir and Westcott, 2000; Struijs et al., 2007), then by concentration or drying. The C18 SPE columns were usually activated with alcohol, generally methanol, and then equilibrated with water (Frank et al., 2004;

Johnsson et al., 2000; Struijs et al., 2007). The extracts, reconstituted in water (Frank et al., 2004; Johnsson et al., 2000; Muir and Westcott, 2000; Struijs et al., 2007), or in 10 % methanol in water (Strandås et al., 2008), were applied to the activated and equilibrated C18 SPE columns. Then, the unwanted compounds were washed out with water (Frank et al., 2004; Johnsson et al., 2000; Struijs et al., 2007), or in some cases with water followed by 30 % methanol in water (Strandås et al., 2008), or with acidified water followed by acidified 20 % ethanol in water (Muir and Westcott, 2000). The wash fractions were discarded; and the SDG was eluted from the C18 SPE columns with pure methanol (Frank et al., 2004; Johnsson et al., 2000; Struijs et al., 2007; Thompson et al., 2006), 30 % ethanol in water (Muir and Westcott, 2000), 40 % methanol in water (Strandås et al., 2008), or 50 % methanol in water (Johnsson et al., 2000). The purified extracts were usually analysed by HPLC (Frank et al., 2004; Johnsson et al., 2000; Muir and Westcott, 2000; Struijs et al., 2007), or by gas chromatography with mass spectrometry (GC-MS) (Thompson et al., 2006). Nuclear magnetic resonance and high performance liquid chromatography with mass spectrometry (HPLC-MS) were also used by Struijs et al. (2007).

The applications of initial purification of SDG extracts by C18 SPE are varied. Muir and Westcott (2000) used SPE for obtaining 80 – 90 % pure SDG for incorporation in bakery products; and for cleaning of extracts before further purification by preparative HPLC in order to obtain > 98 % SDG. Johnsson et al. (2000) used SPE for removing the hydrolysis salts from extracts before HPLC analysis and further purification on silica gel 60 in view of obtaining > 99 % pure SDG. Frank et al. (2004) obtained > 95 % pure SDG for dietary supplementation experiments on rats. Strandås et al. (2008) applied SPE for obtaining high purity SDG for antioxidant capacity assessment; and for cleaning of extracts before further purification by semi-preparative HPLC in view of elucidating the composition and the structure of the flaxseed lignan macromolecule. Thompson et al. (2006) used SPE to clean lignan extracts before and after enzymatic hydrolysis.

With few exceptions, the details pertaining to the efficiency of published SPE procedures for initial purification, and the characteristics of the extracts obtained with them are not known. The recoveries of SDG from the SPE columns, relative to the SDG content of the applied extract, are not reported in the literature. Therefore, it is not possible to appreciate the extent of SDG losses as affected by the various published purification procedures. However, Johnsson et al. (2000) reported > 99.5 % recovery of SDG standard for the SPE procedure they used for desalting flax extracts before HPLC analysis; and SDG concentrations of > 60 % and 80 - 90 % were reported by Westcott and Muir (1998) and Muir and Westcott (2000), respectively, for the initial purification of flaxseed extracts by C18 SPE.

The purpose of this work was to develop a SPE purification technique with a high SDG recovery, and to establish the recovery patterns of SDG, PCouAG, and FerAG as a function of eluent composition, practical eluent groups, and extract concentration of target compounds. The SPE technique was developed using flaxseed extracts obtained with our optimized (2010, 2011b) and validated MAE method (Nemes and Orsat, 2011a) in order to maximize the recovery of pure SDG relative to the SDG content of the flaxseed material used for extraction.

6.3. Materials and Methods

6.3.1. Chemicals

The SDG reference standard of HPLC grade (molecular weight 686.71, purity 97.6 %) was purchased from Chromadex (Santa Ana, CA, USA). The solvents: hexane, methanol, acetonitrile and ethyl acetate were of HPLC grade and were purchased from Fisher Scientific (Ottawa, ON, Canada). Anhydrous ethanol was purchased from Commercial Alcohols (Brampton, ON, Canada). The reagents: sodium hydroxide (purity ≥ 98 %), sulphuric acid (purity 95 – 98 %), phosphoric acid (purity ≥ 85 %) and di-potassium hydrogen phosphate (purity 98 %) were purchased from Sigma-Aldrich (Oakville, ON, Canada).

6.3.2. Flaxseed

Brown flaxseed (*Linum usitatissimum* L.) was purchased from a local grocery store (Montreal, QC, Canada) in 2009. The seeds (100 g) were ground with a coffee grinder then defatted twice with 600 ml hexane under magnetic stirring for 1 h at room temperature. The solvent was filtered under vacuum, and the flaxseed meal was left overnight under the fume hood in order to allow the residual solvent to evaporate (Nemes and Orsat, 2010). The flax meal was ground again with a coffee grinder and the obtained defatted flaxseed meal (DFM) was packed in an air tight plastic bag and kept at -18°C until use.

6.3.3. Microwave-Assisted Extraction

All MAE experiments were carried out with a mono-mode (focused) microwave apparatus (Star System 2, CEM, Mathews, USA; nominal power declared by the manufacturer 800 W, microwave frequency 2.45 GHz) which delivers a maximum power of 710.5 W (section **4.4.1**), as calibrated with the calorimetric method developed specifically for the Star System 2 instrument (**Chapter 4**, section **4.3.1**). The extraction vessels (borosilicate glass, working volume 250 ml) were topped with a Graham-type reflux condenser. Samples of 0.6 and 1.5 g DFM were extracted according to our previously published methodology (Nemes and Orsat, 2011b), which was updated in section **3.5.5**. Briefly, the DFM samples were mixed with 50 ml 0.5M NaOH, and subjected to 22 % power level [156 W as calculated with the calibration in **Equation 4.2** = 7.105 × Power level (%), presented in section **4.4.1**]; the microwave power was applied intermittently (30 s on/off) for 3 min. [Note: Statistically similar results can be obtained if using programmed power levels in the range of 17 to 27 % (section **3.5.5**)]. The final temperature of the extracts, measured with a type K thermocouple (accuracy ± 0.5°C, Fisher Scientific) after briefly stirring the extracts, was 67°C.

6.3.4. Preparation of Microwave-Assisted Extracts for Solid Phase Extraction

The materials and methods used for the preparation of extracts for SPE might affect the recovery of target compounds. The pH of microwave-assisted extracts was adjusted to 3, 5 and 7 with sulphuric or acetic acids and the extracts were used for preliminary SPE tests. Based on the results presented below in the section **6.4.1**, the following preparation procedure was adopted. The water soluble proteins and carbohydrates from the extracts were precipitated through acidification to pH 3 with 5.55 ml 5N H_2SO_4, and addition of 100 ml methanol followed by magnetic stirring for 15 min at 300 rpm. The solid and liquid phases were separated by centrifugation for 10 min at 3000 rpm (Centrific centrifuge, Fisher Scientific). The liquid phase was concentrated to about 25 ml by evaporation under vacuum at 65°C and 195 rpm using a Buchi Rotavapor 205 equipped with a B490 heating bath. The pH of the concentrated aqueous extract was adjusted to 5 by addition of 0.2 ml of 2 M NaOH while measuring the pH with an Accumet 25 instrument (Fisher Scientific), then the volume was brought to 50 ml with double de-ionized water (Simplicity 185, Millipore). An aliquot of 0.5 ml extract was taken aside for HPLC analysis, and the rest of the extract was transferred into a 100 ml syringe fitted with a polyvinylidene difluoride (PVDF) 0.45 µm syringe membrane filter (Millex R-HV, Fisher Scientific). The extract was loaded onto the conditioned and equilibrated SPE funnel directly with the syringe at a rate of 1-2 drops/s.

6.3.5. Solid Phase Extraction

The SDG was separated from the salts resulting from hydrolysis and other low molecular weight polar compounds using SPE VersaPure pre-packed Buchner funnels (internal diameter 90 mm, height 48 mm; Sigma-Aldrich) for large scale purification of aqueous extracts containing polyphenols. The SPE phase (DPA-6S polyamide resin, 50 g, particle size 50 - 180 µm, pH 7.4) absorbed polar and mid-polar compounds by reversed phase mechanism through hydrogen bonding

between the hydroxyl groups of the compounds of interest and the amide groups of the resin. The SPE funnel was fitted onto a vacuum filtration flask (500 ml). The flow rate was maintained at 1 - 2 drops/s throughout the loading of extracts and eluents, as per the recommendations of the manufacturer. The SPE funnel was conditioned with 300 ml of anhydrous ethanol and then equilibrated with 600 ml double de-ionized water. The 0.45 µm filtered extracts were loaded onto the SPE funnel. Then, based on the results of the SPE preliminary tests (section **6.4.1**), the funnel was washed with 300 ml of double de-ionized water to remove the hydrolysis salts and other unwanted compounds. The SDG was eluted with 300 ml of 10, 20, 30, 40, 50 % ethanol in double de-ionized water, and then the SPE funnel was cleaned with 100 % ethanol. The eluates were concentrated to about 50 ml by evaporation under vacuum at 65°C and 195 rpm. An aliquot of 2 ml from each eluate was subjected to high performance liquid chromatography (HPLC) analysis. After each purification experiment, the SPE funnel was regenerated with 300 ml ethyl acetate.

6.3.6. High Performance Liquid Chromatography Analysis

The contents of SDG in the aqueous microwave-assisted extracts prepared for SPE, and the SPE eluates were analysed in triplicates (coefficients of variation < 5 %) using an HPLC Agilent 1100 series controlled by the Chemstation software [Rev. B.01.03 (204), Agilent Technologies]. The chromatograms were recorded at 280 nm using a variable wavelength detector. The quantification of SDG was done as previously reported by us (Nemes and Orsat, 2011a). The separation was carried out on a reversed phase C18 column (particle size 5 µm , length 25 cm, internal diameter 4.6 mm internal; Discovery, Sigma-Aldrich) fitted with a guard cartridge (particle size 5 µm, length 2 cm, internal diameter 4 mm; Supelguard, Sigma-Aldrich) at 25°C at a flow rate of 1 ml/min using a gradient method according to which the solvent A decreased from 100 to 50 % and solvent B increased from 0 to 50% from min 0 to min 50. The solvent A was 0.01 M phosphate buffer with a pH of 2.8 and a content of acetonitrile of 5 %, and

solvent B was acetonitrile. The standard curve for SDG was built with 6 levels of lignan concentrations ranging from 5 to 200 µg/ml methanol (coefficient of determination R^2 > 0.999). SDG eluted at about 19.5 min. The quantification equation was: SDG=peak height/0.4494. The PCouAG and FerAG were identified based on resemblance with previously published chromatograms (Eliasson et al., 2003; Johnsson et al., 2000; Nemes and Orsat, 2010); their respective elution times were 11 and 12.5 min (**Figure 6.1**); their quantification was done in the absence of reference standards based on their respective peak heights in terms of milli absorbance units (mAU).

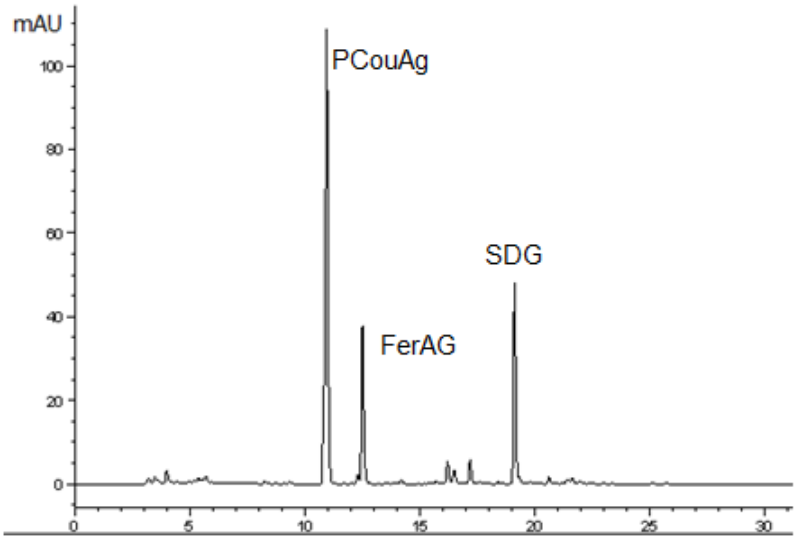

Figure 6.1. Model of HPLC chromatogram for microwave-assisted flaxseed extract

6.3.7. Experimental Design and Statistical Analysis

Five SPE tests were carried out for samples of 0.6 g DFM, and three SPE tests for samples of 1.5 g DFM. Regression models were fitted for the observed recovery yields of SDG, PCouAG and FerAG using the PROC REG procedure, and the predicted values generated with the regression models were correlated with the observed ones using the PROC CORR of SAS 9.2 TS2M2 (SAS Institute Inc.,

Cary, NC, USA). The same analyses were carried out with JMP 8 (SAS Institute Inc., Cary, NC, USA) in order to generate the graphics. The significance of the tests was established at $p < 0.05$ in all cases.

6.4. Results and Discussion

6.4.1. Preliminary Solid Phase Extraction Tests

According to the literature the pH of the flaxseed extracts purified by SPE was either acidic (pH 3) (Johnsson et al., 2000; Strandås et al., 2008; Westcott and Muir, 1998) or neutral (Muir and Westcott, 2000; Struijs et al., 2007; Thompson et al., 2006). The acids used for this purpose included acetic acid (Muir and Westcott, 2000; Thompson et al., 2006), hydrochloric acid (Hyvarinen et al., 2006) and sulphuric acid (Johnsson et al., 2000; Strandås et al., 2008). Sulphuric acid was preferred in this work as acetic acid caused the denaturation of PCouAG and FerAG during the concentration of extracts by vacuum evaporation, which caused split peaks in the HPLC chromatograms. Preliminary tests were carried out in order to assess the effects of pH 3, 5 and 7 on the recovery of SDG. It was observed that the SDG from a pH 3 extract (acidification with 5.55 ml of 5 N H_2SO_4) was not well retained by the SPE bonded phase as nearly 60 % of it was lost in the water eluate. Another extract, neutralised with 5 ml of 5 N H_2SO_4, could not be filtered before SPE as it clogged the membrane filter due to inefficient precipitation of water soluble proteins and carbohydrates. The submicron filtration of extracts is desired in order to avoid the clogging of the SPE funnel and to prolong its life. Based on these findings, it was hypothesised that a two-stage pH adjustment could maximize the recovery of SDG. At stage 1, the acidification to pH 3 would ensure an efficient cleaning of the extract; and at stage 2, the increasing of pH to 5 or 7 could result in improved retention of SDG onto the bonded SPE phase. Subsequently, the pH of two extracts was initially adjusted to 3 (addition of 5.55 ml 5 N H_2SO_4), and then to 5 or 7 (addition of 0.2 or 0.4 ml 2 M NaOH). The highest SDG recovery > 97 % was achieved for the pH 5 extract as opposed to 94 % for the pH 7 extract. The technical

specifications of the SPE funnel indicated that 5 – 20 % alcohol in water could be used for removing the unwanted compounds before eluting the analytes of interest. In two preliminary tests the SPE funnel was washed with 5 % ethanol or 10 % ethanol in water after applying the extract; the amounts of SDG brought out by these eluents were 31.8 and 39.5 % from the total added SDG, respectively. It was concluded that aqueous ethanol was not an appropriate solvent for the washing step. Based on these results and the literature (Frank et al., 2004; Johnsson et al., 2000; Struijs et al., 2007) water was used as wash-eluent for the subsequent SPE tests.

6.4.2. Solid Phase Extraction of Secoisolariciresinol Diglucoside from 0.6 g Defatted Flaxseed Meal Samples

Five microwave-assisted flaxseed extracts obtained from 0.6 g DFM were subjected to SPE purification. An aliquot of each extract was analysed by HPLC in order to quantify its content in SDG, PCouAG and FerAG. The SDG recovered with each SPE eluate was calculated in percentage relative to its amount in the extract loaded onto the funnel (about 12 mg SDG). Predictive models were fitted to the SDG recovery data as a function of SPE eluent concentration in ethanol (10, 20, 30, 40 and 50 %) using the PROC REG regression procedure of SAS. The best fit to the data was obtained with a 2^{nd} degree polynomial model (**Equation 6.1**) which had R^2 = 0.943 and R^2adj. = 0.938. There were 25 data points, and the degrees of freedom (DF) were divided between the model – 2 DF, the intercept – 1 DF, and the error term – 22 DF. The model was highly significant ($p < 0.0001$) as can be seen from the analysis of variance presented in **Table 6.1.** The regression coefficients (parameter estimates) used to construct the predictive model presented in **Equation 6.1** ($p < 0.0001$) are shown in **Table 6.2**. However, the relative standard errors (RSE = standard error × 100/ absolute value of the parameter estimate) were high; they ranged between 5.7 and 14.7 %, which translated into wide confidence intervals for the individual predictions. The standard error is an estimation of the standard deviation of the calculated

regression coefficients (parameter estimates); it gives information about the variability around the regression line, and the accuracy of the prediction. Parameter estimates that have large RSEs are considered less accurate.

$$Eluted\ SDG\ (\%) = 69.2729 - 3.0502 \times SPE_{eluent} + 0.0334 \times SPE_{eluent}^{2}$$

Equation 6.1

Table 6.1. ANOVA for the 2nd degree polynomial predictive model for SDG elution as a function of SPE eluent concentration in ethanol

Source	DF	Sum of Square	Mean Square	F Value	Prob > F
Model	2	6234.5596	3117.2789	182.93	< .0001
Error	22	374.9069	17.0412		
Corrected Total	24	6609.4665			

Table 6.2. Parameter estimates for the quadratic predictive model for SDG elution as a function of SPE eluent concentration in ethanol

Variable	DF	Parameter Estimate	Standard Error	T Value	Prob > |t|
Intercept	1	69.2729	3.9595	17.5	< .0001
SPE-eluent	1	-3.0502	0.3017	-10.11	< .0001
SPE-eluent^2	1	0.0334	0.0049	6.78	< .0001

The line of fit for eluted SDG obtained with the predictive model (**Equation 6.1**) is shown within confidence curves for the predicted SDG means that border the darker shaded area. The confidence curves are in turn shown within upper and lower 95 % confidence limits for the individual SDG predicted values that border the lighter shaded area (**Figure 6.2**). These confidence limits reflected the variations in the predictions caused by the magnitude of the standard errors of the

regression coefficients. The highest variation was due to the 2nd order estimate (RSE = 14.7 %) which caused the confidence limits to stretch around the prediction area for the SPE eluents containing 40 and 50 % ethanol. Most of the applied SDG eluted in decreasing amounts with the SPE eluents containing 10, 20 and 30 % ethanol in water. The proportions of SDG eluted with 40 and 50 % ethanol in water were rather negligible.

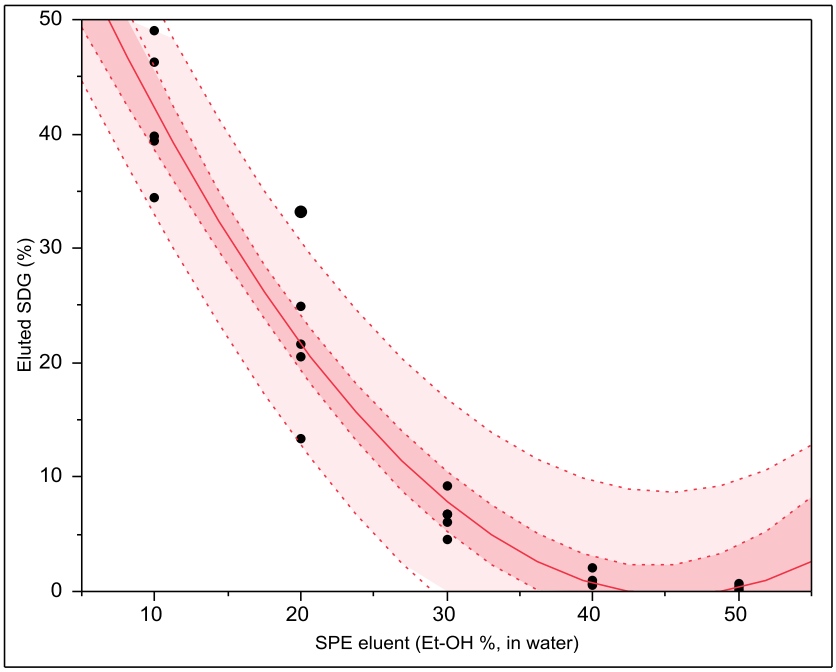

Figure 6.2.The SDG elution profile as a function of SPE eluent concentration in ethanol

The correlation of the observed and predicted SDG recovery values obtained as a function of SPE eluent concentration in ethanol (10 - 50 %) was carried out using the PROC CORR of SAS as a means of further assessing the goodness of fit of the predictive model (r = 0.971; $p < 0.0001$). The data points are represented within a 95 % confidence ellipse (**Figure 6.3)**, which is a graphical indicator of correlation; the more elongated the ellipse (collapsed diagonally) the stronger the correlation of the two variables. An ellipse approaching circular in shape is an indicator of weak correlation. Based on these results it can be concluded that the 2nd degree polynomial predictive model fitted well the SDG recovery data.

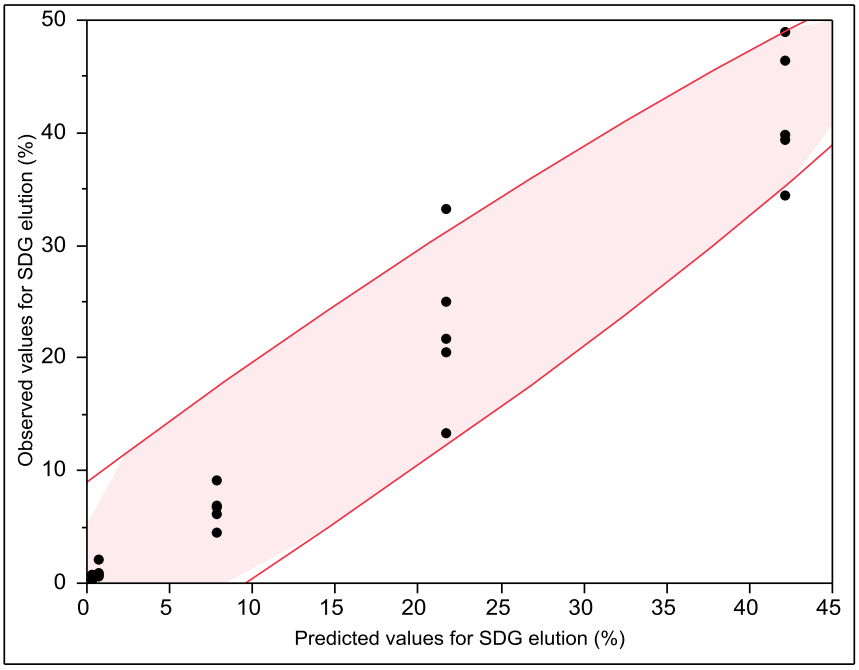

Figure 6.3. Scatter plot with 95 % confidence ellipse for observed versus predicted SDG elution values (%) obtained as a function of eluent concentration in ethanol

The mean values of observed SDG recoveries are presented in **Table 6.3**, along with the predicted SDG recovery values generated with the **Equation 6.1**. It is worth noting that the SPE procedures used in this work were susceptible to error as they did not benefit of accurate, automatic flow rate control. The repeatability of results depended on the way the user appreciated the volume of the collected SPE eluates in vacuum filtration flasks with more or less accurate graduations. Due to these experimental conditions, it was hypothesised that small amounts of SDG "migrated" between two subsequent elution steps and caused the high standard deviation (STDV) values presented in **Table 6.3**. SDG losses occurred mostly in the water SPE eluate (0 % ethanol) and only trace amounts were recovered from the cleaning of the SPE funnel with 100 % ethanol. The regression model from **Equation 6.1** did not allow for the prediction of these losses, and as a consequence, the total predicted recovery could not be estimated and checked against the observed total SDG recovery. The model was useful

however for estimating the pattern of the SDG recovery for the SPE eluents containing 10 – 50 % ethanol.

Table 6.3. The SDG recovery profile as a function of SPE eluent concentration in Et-OH, and practical elution groups for samples of 0.6 g DFM

SPE eluent (Et-OH % in water)	SDG elution yield (% from the applied quantity)		[1]Predicted SDG elution yield (%)	[2]Predicted SDG elution by practical elution group (%)
	Mean (N=5)	STDV		
0	25.0	2.81	-	25.0
10	41.8	5.87	42.1	
20	22.7	7.21	21.6	71.2
30	6.66	1.69	7.86	
40	1.27	0.744	0.757	
50	0.289	0.247	0.345	1.64
100	0.0792	0.0253	-	
Total recovery	97.8	2.47	-	97.8

[1]Predicted values generated with **Equation 6.1**.

[2]Predicted values generated with **Equation 6.2**.

A more practical way of estimating the recoveries and the losses of SDG from the SPE funnel was required. For this purpose the data observed with the individual SPE eluent fractions were arranged according to three practical groups defined as follows; group 1, included the water eluent; group 2, pooled the 10, 20 and 30 % ethanol eluents; and group 3, pooled the 40, 50, and 100 % ethanol eluents. From a practical point of view, groups 1 and 3 gave information on the losses of SDG, and group 2 estimated the percentage of useful recovered SDG. If desired, the water eluent can be concentrated and subjected to SPE purification a second time.

One such test was carried out and it was observed that the SDG recovery pattern was similar to that presented in **Figure 6.2**. However, such an approach would involve a waste of efforts and solvents as the water eluate contained about 3 mg of SDG only.

A 2nd degree polynomial regression model (**Equation 6.2**) was fitted to the data as a function of three practical elution groups using the PROC REG procedure of SAS. The analysis of variance is shown in **Table 6.4**; the predictive model was highly significant ($p < 0.0001$) with $R^2 = 0.992$ and R^2adj. = 0.991. The 15 degrees of freedom were divided between the model – 2 DF, the error – 12 DF, and the intercept – 1 DF. The parameter estimates shown in **Table 6.5** were highly significant ($p < 0.0001$) and were used to construct the predictive model from **Equation 6.2**. The redefinition of the SPE eluent regressor in terms of practical elution groups caused the RSEs values to be reduced by 52.6 to 72.8 % (RSEs 2.7 – 4 %) which translated into more accurate predictions falling into narrow 95 % confidence limits.

$$Eluted\ SDG\ (\%) = -136.8085 + 219.6495 \times SPE_{group} - 57.8341 \times SPE_{group}^2$$

Equation 6.2

The line of fit obtained with the predictive model (**Equation 6.2**) is shown in **Figure 6.4** within confidence curves for the predicted mean SDG values (darker shade) and upper and lower 95 % confidence limits for the individual predicted SDG values (lighter shade).

Table 6.4. ANOVA for the 2nd order polynomial predictive model for SDG elution as a function of three practical SPE elution groups

Source	DF	Sum of Square	Mean Square	F Value	Prob > F
Model	2	12515.129	6257.564	781.499	< .0001
Error	12	96.086	8.007		
Corrected Total	14	12611.214			

Table 6.5. Parameter estimates for the 2nd degree polynomial predictive model for SDG elution as a function of three practical SPE elution groups

Variable	DF	Parameter Estimate	Standard Error	T Value	Prob > |t|
Intercept	1	-136.8085	5.5161	-24.8	< .0001
SPE-group	1	219.6495	6.2638	35.07	< .0001
SPE-group^2	1	-57.8341	1.5499	-37.32	< .0001

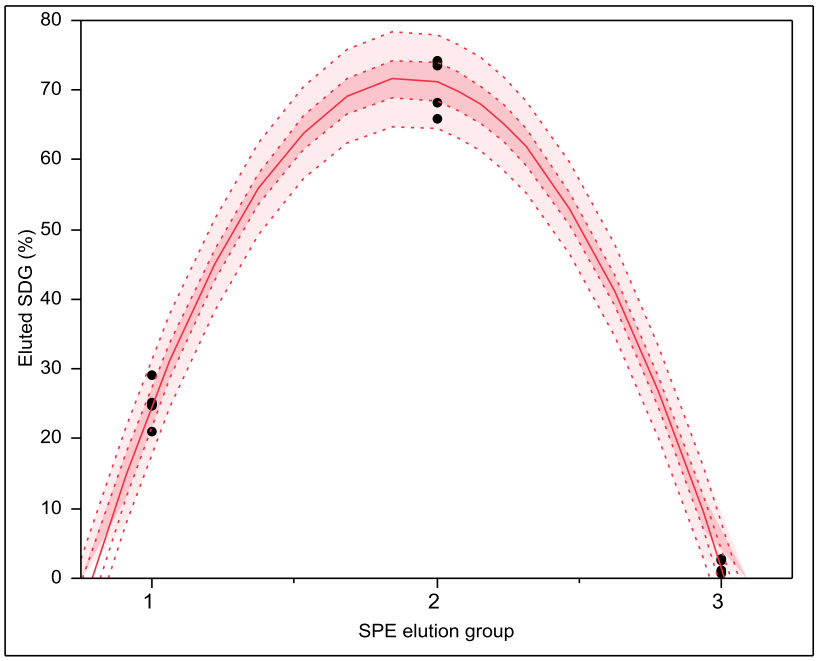

Figure 6.4. The SDG elution profile as a function of three practical SPE elution groups

The predicted SDG recoveries (25.0, 71.2 and 1.64 % for groups 1, 2 and 3, respectively) are shown in the last column in **Table 6.3**; they coincided with the observed recovery values (sum of means for the SPE eluents in groups 2 and 3). Based on these results it was concluded that it was preferable to model the SDG recoveries as a function of practical SPE elution groups; and accurate predictions were obtained as a result of reducing the variation caused by the migration of SDG between subsequent eluents. This procedure also benefited of highly improved economy in terms of HPLC work time and solvent consumption for the following reasons: analysis of seven SPE eluates in triplicates required a total of 21 HPLC injections, whereas three eluate groups to be analysed in triplicates required a total of 9 HPLC injections. In addition, the procedure using practical elution groups has an excellent repeatability as demonstrated by the accuracy of the predictions.

The correlation of the observed with the predicted SDG recovery values, obtained as a function of elution groups, is shown in **Figure 6.5**. The elongated, diagonally collapsed shape of the 95 % confidence ellipse indicated a strong correlation, also confirmed by the highly significant ($p < 0.0001$) Pearson's correlation coefficient $r = 0.996$. The observed and predicted total SDG recoveries from the SPE funnel were 97.8 % (**Table 6.3**). The 2.2 % losses were likely occurring during the transfer, concentration and further preparation of eluates for HPLC analysis.

This work provides an initial report on the efficiency of purification of SDG from flaxseed microwave-assisted extracts using simple and accessible SPE materials and procedures. Modeling the observed recovery data as a function of individual SPE eluents helped understanding the influence of the eluents composition on the SDG recovery. More accurate SDG recovery patterns were generated by modeling the data as a function of practical SPE elution groups. The latter approach is unique and useful for researchers since it addressed several key points of experimental organization.

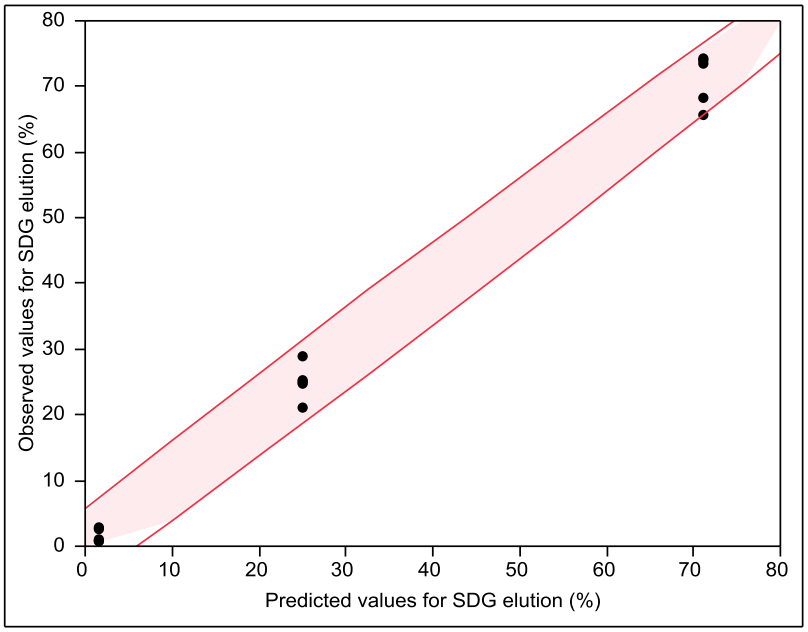

Figure 6.5. Scatter plot with 95 % confidence ellipse for the observed versus predicted SDG elution values (%) as a function of three practical elution groups

6.4.3. Solid Phase Extraction of *P*-Coumaric Acid Glucoside from 0.6 g Defatted Flaxseed Meal Samples

The same SPE replicates used for modeling the elution profile of SDG were used for fitting predictive models for the PCouAG recovered with 10 to 50 % ethanol and with the three practical elution groups (as defined above for SDG). The quantification of PCouAG in the extracts loaded on the SPE funnel and in each eluate was done in the absence of PCouAG standard compound in terms of peak height (absorbance, mAU). The recovery values were expressed in percentage relative to the amount of PCouAG loaded on the SPE funnel. The best fit to the observed data was obtained with a 3rd degree polynomial predictive model presented in **Equation 6.3**. The model was highly significant ($p < 0.0001$; **Table 6.6)**, and had $R^2 = 0.879$, and R^2adj. = 0.863. The parameter estimates ($p \leq 0.0011$) used to construct the predictive model from **Equation 6.3** are presented in **Table 6.7**. The RSEs ranged from 10 to 26.4 %. The highest

variability in this system was introduced by the 2nd and the 3rd order estimates as indicated by their respective RSEs of 21.7 and 26.5 %.

$$Eluted\ PCouAG\ (\%) = 31.5359 - 2.4558 \times SPE_{eluent} + 0.07016 \times SPE_{eluent}^{2} - 0.000637 \times SPE_{eluent}^{3}$$

Equation 6.3

Table 6.6. ANOVA for the 3rd degree polynomial predictive model for PCouAG elution as a function of SPE eluent concentration in ethanol

Source	DF	Sum of Square	Mean Square	F Value	Prob > F
Model	3	313.8399	104.6133	51.26	< .0001
Error	21	42.5871	2.0408		
Corrected Total	24	356.697			

Table 6.7. Parameter estimates for the 3rd degree polynomial predictive model for PCouAG elution as a function of SPE eluent concentration in ethanol

Variable	DF	Parameter Estimate	Standard Error	T Value	Prob > |t|
Intercept	1	31.5359	3.1429	10.03	< .0001
SPE-eluent	1	-2.4558	0.4108	-5.98	< .0001
SPE-eluent^2	1	0.07016	0.0152	4.6	0.0002
SPE-eluent^3	1	-0.000637	0.000168	-3.79	0.0011

The PCouAG recovery pattern modeled as a function of SPE eluent concentration in ethanol is presented in **Figure 6.6**. The line of fit is framed by confidence curves for the predicted PCouAG mean values (darker shade), and upper and lower 95 % confidence limits for the individual predicted PCouAG values (lighter

shade). As it was the case with SDG (**Figure 6.2**), the width of the 95 % confidence limits was due to the increased standard errors for the parameter estimates.

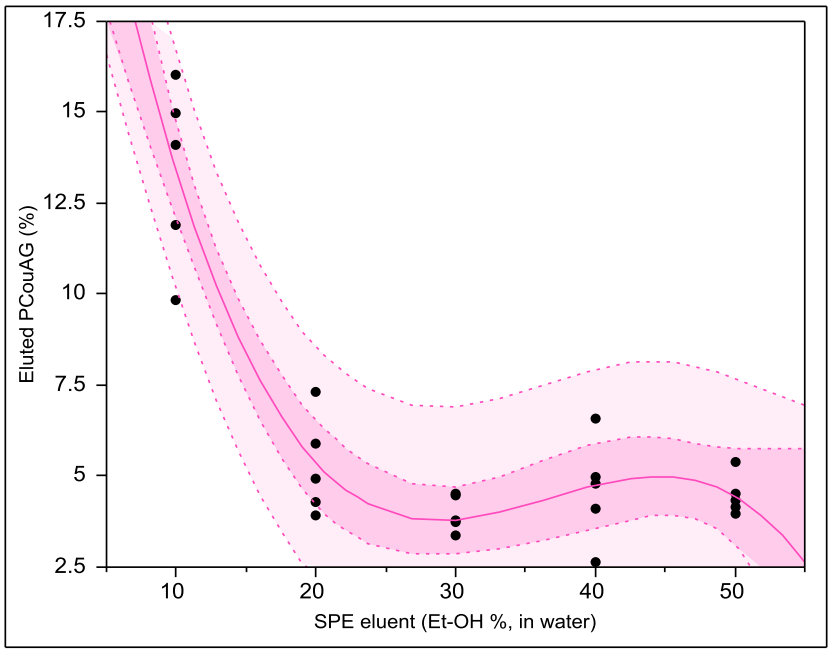

Figure 6.6. The PCouAG elution profile as a function of SPE eluent concentration in ethanol

However, the correlation of the predicted with the observed PCouAG recovery values shown in **Figure 6.7** was highly significant ($p < 0.0001$) with a Pearson's correlation coefficient r = 0.938. A close agreement between the observed and the predicted recovery values of PCouAG obtained as a function of eluent concentration in ethanol can also be observed in **Table 6.8**. Unlike SDG, most of the PCouAG was found in the water eluate indicating that the low molecular weight polar compounds were separated from SDG at the early stages of purification.

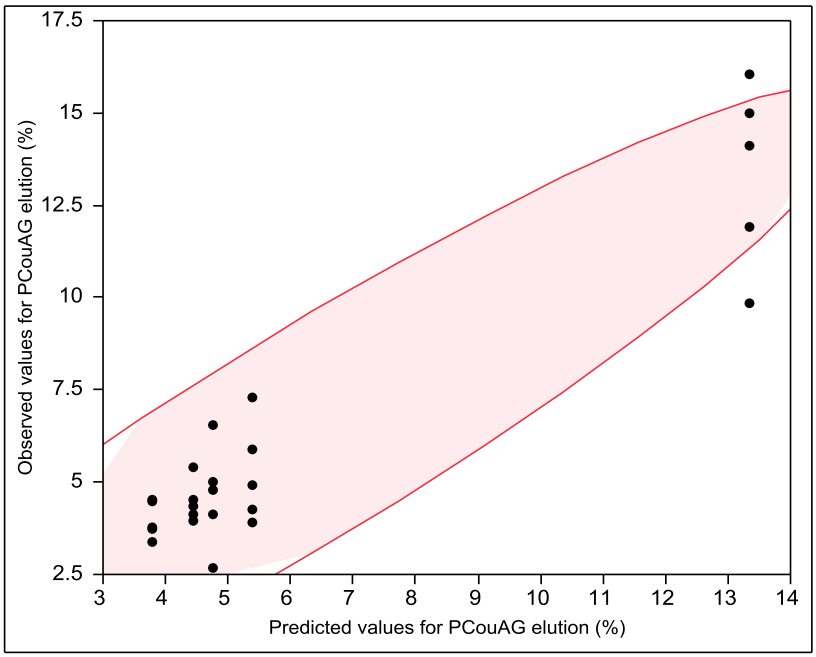

Figure 6.7. Scatter plot with 95 % confidence ellipse for the observed versus predicted PCouAG elution values (%) as a function of SPE eluent concentration in ethanol

Table 6.8. The PCouAG recovery profile as a function of SPE eluent concentration in ethanol, and practical elution groups for samples of 0.6 g DFM

SPE eluent (Et-OH % in water)	PCouAG elution yield (% from the applied quantity)		[1]Predicted PCouAG elution yield (%)	[2] Predicted PCouAG elution by practical elution group (%)
	Mean (N=5)	STDV		
0	62.5	2.601	-	62.5
10	13.4	2.49	13.4	
20	5.25	1.36	5.38	22.6
30	3.98	0.499	3.79	

Table 6.8 continues on the next page.

Table 6.8 continued

SPE eluent (Et-OH % in water)	PCouAG elution yield (% from the applied quantity)		[1]Predicted PCouAG elution yield (%)	[2] Predicted PCouAG elution by practical elution group (%)
	Mean (N=5)	STDV		
40	4.62	1.42	4.75	
50	4.48	0.549	4.44	11.0
100	1.86	0.884	-	
Total recovery	96.1	3.70	-	96.1

[1] Predicted values generated with **Equation 6.3**.

[2] Predicted values generated with **Equation 6.4**.

In order to obtain a complete recovery pattern for the PCouAG data, the SPE eluents were arranged in three practical groups as described above for SDG, and a 2nd order polynomial predictive model was fitted. The model shown in **Equation 6.4** was highly significant (p<0.0001; **Table 6.9**) with R^2 = 0.988 and R^2adj. = 0.986.

$$Eluted\ PCouAG\ (\%) = 130.5555 - 82.1728 \times SPE_{group} + 14.1031 \times SPE_{group}^2$$

Equation 6.4

The parameter estimates were highly significant (p < 0.0001; **Table 6.10**) and were used to build the 2nd order polynomial predictive model shown in **Equation 6.4**. The RSEs for the intercept, the linear and quadratic terms were 4.11, 7.41 and 10.9 %, respectively; they were reduced by 58.9 % by comparison with the RSEs of the parameter estimates presented in **Table 6.7**.

Table 6.9. ANOVA for the 2nd order polynomial predictive model for PCouAG

Source	DF	Sum of Square	Mean Square	F Value	Prob > F
Model	2	7299.0422	3649.5211	482.21	< .0001
Error	12	90.8196	7.5683		
Corrected Total	14	7389.8618			

Table 6.10. Parameter estimates for the 2nd order polynomial predictive model for PCouAG as a function of three practical SPE elution groups

Variable	DF	Parameter Estimate	Standard Error	T Value	Prob > |t|
Intercept	1	130.5555	5.3628	24.34	< .0001
SPE-group	1	-82.1728	6.0897	-13.49	< .0001
SPE-group^2	1	14.1031	1.5068	9.36	< .0001

The reduced variation in the data was reflected by the narrow confidence curves (darker shade) for the predicted PCouAG means and the narrow upper and lower 95 % confidence limits for the individual predictions (lighter shade) in **Figure 6.8**. Once again, the predicted recoveries coincided with the observed mean recovery for group 1 (62.5 %), and the sum of means for groups 2 and 3 (22.6 and 11.0 %, respectively) proving that the SPE data was more accurately modeled as a function of practical elution groups. The correlation of the observed and the predicted PCouAG recoveries is shown in **Figure 6.9**. The 95 % confidence ellipse is elongated and collapsed diagonally indicating a strong linear relationship between the two variables. Indeed, the Pearson's correlation coefficient was r = 0.994 at $p < 0.0001$.

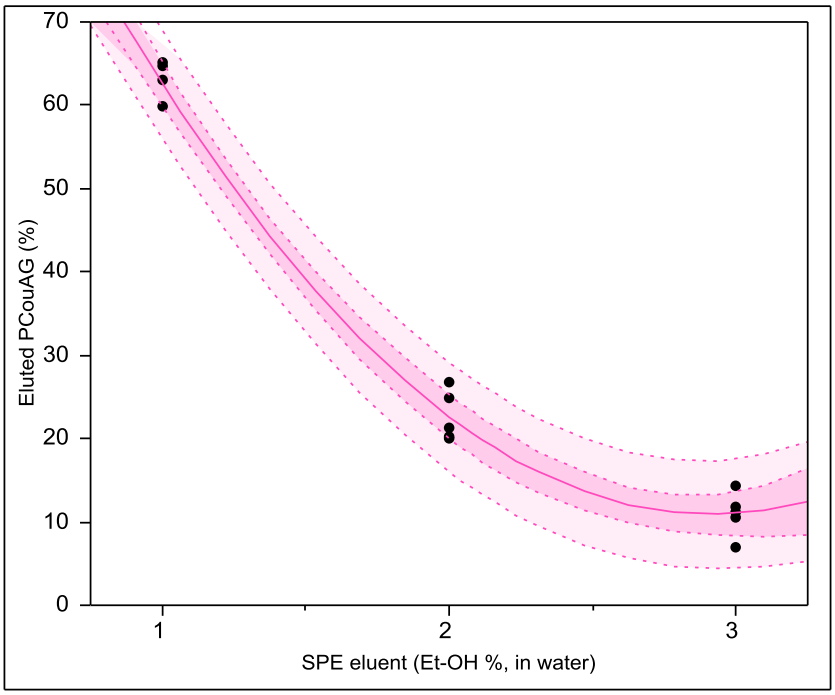

Figure 6.8. The PCouAG elution profile as a function of three practical SPE elution groups

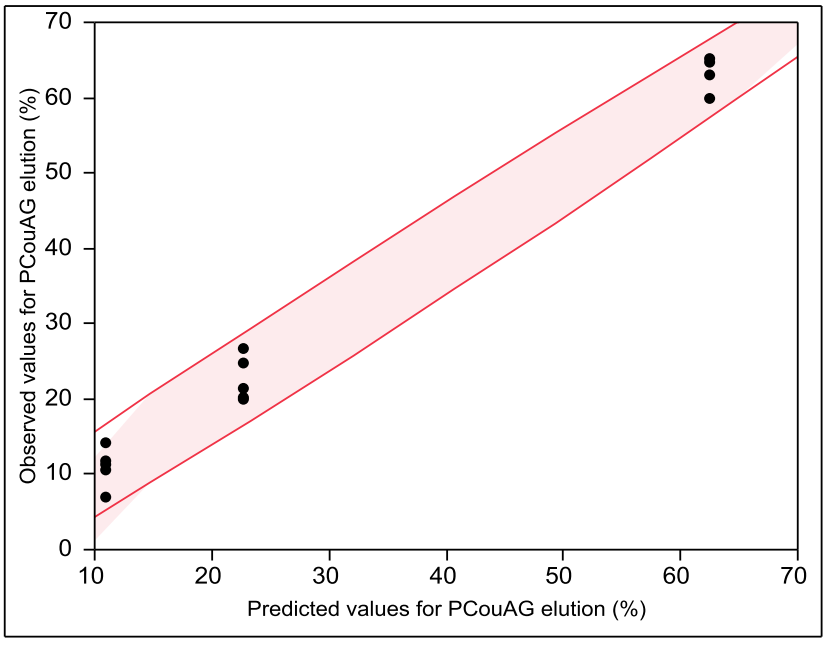

Figure 6.9. Scatter plot with 95 % confidence ellipse for observed versus predicted PCouAG elution values (%) as a function of practical SPE elution groups

The purpose of modeling the elution pattern of PCouAG was to assess the ability of the SPE packed bed to separate the lower molecular weight polar compounds, such as PCouAG and FerAG, from SDG which has higher molecular weight and moderate polarity. As expected, the greater amount of PCouAG was contained in the water eluate (elution group 1), unlike SDG which was eluted by 10, 20 and 30 % ethanol (elution group 2). It can be concluded that the SPE packed bed had a good ability of separating SDG from lower molecular weight polar compounds.

6.4.4. Solid Phase Extraction of Ferulic Acid Glucoside from 0.6 g Defatted Flaxseed Meal Samples

The SPE recovery patterns of FerAG and PCouAG were expected to be alike as both compounds have similar polarities and eluted close to each other in HPLC chromatograms. This hypothesis was confirmed by the results presented below. The same SPE replicates used above for fitting regression models for SDG and PCouAG were used for FerAG as well. The best fit to the data was achieved with a 3rd degree polynomial model (**Equation 6.5**), which was highly significant (p < 0.0001; **Table 6.11**) and had with R^2 = 0.913 and R^2adj. = 0.900. The parameter estimates used for building the predictive model (**Equation 6.5**) were highly significant ($p \leq 0.0007$; **Table 6.12**). The RSEs of the intercept, linear, quadratic and cubic terms were 9, 16, 21 and 25 %, respectively. The predictions were less accurate in the region of curvature as it was previously seen for SDG and PCouAG for the modelling as a function of the SPE eluent concentration in ethanol.

$$Eluted\ FerAG\ (\%) = 30.1429 - 2.2891 \times SPE_{eluent} + 0.0649 \times SPE_{eluent}^2 - 0.000601 \times SPE_{eluent}^3$$

Equation 6.5

Table 6.11. ANOVA for the 3rd degree polynomial predictive model for FerAG as a function of SPE eluent concentration in ethanol

Source	DF	Sum of Square	Mean Square	F Value	Prob > F
Model	3	357.6644	119.2215		< .0001
Error	21	34.2501	1.6309		
Corrected Total	24	391.9145			

Table 6.12. Parameter estimates for the 3rd degree polynomial predictive model for FerAG as a function of eluent concentration in ethanol

Variable	DF	Parameter Estimate	Standard Error	T Value	Prob > |t|
Intercept	1	30.1429	2.8096	10.73	< .0001
SPE-eluent	1	-2.2891	0.3673	-6.23	< .0001
SPE-eluent^2	1	0.0649	0.0136	4.76	0.0001
SPE-eluent^3	1	-0.000601	0.000151	-3.99	0.0007

The recovery pattern of FerAG (**Figure 6.10**) resembled that of PCouAG (**Figure 6.6**) indicating similarity of interactions with the SPE solid and mobile phases. The two compounds were not strongly retained by the packed bed and eluted early, mainly with water, allowing for obtaining subsequent eluates rich in SDG and poor in PCouAG and FerAG. Despite the wide 95 % confidence interval (lighter shade; **Figure 6.10**) for the individual predicted FerAG recoveries, these were strongly correlated with the observed recovery values ($r = 0.955$; $p < 0.0001$) as depicted by the elongated, diagonal, 95% confidence ellipse shown in **Figure 6.11**.

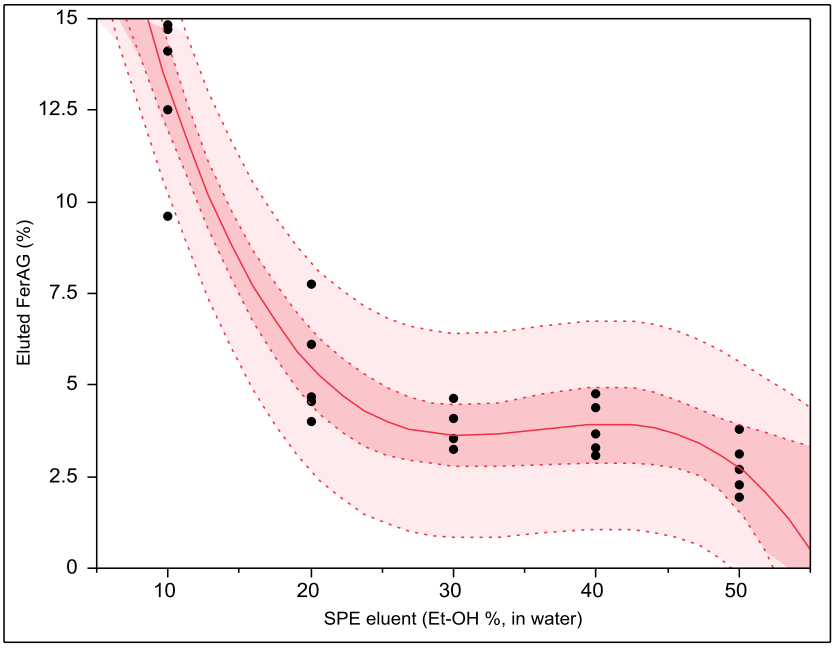

Figure 6.10. The FerAG elution profile as a function of SPE eluent concentration in ethanol

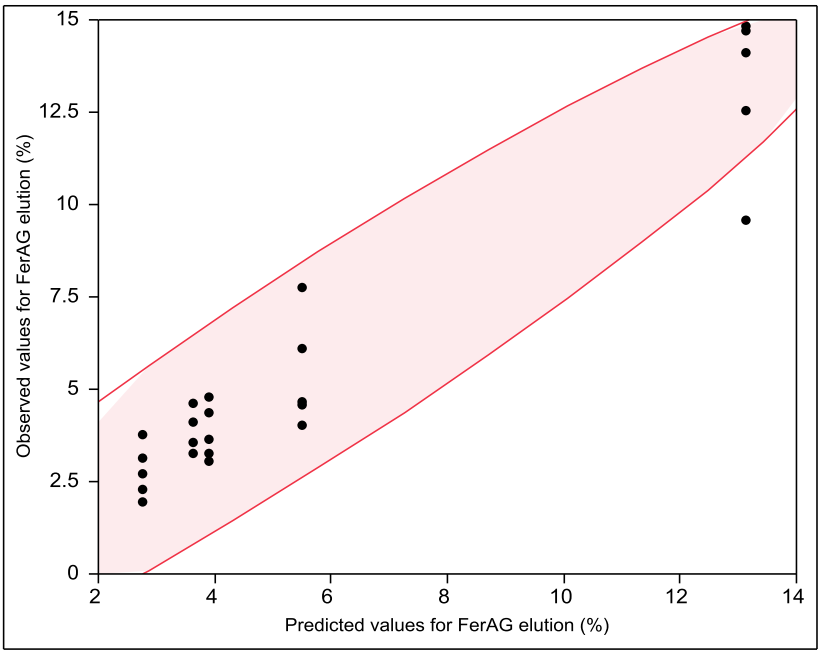

Figure 6.11. Scatter plot with 95 % confidence ellipse for observed versus predicted FerAG elution values (%) as a function of eluent concentration in ethanol

The FerAG mean recovery values obtained as a function of SPE eluent concentration in ethanol, along with their standard deviations are shown in **Table 6.13**. As it was the case with PCouAG, most of the FerAG eluted with water (62.1 %). However, important losses (7.5 %) were recorded for FerAG. These could no longer be attributed only to the manipulations and the preparation of eluates in view of HPLC analysis. It is possible that small amounts of FerAG were degraded during eluates concentration by vacuum evaporation. The predicted values obtained with **Equation 6.5** were in close agreement with the observed ones.

Table 6.13. The FerAG recovery profile as a function of SPE eluent concentration in ethanol

SPE eluent (Et-OH % in water)	FerAG elution yield (% from the applied quantity)		[1] Predicted FerAG elution yield (%)	[2] Predicted FerAG elution by practical elution group (%)
	Mean (N=5)	STDV		
0	62.1	2.66	-	62.1
10	13.2	2.19	13.1	
20	5.42	1.51	5.502	22.3
30	3.75	0.603	3.63	
40	3.83	0.73	3.91	
50	2.77	0.724	2.75	8.06
100	1.46	0.241	-	
Total recovery	92.5	4.35	-	92.5

[1] Predicted values generated **Equation 6.5**.

[2] Predicted values generated with the **Equation 6.6**.

A 2nd degree polynomial predictive model (**Equation 6.6**) was fitted for the observed FerAG recovery data as a function of practical SPE elution groups. The

model was highly significant ($p < 0.0001$; **Table 6.14**) and had $R^2 = 0.981$ and R^2adj. = 0.977. The parameter estimates used for constructing the predictive model from **Equation 6.6** were highly significant ($p < 0.0001$; **Table 6.15**). The RSEs for the intercept, the linear and the quadratic parameter estimates were 5.5, 10.2 and 15.5 %, respectively; these were reduced by 38 - 38.9 % as opposed to those presented above for modeling of FerAG recoveries as a function of eluent concentration in ethanol.

$$Eluted\ FerAG\ (\%) = 127.2783 - 779379 \times SPE_{group} + 12.7325 \times SPE_{group}^2$$

Equation 6.6

Table 6.14. ANOVA for the 2nd degree polynomial predictive model for FerAG as a function of three practical SPE elution groups

Source	DF	Sum of Square	Mean Square	F Value	Prob > F
Model	2	7832.6882	3917.3441	302.51	< .0001
Error	12	155.3924	12.9494		
Corrected Total	14	7990.0806			

Table 6.15. Parameter estimates for the 2nd order polynomial predictive model for FerAG, as a function of three practical SPE elution groups

Variable	DF	Parameter Estimate	Standard Error	T Value	Prob > |t|
Intercept	1	127.2783	7.0148	18.14	< .0001
SPE-group	1	-77.9379	7.9657	-9.78	< .0001
SPE-group^2	1	12.7325	1.9709	6.46	< .0001

The line of fit generated with **Equation 6.6**, is shown in **Figure 6.12** framed by confidence curves for the predicted FerAG recovery means (darker shade) and

lower and upper 95 % confidence limits for the predicted individual FerAG recovery values (lighter shade). The observed and predicted FerAG recoveries obtained as a function of elution group were strongly correlated (r = 0.99; $p < 0$ .0001) as depicted by the elongated, collapsed diagonally, 95 % confidence ellipse from **Figure 6.13**.

The amount of SDG per extract loaded onto the SPE funnel was about 12 mg. The separation of SDG from other compounds present in the extract was possible due to their respective selective interactions with the SPE packed bed and the elution solvents. For example, the SDG was retained by the polyamide resin (packed bed) through a reversed phase mechanism, according to which hydrogen bonds were formed between the hydroxyl groups of SDG and the amide groups of the resin. SDG has more hydroxyl groups available for hydrogen bonding than PCouAG and FerAG. This allowed for the early elution of PCouAG and FerAG with water; 62.5 and 62.1 % of the initial amounts loaded onto the SPE funnel were eluted, respectively. At the same time, the water eluent brought out only 25 % of the loaded SDG. Most of the applied SDG was eluted by the SPE group 2, 71.2 %, which corresponded to a yield of 8.54 mg SDG. This SPE group, rich in SDG, was poor in PCouAG and FerAG; it contained 22.6 and 22.3 % of the initially loaded amounts, respectively. The SPE group 2 was also free of hydrolysis salts, which are polar compounds that were not retained by the SPE bonded phase and were washed out with the water eluent. In fact, the standard method for removing salts from aqueous natural extracts uses reversed phase SPE; the salts are washed out with water, and the natural compounds are eluted with a solvent system containing organic solvents (Shimizu, 1998; Stead, 1998). The pooled eluates from SPE group 2 can be used for further experiments such as purity tests, antioxidant capacity, and SDG stability during various storage conditions.

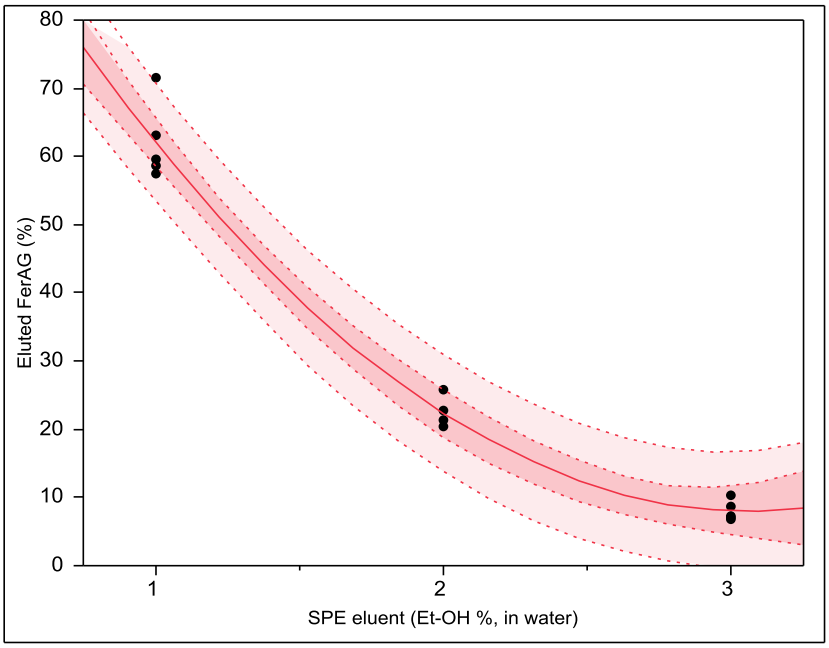

Figure 6.12. The FerAG elution profile as a function of function of SPE practical elution groups

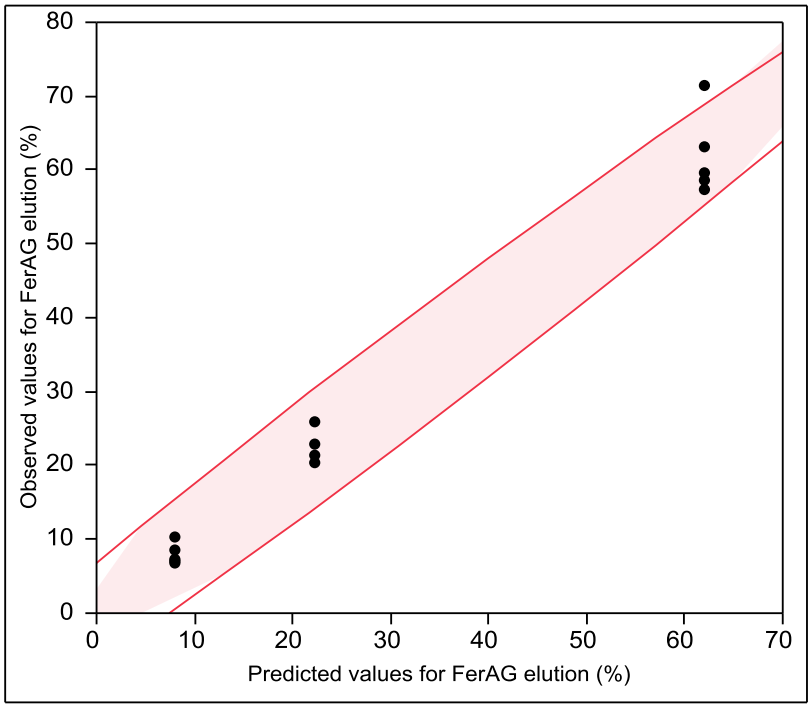

Figure 6.13. Scatter plot with 95 % confidence ellipse for observed versus predicted FerAG elution values (%) as a function of SPE practical elution groups

The novelty of this work consists in the generation of detailed elution patterns of SDG, PCouAG and FerAG through statistical modeling as a function of SPE eluent concentration in ethanol, and as a function of practical SPE elution groups. Although purification procedures for SDG by SPE have been reported in the literature, the details regarding the losses of compounds were generally not reported and the statistical modeling of the elution patterns were lacking. The following SDG recovery yields, relative to the amount of DFM used for purification experiments were computed based on published data: 0.95 and 0.97 % (Frank et al., 2004), 0.117 % (Johnsson et al., 2000), and 0.6 % (Strandås et al., 2008). The relative recovery yield achieved in this work was 1.42 % and corresponded to the 8.54 mg SDG purified from 0.6 g DFM. The total SDG recovery from the SPE funnel was 97.8 %, the remainder of 2.2 % was assumed to be lost during the transfer, concentration and preparation of eluates for HPLC analysis. However, judging from the greater losses recorded for PCouAG and FerAG of 3.9 and 7.5 %, respectively, these compounds appear to be susceptible to degradation or strong adherence to the walls of the evaporation flasks during the concentration under vacuum of eluates.

6.4.5. Solid Phase Extraction of Secoisolariciresinol Diglucoside, *P*-Coumaric Acid Glucoside and Ferulic Acid Glucoside from 1.5 g Defatted Flaxseed Meal Samples

Three SPE tests using MAE extracts obtained from 1.5 g DFM were carried out in order to verify if the recoveries of SDG, PCouAG and FerAG would follow the same patterns as those presented for the SPE tests from MAE extracts obtained from 0.6 g DFM. A 2nd degree polynomial predictive model (**Equation 6.7**) was fitted to the SDG data grouped by SPE practical elution groups. The model was highly significant ($p < 0.0001$) with $R^2 = 0.971$ and R^2adj. = 0.961. The parameter estimates used to build the predictive model from **Equation 6.7** were significant ($p \leq 0.0017$) as it can be observed from **Table 6.16**. The RSEs for the intercept, linear and quadratic terms were 18.49, 9.86, and 8.24 %, respectively.

$$Eluted\ SDG\ (\%) = -68.8018 + 146.556 \times SPE_{group} - 40.9369 \times SPE_{group}^2$$

Equation 6.7

Table 6.16. Parameter estimates for the 2nd degree polynomial predictive model for SDG elution from samples of 1.5 g DFM, as a function of three practical SPE elution groups

Variable	DF	Parameter Estimate	Standard Error	T Value	Prob > |t|
Intercept	1	-68.8018	12.7230	-5.41	0.0017
SPE-group	1	146.5560	14.4476	10.14	< .0001
SPE-group^2	1	-40.9369	3.3749	-11.45	< .0001

The recovery pattern of SDG from samples of 1.5 g DFM is presented in **Figure 6.14**. Increasing the sample size from 0.6 to 1.5 g DFM translated into increased losses of SDG into the water eluate (25 vs. 36.8 %) and decreased recovery of SDG in the SPE group 2 (71.2 vs. 60.6 %).

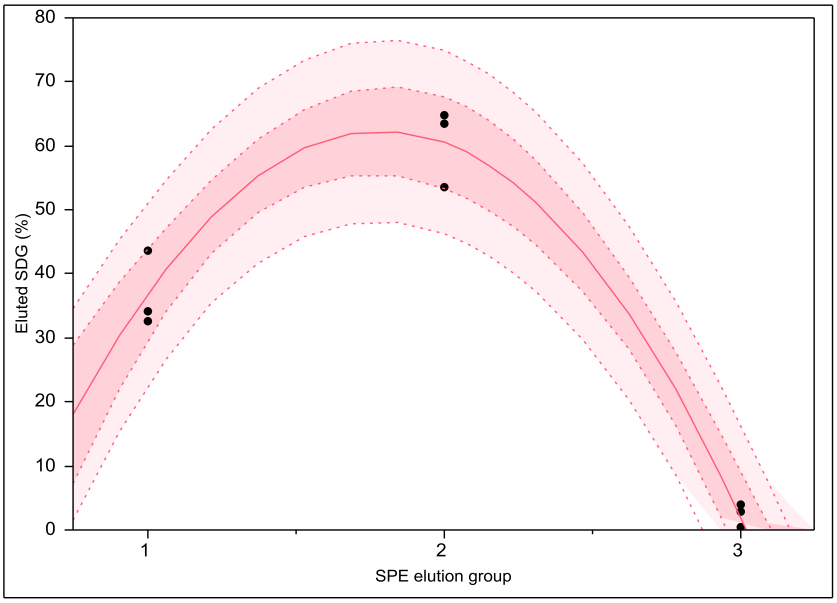

Figure 6.14. The SDG elution profile from samples of 1.5 g DFM as a function of three practical SPE elution groups

The mean observed values for SDG recovery (%) and their respective standard deviations for the SPE groups 1, 2 and 3 were: 36.8 ± 5.92, 60.6 ± 6.19, and 2.43 ± 1.83, respectively. The total recovery from the SPE funnel was 99.8 %. As it was the case with the previous modeling as a function of elution group, the predicted values for SDG recovery coincided with the mean observed values. There was a strong correlation between the observed and predicted SDG values; the Pearson correlation coefficient was r = 0.985 ($p < 0$ .0001).

Second degree polynomial predictive models were fitted for the observed values of eluted PCouAG ($p = 0.0117$; $R^2 = 0.892$) and FerAG ($p = 0.0017$; $R^2 = 0.959$). The predicted recovery values coincided with the observed mean values, with which they were well correlated for both PCouAG (r = 0.945; $p = 0.0014$) and FerAG (r = 0.979; $p = 0.0001$). The predicted values for PCouAG and FerAG in order of elution group were: 36.2, 45.2, 17.8 % and 39.2, 45.6 and 14.1 %, respectively. Increasing the sample size reduced the yields of both compounds eluted by the water SPE eluent, which caused the SPE group 2 to be richer in PCouAG and FerAG than the group 2 obtained with 0.6 g DFM. About 33 mg SDG were loaded onto the SPE funnel with each MAE extract obtained from 1.5 g DFM. The SPE elution group 2 recovered 60.6 % of the initial SDG, which corresponded to about 20 mg SDG, and 45.2 and 45.6% of the initial PCouAG and FerAG, respectively.

The total recoveries from the SPE funnel were 99.2 % for PCouAG and 98.8 % for the FerAG. These high recoveries refuted the hypothesis set earlier, according to which the lower recoveries for PCouAG, and especially for FerAG, with the SPE tests using 0.6 g DFM were due to the degradation of compounds during the concentration of eluates by vacuum evaporation. A more plausible hypothesis for explaining the lower recoveries would be that PCouAG and especially FerAG had a stronger ability than SDG to stick to the walls of the vacuum evaporation flasks. Such losses could be reduced by sonicating the vacuum evaporation flasks in order to dislodge the compounds that had the tendency to stick to the walls of the flask.

The SPE procedure for the purification of MAE from 1.5 g DFM is very attractive for producing about 20 mg SDG/test, free of hydrolysis salts for further experiments such as compound stability during storage, antioxidant activity and possibly for the formulation of functional foods enriched in SDG.

The possible applications of the results reported in this paper, besides carrying out further experiments at laboratory scale as mentioned above, include designing simplified work plans for pilot scale SPE studies with improved efficiency and economy. The preparation of flaxseed extracts prior to pilot scale SPE purification by adjusting the pH of extracts in two stages impacts both aspects. An initial pH adjustment to 3 is required for cleaning the flaxseed extracts by precipitating the water soluble carbohydrates and proteins which, if not removed, could clog the packed SPE bed and decrease its life. Then, the pH of the flaxseed extracts is adjusted to 5 before their loading onto the SPE funnel in order to reduce the losses of SDG in the wash-water eluate. The recovery patterns of SDG generated with the regression equations as a function of three practical elution groups, demonstrated that important losses of SDG occur in the wash water eluate (SPE group 1). Prior to scaling up the SPE of SDG, it would be interesting to compare different SPE columns designed to adsorb phenolic compounds through reversed phase mechanisms, and select those which minimize these losses. Another important factor to be considered for the optimization of pilot scale SPE of SDG is the concentration of flaxseed extracts in SDG, PCouAG and FerAG. As demonstrated in this chapter, using more concentrated extracts does increase the yield of purified SDG but also increases the loss of SDG in the SPE group 1. A simplified experimental design for the pilot scale studies would involve gathering the data according to three practical SPE elution groups: group 1 – contains the water eluate, group 2 – pools the eluates containing 10, 20 and 30 % Et-OH; and group 3 – pools the eluates containing 40, 50 and 100 % Et-OH. The benefits of applying such an experimental design are two-folded; (1) the eluates post treatment is much simplified, which results in significant reductions in the time and the cost of chromatographic analysis; and (2) the experimental errors due to

the use of non-automated SPE set-ups are controlled thus ensuring good repeatability of replicated experiments.

6.5. Conclusions

The elution patterns of SDG, PCouAG and FerAG were accurately predicted with 2nd degree polynomial regression models as a function of three practical SPE elution groups. The recovery patterns generated as a function of SPE eluent concentration in ethanol were relatively accurate and provided detailed information about the elution behaviour of the three compounds. There were no clear boundaries for the elution of the three compounds; small amounts "migrated" into the subsequent eluates as a consequence of errors induced by using non-automated SPE setups. These errors translated into high standard deviation values for the mean responses recorded as a function of eluent concentration in ethanol. However, this variation could be controlled in part by arranging and analysing the data as a function of practical elution groups. The predictive models gained in accuracy; the predicted values fell into narrow 95 % confidence intervals, and coincided with the mean observed values for the responses. The MAE extracts purified by SPE were free of leftover salts from hydrolysis, and provided SDG amounts (8.54 or 20 mg from 0.6 and 1.5 mg of defatted flaxseed meal, respectively) useful for further applications. The size of samples used for producing the MAE extracts for SPE affected the proportion of the three compounds recovered with the SPE elution group 2. This proved that the elution behaviour of the three compounds varied as a function of eluent composition and extracts concentration in target compounds. The results presented in this chapter are useful for designing simplified future pilot scale SPE studies for the purification of SDG from flaxseed extracts with improved efficiency and economy.

Connecting Statement to Chapter 7

In **Chapter 6**, an affordable, non-automated and vacuum driven solid phase extraction (SPE) method was developed for removing the chemicals used for extraction from microwave-assisted flaxseed extracts. The recovery yields of secoisolariciresinol (SDG), *p*-coumaric acid glucoside, and ferulic acid glucoside were accurately modeled. The regression models were used to predict the losses and the useful amounts of SDG obtained with the SPE method.

In **Chapter 7**, the SPE method is used in order to assess if the results of the enzymatic hydrolysis of the flaxseed extracts are significantly affected by the presence or absence of extraction chemicals. In other words, microwave-assisted flaxseed extracts were purified by SPE before being hydrolysed enzymatically, in order to test the hypothesis according to which the activity of the enzymes might be negatively affected by the presence of sodium hydroxide and sulphuric acid.

Chapter 7 is written in manuscript style and is soon to be submitted for publication as follows.

- Nemes, S.M., Orsat, V., Raghavan, G.S.V., 2012. Development of an Enzymatic Hydrolysis Method for the General Quantification of Lignan Aglycones in Foods Using Microwave-Assisted Flaxseed Extracts as Reference Model

Some of the results presented in **Chapter 7** have been disseminated through conference presentations.

- Nemes, S.M., Orsat, V., Raghavan, G.S.V., 2010. Microwave-Assisted Technology for Quantitative Analysis of Lignans in Foods, The XVIIth World Congress of the International Commission of Agricultural

Engineering CIGR, hosted by the Canadian Society for Bioengineering (CSBE/SCGAB), Québec City, Canada, June 13-17.

- Nemes, S.M., Orsat, V., Raghavan, G.S.V., 2010. Optimization of Enzymatic Hydrolysis of Plant Lignans for Analytical Purposes, The Northeast Agricultural and Biological Engineering Conference NABEC, Geneva, NY, USA, July 18-21.

Chapter 7 - Development of an Enzymatic Hydrolysis Method for the General Quantification of Lignan Aglycones in Foods Using Microwave-Assisted Flaxseed Extracts as Reference Model

7.1. Abstract

The coupling of an optimized microwave-assisted extraction method (MAE) with a subsequent enzymatic hydrolysis (EH) method is proposed for the general extraction of lignan aglycones from plant samples. Flaxseed extracts, obtained by MAE, with known contents of secoisolariciresinol diglucoside (SDG) were used as reference model. The optimized EH conditions were developed by means of screening designs and comparative studies using the factors: concentration of sodium acetate buffer pH 5 (0.01 and 0.1 M), concentrations of β-glucuronidase and cellulase enzymes (5 – 300 U/mg sample), with/without prior solid phase extraction (SPE) purification of extracts, and the time of incubation at 37°C (24 and 48 h). The SDG from the flaxseed extracts was hydrolysed in proportions of 95.6 % and 98.7 % after 48 h of incubation in 0.01 M sodium acetate buffer with 40 and 100 U (depending on the batch of enzyme) of crude β-glucuronidase/mg sample, and with 300 U of purified β-glucuronidase/mg sample, respectively. The degradation of secoisolariciresinol (SECO) to lignan artefacts could not be prevented as it was caused by both the buffer and the enzymes. The SECO recovery yields were 86 % when 40 and 100 U enzyme/mg sample were used, and 66.3 % when 300 U enzyme/mg sample were used. The effects of the studied factors on the yields of the EH of flaxseed extracts are described in this chapter.

Key words: method development, phytoestrogen, aglycone recovery, lignan aglycone, degradation, lignan artefact

7.2. Introduction

The increased interest in quantifying lignans in plant foods in the past two decades resulted in the publication of a variety of extraction methods that were applied for the general quantification of lignans in beverages, fruits, vegetable, grains, nuts and oil seeds (Kuhnle et al., 2009; Kuhnle et al., 2008; Milder et al., 2005; Milder et al., 2004; Penalvo et al., 2008; Penalvo et al., 2005; Smeds et al., 2007; Thompson et al., 2006). Accurate measurements of lignan contents in foods are useful for studying the effects of lignan-rich diets on various diseases. The flaxseed lignan, secoisolariciresinol diglucoside (SDG), has been shown to protect against cardiovascular diseases, the metabolic syndrome and breast cancer; these health benefits are due to the conversion of SDG to mammalian lignans in the human digestive tract (Adolphe et al., 2010; Peterson et al., 2010).

It is known that in plants, lignans occur conjugated to one or more sugars and are generally referred to as lignan glucosides (Milder et al., 2004) or to phenolic and/or fatty acids (Plattner and Powell, 1978; Powell and Plattner, 1976). Therefore, in order for an extraction method to be generally applicable for lignan quantification in a variety of plant samples, it has to initially cause the cleavage of ester bonds, and then break the glucosidic bonds in order to release the lignan aglycones.

Lignan glucosides can be extracted by: a) direct alkaline hydrolysis at room temperature [1 M NaOH, 1 h; (Eliasson et al., 2003)] or in a microwave environment [0.5 M NaOH, 3 min at 156 W with intermittent microwave power application (Nemes and Orsat, 2011a, b); method updated in section **3.5.5**]; b) extraction with aqueous methanol (70 % methanol, two extraction steps of 2 h at 60 - 70°C) followed by alkaline hydrolysis [1 M NaOH, 3 h at room temperature; (Thompson et al., 2007; Thompson et al., 2006)]; and c) methanolysis [0.3 M NaOH in 70 % methanol, 1 h at 60°C; (Milder et al., 2005; Penalvo et al., 2008; Penalvo et al., 2005; Smeds et al., 2007)]. In terms of

extraction efficiency of lignan glucosides, the optimised microwave-assisted extraction (MAE) method reported by Nemes and Orsat (2011a, b) resulted in 6, 21.4 and 26.6 % higher extraction yields than the methods reported by Eliasson et al. (2003), Smeds et al. (2007), and Thompson et al. (2006), respectively.

Lignan aglycones can be obtained by hydrolysing the lignan glucosides with: a) hot acid [1 M HCl, 1 h at 95°C; (Sicilia et al., 2003)], or b) enzymatic preparations of β-glucuronidase , β-glucosidase or cellulase, used alone or mixed (Kraushofer and Sontag, 2002; Milder et al., 2005; Penalvo et al., 2008; Penalvo et al., 2005; Smeds et al., 2007; Smeds et al., 2009; Thompson et al., 2007; Thompson et al., 2006). The preferred enzyme type appeared to be β-glucuronidase as it released higher amounts of lignan aglycones than β-glucosidase (Milder et al., 2004) and cellulase (Kosińska et al., 2011b). The enzymatic hydrolysis (EH) methods cannot be compared among themselves in terms of completion of hydrolysing the lignan glucosides to aglycones, as the hydrolysis efficiencies were often not reported, and the recoveries of lignan glucosides and/or aglycones were most likely affected by losses due to subsequent cleaning of extracts by liquid/liquid extraction or solid phase extraction (SPE). Moreover, the details pertaining to the development and optimization of the various enzymatic hydrolysis methods, and the exact hydrolysis conditions (such as units of enzyme/mg sample, volume of reaction, and the time of incubation) are not always reported. It has been shown however, that the EH was preferred to acid hydrolysis as it caused less degradation of lignan aglycones (Oomah and Hosseinian, 2008; Sicilia et al., 2003).

It is not clear how the EH conditions affect the released lignan aglycones; it is possible that they are degraded to lignan artefacts by the enzyme. Milder et al. (2004) reported 93 ± 5 % recovery for secoisolariciresinol (SECO) standard incubated with β-glucuronidase in sodium acetate buffer (0.05 M, pH 5) for 16 h at 37°C. Kraushofer and Sontag (2002) reported 75.5 ± 8 % recovery of SECO standard subjected to methanolysis (1 M NaOH in anhydrous methanol, 2.5 h of

sonication) and incubated with cellulase in sodium acetate buffer (0.1 M, pH 5) for 22 h at 37°C. According to Penalvo et al. (2005), Milder et al. (2005) and Thompson et al. (2006), the SECO released from flaxseed extracts after EH amounted to 97, 98 and 99 % of the total of lignan aglycones, respectively; the remaining percentage leading to 100 being made of matairesinol, lariciresinol and pinoresinol. For obtaining an accurate quantification of lignan aglycones in a variety of plant samples, the following lignans should be analysed in addition to the four mentioned above: medioresinol and syringaresinol [found in abundance in cereals; (Penalvo et al., 2005)], and isolariciresinol and demethoxy-secoisolariciresinol [lignan artefacts caused by EH conditions; (Sicilia et al., 2003)].

The precision of results for the various lignan aglycones obtained with EH have been shown to vary widely among different types of samples. Coefficients of variation (CV) of 6.6 - 32.5 % for duplicate analyses of different types of samples in the same day, and 6 - 20.8 % for duplicate analyses of various samples at different days have been reported by Milder et al. (2004); the lower CV values are for pinoresinol and lariciresinol, and the higher CV values are for SECO. Mean values per sample have been accepted if the CVs were < 15 % (Penalvo et al., 2008), and < 20 % (Milder et al., 2005).

The efficiency and accuracy of lignan quantification in foods depends on the coupling of an optimized method of initial hydrolysis in order to release lignan glucosides, with an optimized method of final EH in order to release lignan aglycones. The coupling of these types of hydrolysis methods is supported by the literature. The EH is preferred to acid hydrolysis to release lignan aglycones with a minimum of lignan artefacts formation (Kraushofer and Sontag, 2002; Sicilia et al., 2003). The EH must be preceded by an alkaline hydrolysis as it is not suitable to release lignan aglycones directly from complex lignan molecules (Kraushofer and Sontag, 2002; Milder et al., 2004), fact also supported by the higher lignan aglycone yields reported by Milder et al. (2005), Penalvo et al. (2005), and

Thompson et al. (2006) as opposed to the lower yields reported by Kuhnle et al. (2008) and Kuhnle et al. (2009).

The objectives of the current work are to find the optimum EH conditions to be used for converting lignan glucosides from microwave-assisted extracts to lignan aglycones in order to allow for the general quantification of lignans in a broad range of plant samples. Flaxseed was used as the model plant matrix as it is the richest food in SDG lignan, and also because EH converts SDG to SECO, which is then degraded in part to other lignan aglycones. Moreover, Milder et al. (2004) have used flaxseed, bread and broccoli as model products to optimise an EH method, and have found that the EH conditions that maximised the yields of lignan aglycones from flaxseed applied as well to bread and broccoli. Based on the literature (Kraushofer and Sontag, 2002; Milder et al., 2005; Milder et al., 2004; Penalvo et al., 2005; Thompson et al., 2006), it is hypothesised that SECO will account for 75.5 to 99 % of the total lignan aglycones in microwave-assisted flaxseed extracts subjected to EH. Further, the optimization of EH in terms of SECO maximization will be challenging, although it will simplify the task at the same time, as SECO yields have been shown to vary widely for duplicate analyses of various samples [CVs of 20.8 – 32.5 % (Milder et al., 2004)].

In order to achieve the set objectives, microwave-assisted flaxseed extracts were subjected to EH with β-glucuronidase with/without prior purification by SPE under various combinations of the following factors: units of enzyme/mg sample [5 to 300 U/mg defatted flaxseed meal (DFM)], sodium acetate buffer concentrations (0.01 and 0.1 M, pH 5), and time of incubation (24 and 48 h). As starting point (initial design), the factors' levels used were 5 U enzyme/mg DFM and 0.1 M buffer concentration as per the work of Thompson et al. (2006); and 40 U enzyme/mg DFM and the 0.01 M buffer concentration as per the work of Smeds et al. (2007); both publications being identified as trend-setting in the field of lignan quantification (Schwartz et al., 2009). The efficiencies of the enzymes β-glucuronidase, cellulase and a mixture of the two to convert the SDG from the

extracts to SECO were compared. The effects of EH conditions on the recovery of SECO standard were also studied.

7.3. Materials and Methods

7.3.1. Chemicals

The lignan standards used were of high performance liquid chromatography (HPLC) grade; the secoisolariciresinol diglucoside (SDG, molecular weight 686.7, purity 97.6 %) and the anhydro-secoisolariciresinol (ANSECO, molecular weight 344.4, purity > 99 %) were purchased from Chromadex (Santa Ana, CA, U.S.A.); the secoisolariciresinol (SECO, molecular weight 362.4, purity ≥ 95 %) was purchased from Sigma-Aldrich (Oakville, ON, Canada). The solvents: acetonitrile, methanol and hexane of HPLC grade were purchased from Fisher Scientific (Ottawa, ON, Canada). Anhydrous ethanol was purchased from Commercial Alcohols (Brampton, ON, Canada). The reagents: sodium hydroxide ≥ 98 %, sulphuric acid 95 - 98 % ACS, phosphoric acid ≥ 85 %, glacial acetic acid > 99 %, sodium acetate anhydrous, and di-potassium hydrogen phosphate 98 % were purchased from Sigma-Aldrich (Oakville, ON, Canada). Enzyme preparations of β-glucuronidase from *Helix pomatia* type HP-2 (2 ml crude aqueous solution containing 104130 U β-glucuronidase and 835 U sulfatase/ml), and type H-1 (partially purified powder containing 100000 U β-glucuronidase and > 3300 U sulfatase) were purchased from Sigma-Aldrich (Oakville, ON, Canada); Cellulysin cellulase from *Trichoderma viride* (powder containing 100000 U cellulase) was purchased from EMD (Mississauga, ON, Canada).

7.3.2. Flaxseed

Defatted flaxseed meal (DFM) was obtained by grinding and defatting brown flaxseed (*Linum usitatissimum* L.) purchased from a local grocery store (Montreal, QC, Canada). One hundred grams of flaxseed meal were defatted twice with 600 ml hexane under magnetic stirring for 1 h at room temperature.

The solvent was removed by filtration under vacuum; then, the flaxseed meal was left overnight under the fume hood in order to allow the residual hexane to evaporate (Nemes and Orsat, 2010). The flax meal was ground again with a coffee grinder and the obtained DFM was packed in an air tight plastic bag and kept at -18°C until further use. The SDG content of the DFM was 22.9 ± 0.26 mg/g as determined by MAE.

7.3.3. Microwave–Assisted Extraction

The extraction of SDG from DFM was carried out in a 250 ml borosilicate glass vessel, topped with a Graham-type reflux condenser, which was inserted in a mono-mode microwave apparatus (Star System 2, CEM, Mathews, USA; nominal power declared by the manufacturer 800 W, microwave frequency 2.45 GHz) which delivers a maximum power of 710.5 W (section **4.4.1**), as calibrated with the calorimetric method developed specifically for the Star System 2 instrument (**Chapter 4**, section **4.3.1**). Samples of 0.5 and 0.6 g DFM were extracted according to our previously published methodology (Nemes and Orsat, 2011b) which was updated in section **3.5.5**. Briefly, the samples were hydrolysed with 50 ml of 0.5 M NaOH at 22 % power level [156 W as calculated with the calibration in **Equation 4.2** = 7.105 × Power level (%), presented in section **4.4.1**]; the microwave power was applied intermittently (30 s on/off) for 3 min. A type K thermocouple (accuracy ± 0.5°C, Fisher Scientific) was used to measure the final temperature of the extracts after briefly stirring them. The temperature of the extracts rose from room temperature (22 – 23°C) to about 67°C over the 3 min span.

7.3.4. High Performance Liquid Chromatography Analysis

The extracts were analysed by high performance liquid chromatography (HPLC) in triplicates with an Agilent 1100 series instrument controlled by the Chemstation software [Rev. B.01.03 (204), Agilent Technologies]. The

chromatograms were recorded at 280 nm using a variable wavelength detector. The SDG, SECO, and ANSECO were eluted at 25°C on a reversed-phase C18 column (particle size 5 µm, length 25 cm, internal diameter 4.6 mm; Discovery, Sigma-Aldrich) fitted with a guard cartridge (particle size 5 µm, length 2 cm, internal diameter 4 mm; Supelguard, Sigma-Aldrich). The analysis method was slightly modified from Johnsson et al. (2000), and used a linear gradient of the solvents A - 0.01 M phosphate buffer with a pH of 2.8 containing 5 % acetonitrile, and - B acetonitrile. The solvent A decreased from 100 to 50 %, and the solvent B increased from 0 to 50 % at a flow rate of 1 ml/min as previously reported by Nemes and Orsat (2011a). In this system SDG, SECO and ANSECO eluted at 19.5, 26.5 and 38.5 min, respectively. Standard curves were built using six levels of lignan concentrations ranging from 5 to 200 µg lignan/ml methanol; in all cases the coefficients of determination (R^2) were > 0.999. The following equations were used for quantification: SDG = peak height/0.4494, SECO = peak height/1.054, and ANSECO = peak height/0.8065. The *p*-coumaric acid glucoside (PCouAG) and ferulic acid glucoside (FerAG) were identified as previously reported by Nemes and Orsat (2011c) based on resemblance with previously published chromatograms (Eliasson et al., 2003; Johnsson et al., 2000; Nemes and Orsat, 2010); and were quantified in terms of milli absorbance units (mAU) based on their peak heights; their respective elution times were 11 and 12.5 min. Before injection into the HPLC, the water soluble carbohydrates and proteins contained in the extracts were removed by precipitation under magnetic stirring for 15 min after pH adjustment to 3 with 5.55 ml of 5 N H_2SO_4 and addition of 2 ml methanol/ml of extract, followed by centrifugation at 3000 rpm for 10 min (Spinette centrifuge, International Equipment Company; Needham Heights, MA, USA). Then, the extracts were filtered through 0.22 µm nylon syringe filters (13 mm diameter, Whatman Puradisc) and analysed by HPLC.

7.3.5. Solid Phase Extraction Purification of Flaxseed Extracts

Extracts obtained by MAE from 0.6 g DFM were cleaned of the salts resulting from hydrolysis by SPE purification using a method previously developed by Nemes and Orsat (2011c). Briefly, the pH of the extracts was adjusted in two steps, initially to 3 as described above (section **7.3.4**); then, the methanol was removed from the extracts by evaporation under vacuum (65°C, 195 rpm; Buchi Rotavapor 205 equipped with a B490 heating bath) and the pH was brought to 5 by addition of 0.2 ml of 2 M NaOH. Then, the volume of the extracts was adjusted to 50 ml with double de-ionized water (Simplicity 185, Millipore), and they were transferred into 100 ml syringes fitted with polyvinylidene difluoride (PVDF) 0.45 µm syringe membrane filters (Millex R-HV, Fisher Scientific). The extracts were loaded onto conditioned and equilibrated SPE funnels (packed bed 50 g of DPA-6S polyamide resin, particle size 50 - 180 µm, pH 7.4; internal diameter 90 mm, height 48 mm; VersaPure pre-packed Buchner funnels, Sigma-Aldrich), fitted onto 500 ml vacuum filtration flasks, directly with the syringe at a rate of 1 - 2 drops/s. The conditioning and equilibration of the SPE funnel were done with 300 ml of anhydrous ethanol and 600 ml double de-ionized water, respectively. After loading the extract, the funnel was washed with 300 ml of double de-ionized water to remove the salts resulting from hydrolysis and other unwanted low molecular weight compounds. The SDG was eluted with 300 ml of 10, 20, 30, 40, 50 % ethanol in double de-ionized water, and then the SPE funnel was cleaned with 100 % ethanol. The eluates were concentrated to about 50 ml by evaporation under vacuum at 65°C and 195 rpm. An aliquot of 2 ml from each eluate was subjected to HPLC analysis. After each purification experiment, the SPE funnel was regenerated with 300 ml ethyl acetate.

7.3.6. Enzymatic Hydrolysis of Flaxseed Extracts and Secoisolariciresinol Standard

The EH experiments were carried out using microwave-assisted extracts obtained from 0.5 and 0.6 g DFM (without and with prior SPE purification, respectively), and SECO standard. The volume of the EH extracts was 3 ml for the experiments using microwave-assisted extracts, and 2 ml for the experiments using SECO standard. The EH extracts were incubated at 37°C in a water bath. The extracts from 0.5 g DFM were prepared for EH as follows. The water soluble carbohydrates and proteins were precipitated under magnetic stirring (15 min, 300 rpm) after adjusting the pH to 5 with 2 ml glacial acetic acid and adding 100 ml methanol; then, the liquid and solid phases were separated by centrifugation (10 min, 3000 rpm). The extracts were concentrated to about 1 ml by evaporation under vacuum (65°C, 195 rpm), and then reconstituted in sodium acetate buffer (pH 5, 0.01 or 0.1 M, according to the experimental designs presented in section **7.3.7**) in order to obtain a sample to buffer ratio of 25 mg DFM/ml. Two ml of buffered microwave-assisted extract containing about 1 mg SDG was used per EH experiment. The microwave-assisted extracts from 0.6 g DFM were used to obtain 10, 20 and 30 % ethanol SPE eluates, which when pooled contained about 8.54 mg SDG [corresponds to 71.2 % of the SDG contained in the extract subjected to purification (Nemes and Orsat, 2011c)]. The pooled eluates were concentrated to about 1 ml, then diluted to 8.5 ml with sodium acetate buffer (pH 5, 0.01 or 01 M, according to the experimental design presented in section **7.3.7**). One ml of purified microwave-assisted extract containing about 1 mg SDG was used per EH experiment. Due to the high cost of pure lignan standard, only 20 µg of SECO were used per EH experiment. Before injection into HPLC the EH extracts were diluted to 4 - 5 ml with methanol and filtered through 0.22 µm nylon membrane filters. The EH tests were carried out with: (1) crude solutions of β-glucuronidase for the screening studies, using different batches (purchases) of enzymes per experimental design; (2) one batch of partially purified β-glucuronidase was used for the two comparative studies using SECO standard, and for comparing the

efficiency of purified β-glucuronidase, cellulase and a mix of the two enzymes for hydrolysing SDG from microwave-assisted extracts to SECO.

7.3.7. Experimental Design

All experimental designs were analysed with JMP 8.0.2 software (SAS Institute Inc., Cary, NC, USA); the tests were considered significant at $p < 0.05$.

7.3.7.1. 1st Screening Design

Full factorial screening designs are generally used for assessing the magnitude and the directions of factors' effects on one or more response variables; they are economical, as the replication of experimental runs is not necessary in order to perform the analysis of variance (ANOVA) (Bezerra et al., 2008; Lundstedt et al., 1998). A two-level, full factorial, un-replicated, screening design was used to study the effects of sodium acetate buffer concentration (pH 5, 0.01 and 0.1 M), enzyme concentration (5 and 40 U β-glucuronidase/mg DFM), and time of incubation (24 and 48 h) on the proportions of SDG (from microwave-assisted extracts) converted into SECO or ANSECO, the total recovered lignan, and the proportions of PCouAG and FerAG not converted to aglycones. The levels of the factors enzyme concentration and buffer concentration were as per the works of Smeds et al. (2007) and Thompson et al. (2006) which were identified as trend-setting in lignan analysis in foods by Schwartz et al. (2009). The 1st screening design used crude aqueous solution of β-glucuronidase (purchase 1).

7.3.7.2. 2nd Screening Design

A two-level, full factorial, un-replicated, screening design was used to study the effects of SPE purification (0 – no purification, 1 – SPE purification), sodium acetate buffer concentration (pH 5, 0.01 and 0.1 M), enzyme concentration (50 and 100 U β-glucuronidase/mg DFM), and time of incubation (24 and 48 h)

on the proportions of SDG (from microwave-assisted extracts) converted into SECO or ANSECO, the total recovered lignan, and the proportions of PCouAG and FerAG not converted to aglycones. The 2nd screening design used crude aqueous solution of β-glucuronidase (purchase 2).

7.3.7.3. Comparative Studies

The comparative studies used full factorial replicated designs. One study was conducted in order to compare the capacities of partially purified β-glucuronidase (300 U/mg DFM), cellulase (300 U/mg DFM), and a mixture of the two enzymes (300 + 300 U/mg DFM) to convert the SDG from microwave-assisted extracts when using 0.01 M, pH 5 sodium acetate buffer and incubations times of 24 and 48 h. In another study, 20 µg SECO standard were incubated for 24 h in sodium acetate buffer (pH 5; 0.01 and 0.1 M) with or without 1500 U of partially purified β-glucuronidase/test in order to assess the effect of these conditions on the recovery (stability) of SECO. This study assumed that the proportion of SECO that could not be identified as such was denatured to other lignan compounds as supported by the recoveries of SECO standard of 75.5 ± 8 % and 93 ± 5 % after incubation with enzyme reported by Kraushofer and Sontag (2002), and Milder et al. (2004), respectively.

7.4. Results and Discussion

7.4.1. 1st Screening Design

The 1st screening design consisted of 8 experimental combinations of the factors: buffer concentration (0.01 and 0.1 M), enzyme concentration (5 and 40 U/mg DFM), and the time of incubation (24 and 48 h). For each experimental combination, 2 ml of microwave-assisted extracts containing 50 mg DFM (1 mg SDG) were incubated with crude solution of β-glucuronidase at 37°C. The effects of these hydrolysis conditions were studied by analyzing the proportions of conversion of SDG to SECO, and the proportions of PCouAG and FerAG left un-

hydrolysed. The amounts of lignans quantified by HPLC were converted to micro-moles (µM) in order to calculate the proportions of hydrolysed or un-hydrolysed SDG in percentages. The results are graphically presented in the prediction profiler in **Figure 7.1**.

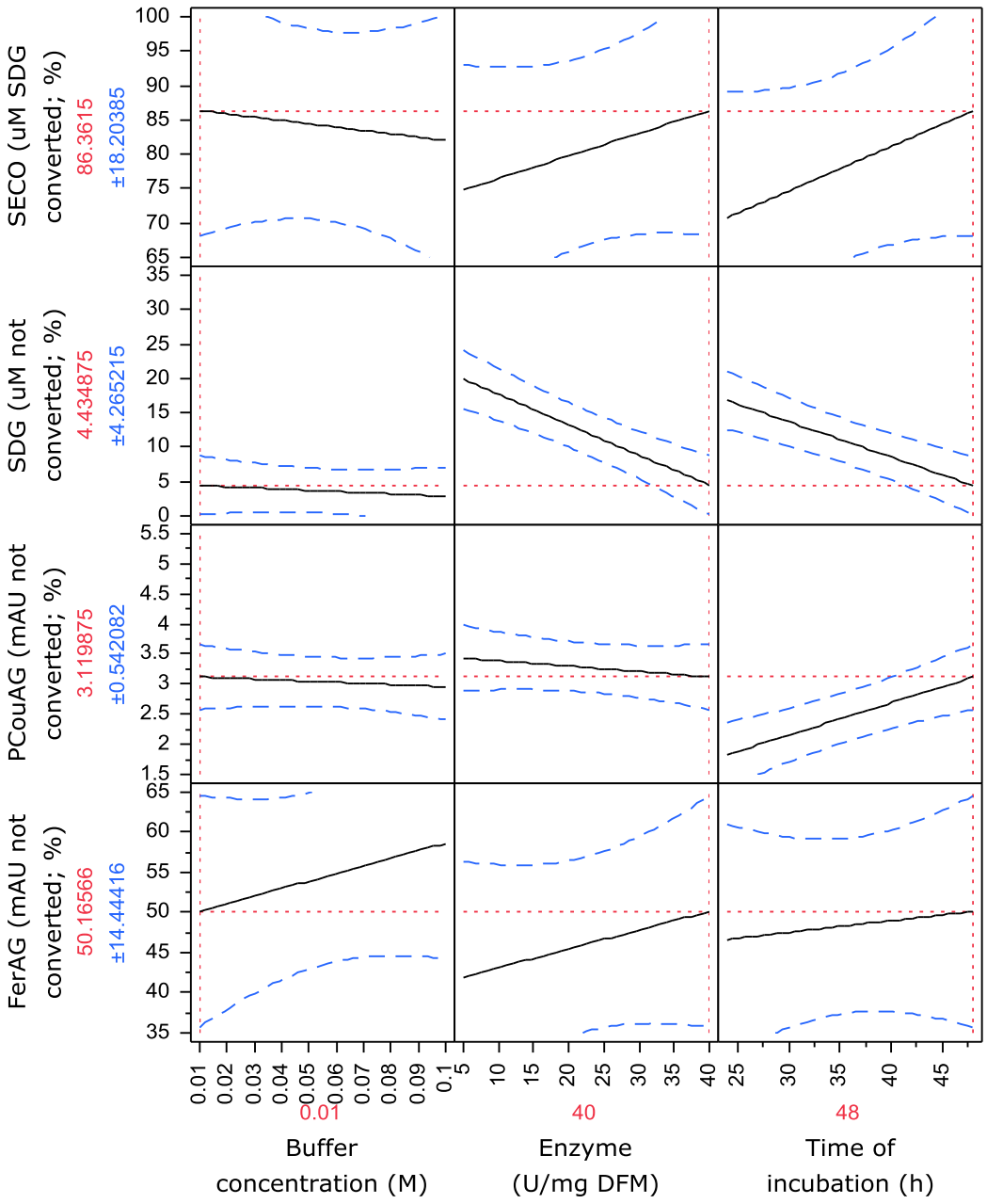

Figure 7.1. The prediction profiler for the 1st screening design

The highest proportion of SDG converted to SECO (86.4 %) was achieved with 40 U of enzyme/mg DFM incubated with 0.01 M buffer for 48 h. The hydrolysis was not complete, as 4.43 % SDG was left intact. Under these hydrolysis conditions the proportions of PCouAG and FerAG left un-hydrolysed were 3.12 and 50.2 %, respectively, indicating that a surplus of enzyme could be useful in case this preferentially hydrolyses substrates other than SDG.

7.4.1.1. Formation of an Intermediary Compound from Secoisolariciresinol Diglucoside, and of Lignan Aglycone Artefacts from Secoisolariciresinol

The lignan artefact ANSECO was found in two extracts in proportions of 0.06 and 0.08 % of the amounts of lignan used for hydrolysis and could not be explained by the experimental conditions. ANSECO is formed through the loss of one water molecule from SECO; and it was usually found when using hydrolysis with hot concentrated (> 1 M) hydrochloric acid (Kraushofer and Sontag, 2002; Liggins et al., 2000; Yuan et al., 2008) but not when using EH (Sicilia et al., 2003). The fact that only trace amounts of ANSECO were found with this work confirms the fact that EH is milder and produces less lignan artefacts than acidic hydrolysis as previously stated in the literature (Oomah and Hosseinian, 2008; Schwartz et al., 2009; Sicilia et al., 2003).

It is worth noting that 9.17 % of the lignan subjected to EH was uncounted for by the sum of quantified SDG and SECO. It was assumed that the 9.17 % represented the sum of intermediary hydrolysis product (secoisolariciresinol monoglucoside, SMG) and lignan aglycones artefacts (possibly matairesinol, pinoresinol, lariciresinol, isolariciresinol, and demethoxy-secoisolariciresinol), that were not identifiable with the HPLC method used in this experiment. The presence of the partially hydrolysed compound, SMG, was reported by Yuan et al. (2008) upon alkaline hydrolysis followed by acidic hydrolysis of the flaxseed lignan macromolecule. Thus, it is hypothesised that SMG was also formed during

the EH of SDG. The assumption that lignan aglycone artefacts were also formed during the EH of microwave-assisted flaxseed extracts was based on results from the literature. For example, it is known that SDG is the only naturally occurring lignan in flaxseed (Attoumbré et al., 2011; Eliasson et al., 2003; Ford et al., 2001; Johnsson et al., 2002; Kamal-Eldin et al., 2001; Kosińska et al., 2011b; Struijs et al., 2009), but the EH of flaxseed extracts has not resulted only in SECO (the natural aglycone obtained from the deglucosilation of SDG) as a final product. In fact, from the total amount of SDG subjected to EH, 97 to 99 % have been hydrolysed to SECO, and 1 to 3 % have been hydrolysed to other lignan aglycones such as: lariciresinol, pinoresinol and matairesinol (Milder et al., 2004; Penalvo et al., 2005; Thompson et al., 2006). Thus, it appears that these three lignan aglycones, often reported in the literature as being naturally occurring flaxseed lignans, were artefacts formed through the degradation of SECO. In addition to these lignan aglycone artefacts, Sicilia et al. (2003) also reported that trace amounts of demethoxy-secoisolariciresinol were formed from the degradation of SECO, and that lariciresinol was further degraded to isolariciresinol. This complicates the task of accurately quantifying lignan aglycones in foods due to the need of using five lignan standards to account for the compounds originating from SDG. However, the EH of flaxseed extracts can be optimized while ignoring the five lignan aglycone artefacts of SECO by focusing on maximizing the yield of SECO.

7.4.1.2. Predictive Models for the 1st Screening Design

The goal of this experiment is to completely hydrolyse the SDG, and in the same time to maximise its conversion to SECO - the natural aglycone form of SDG. Although, as discussed above (section **7.4.1.1**) SECO is further transformed to lignan aglycone artefacts, they were not quantified in this work due to increased costs of HPLC analysis when using a total of eight purified lignan standards (SDG, SECO, ANSECO, matairesinol, pinoresinol, lariciresinol, isolariciresinol, and demethoxy-secoisolariciresinol). Thus, the predictive models for the

minimization of SDG (**Equation 7.1**; $R^2 = 0.993$, $R^2_{adj.} = 0.987$, $p < 0.0001$) and for the maximization of SECO (**Equation 7.2**; $R^2 = 0.984$, $R^2_{adj.} = 0.973$, $p = 0.0005$) were both necessary for describing the complex results of the EH of SDG from flaxseed extracts. The estimated regression coefficients used to build the models from **Equation 7.1** and **Equation 7.2** were computed using the coded levels (-1, 1) for the experimental factors as shown in **Table 7.1** and **Table 7.2**, respectively.

$$SDG\,\% = 20.4839 - 9.7101 \times Enzyme - 9.7034 \times Time + 2.5691 \times Enzyme \times Time$$

Equation 7.1

$$SECO\,\% = 70.2705 + 6.9718 \times Enzyme + 8.5910 \times Time - 1.6478 \times Enzyme \times Time$$

Equation 7.2

Table 7.1. Parameter estimates for the predictive model for the minimization of SDG (1st Screening design)

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	20.4839	0.5974	34.29	<0.0001
Enzyme [(-1) 5, (1) 40 ; U/mg DFM]	-9.7101	0.5974	-16.25	<0.0001
Time of incubation [(-1) 24, (1) 48; h]	-9.7034	0.5974	-16.24	<0.0001
Enzyme (U/mg DFM) × Time of incubation (h)	2.5691	0.5974	4.30	0.0126

Table 7.2. Parameter estimates for the predictive model for the maximization of SECO (1st Screening design)

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	70.2705	0.7056	99.59	<0.0001
Enzyme [(-1) 5, (1) 40 ; U/mg DFM]	6.9718	0.7056	9.88	0.0006
Time of incubation [(-1) 24, (1) 48; h]	8.5910	0.7056	12.18	0.0003
Enzyme (U/mg DFM) × Time of incubation (h)	-1.6478	0.7056	-2.34	0.0798

7.4.1.3. Effects of the Studied Factors on the Enzymatic Hydrolysis Yields for the 1st Screening Design

The buffer concentration and its interactions with the enzyme concentration and time of incubation did not count significantly towards the maximization of SECO through the depletion of SDG. Thus, the models (**Equation 7.1** and **Equation 7.2**) took into account the averaged effects of buffer concentration on the hydrolysis of SDG. However, for the purpose of practical applications of the hydrolysis conditions, and in order to replicate the results shown in **Figure 7.1**, one should use 0.01 M buffer as it gave higher yields than 0.1 M buffer.

The regression coefficient of the Enzyme × Time interaction term was significant in the case of **Equation 7.1** but it was not significant in the case of **Equation 7.2**. This interaction did not have a straight forward interpretation as it included the proportions of SDG partially hydrolysed to SMG and/or the proportions of SECO degraded to aglycone artefacts, the total amount of which could be calculated but the proportions of the individual contributions were not known. The differences between 48 and 24 h of hydrolysis for 5 vs. 40 U of enzyme were 22.7 vs. 12.4 % hydrolysed SDG and 22.1 vs. 15.6 % released SECO, respectively. During the 2nd day of hydrolysis with 40 U of enzyme, 12.4 % SDG was hydrolysed (in addition to the 83.2 % SDG hydrolysed during the 1st day) but the % of additional SECO formed was 15.6, which indicated that the difference of 3.2 % SECO was formed from SMG. This confirms the hypothesis emitted in section **7.4.1.1** that SMG might be formed during the EH of SDG.

7.4.1.4. Conclusions for the 1st Screening Design, and Directions for the 2nd Screening Design

Overall, it appeared that the hydrolysis of SDG from flaxseed extracts proceeded at a high rate during the 1st day and slowed down significantly during the 2nd day of incubation, with more accentuated hydrolysis rates being observed when using

the higher concentration of enzyme (40 vs. 5 U/mg DFM). It is to be concluded that the SDG hydrolysis rate could be increased during the 1st day, by using higher concentrations of enzyme and, at the same time increasing the proportion of SECO formation and reducing the proportion of SDG hydrolysed partially to SMG. Further, using SPE purification prior to EH could be useful for removing non-lignan glucosidic compounds (e.g., PCouAG and FerAG; **Figure 7.1**) that contributed to the reduction of the amount of enzyme available for the hydrolysis of lignan glucosides. In order to study the potential beneficial effects of prior SPE purification on the conversion of SDG to SECO, a new screening design (2nd) is to be carried out using SPE purification and concentrations of enzymes greater than those used in the 1st screening design.

7.4.2. 2nd Screening Design

The 2nd screening design included 16 experimental combinations of the factors: SPE purification (0 – no purification, 1 – SPE purification), buffer concentration (0.01 and 0.1 M), enzyme concentration (50 and 100 U/mg DFM), and the time of incubation (24 and 48 h). Each experiment used 2 ml of microwave-assisted extracts containing 50 mg DFM (1 mg SDG) which were incubated with crude solution of β-glucuronidase (purchase 2) at 37°C. The proportions of conversion of SDG to SECO, and the proportions of PCouAG and FerAG left un-hydrolysed were analysed. The results obtained with the extracts subjected to EH without prior SPE purification (**Figure 7.2**) were similar with those obtained with the 1st screening design (section **7.4.1**; **Figure 7.1**); for example, SECO = 85.9 vs. 86.4 %, SDG = 4.11 vs. 4.43 %, PCouAG = 3.17 vs. 3.12 %, and FerAG = 50.9 vs. 50.2 %, for the 2nd vs. the 1st screening design, respectively. Although it did not affect the EH yields significantly, the preferred buffer concentration was 0.01 M, as it was the case with the 1st screening design.

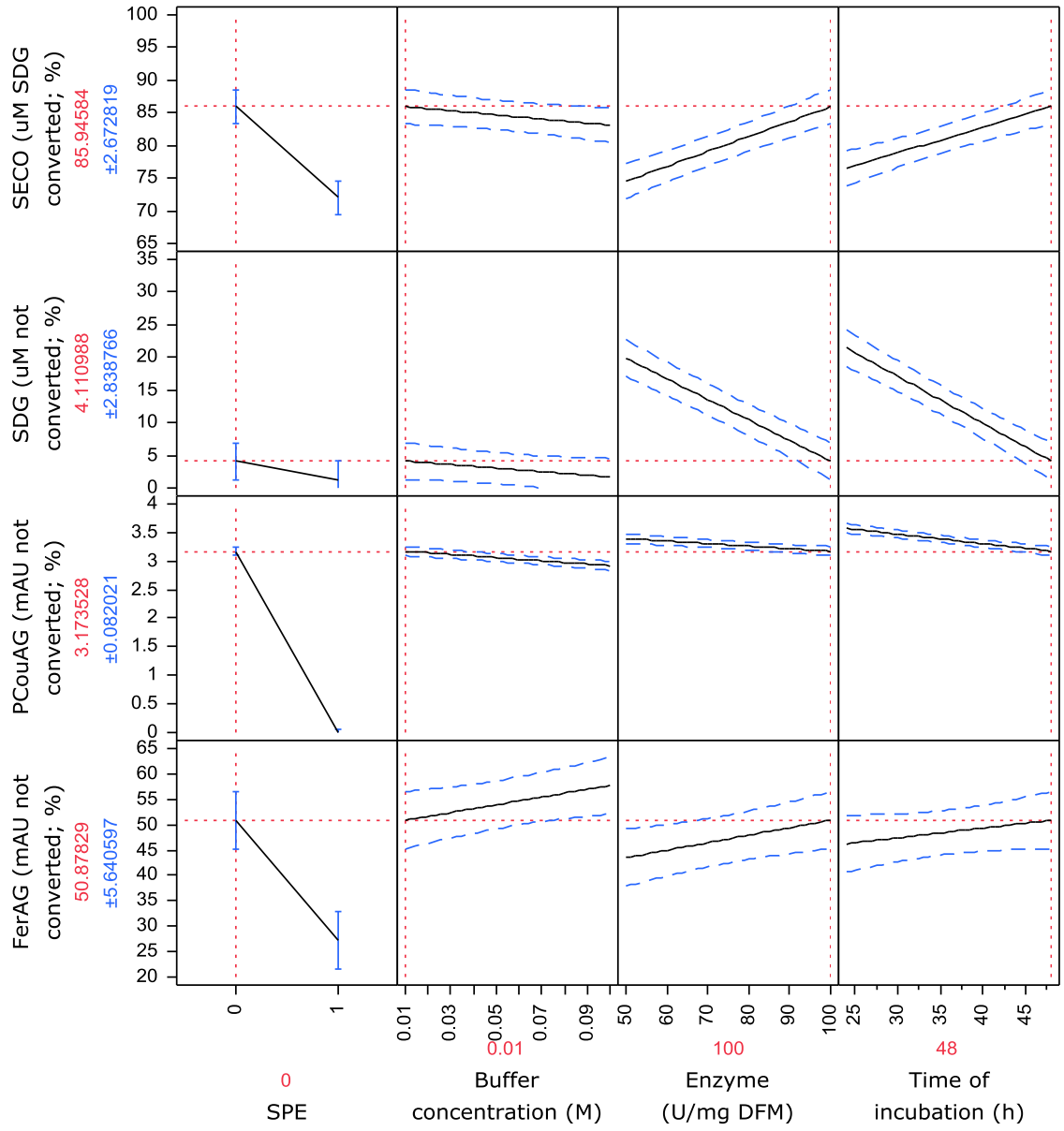

Figure 7.2. The predictions profiler for the 2nd screening design

7.4.2.1. Predictive Models for the 2nd Screening Design

The predictive models for the maximization of SECO (**Equation 7.3**, R^2 = 0.992, $R^2_{adj.}$ = 0.979, p < 0.0001) and the minimization of SDG (**Equation 7.4**; R^2 = 0.996, $R^2_{adj.}$= 0.990, p < 0.0001) were constructed using the estimated regression coefficients presented in **Table 7.3** and **Table 7.4**, respectively. The

Equation 7.3 and **Equation 7.4** were linked by identical hydrolysis conditions (maximization of SECO through the depletion of SDG); thus, the factors with non-significant regression coefficients were kept in **Equation 7.4** because they were significant in **Equation 7.3**. The models used coded levels of 0 and 1 for the SPE factor and -1 and 1 for the other factors.

$$SECO\ \% = 71.7491 + 7.5168 \times SPE - 2.4208 \times Buffer + 7.0579 \times Enzyme + 7.0776 \times Time + 8.1411 \times SPE \times Buffer - 6.6296 \times SPE \times Enzyme - 6.7002 \times SPE \times Time + 1.0038 \times Buffer \times Time - 1.3557 \times Enzyme \times Time$$

Equation 7.3

Table 7.3. Parameter estimates for the predictive model for the maximization of SECO (2nd Screening design)

Term	Estimate	Std Error	t Ratio	Prob > |t|
Intercept	71.7491	0.4885	146.88	<0.0001
SPE[0, 1]	7.5168	0.6908	10.88	<0.0001
Buffer concentration [(-1) 0.01, (1) 0.1; M)	-2.4208	0.4885	-4.96	0.0026
Enzyme [(-1) 50, (1) 100 ; U/mg DFM]	7.0579	0.4885	14.45	<0.0001
Time of incubation [(-1) 24, (1) 48; h]	7.0776	0.4885	14.49	<0.0001
SPE[0, 1]*Buffer concentration (M)	8.1411	0.6908	11.78	<0.0001
SPE[0, 1]*Enzyme (U/mg DFM)	-6.6296	0.6908	-9.60	<0.0001
SPE[0, 1]*Time of incubation (h)	-6.7002	0.6908	-9.70	<0.0001
Buffer concentration (M)*Time of incubation (h)	1.0038	0.3454	2.91	0.0271
Enzyme (U/mg DFM)*Time of incubation (h)	-1.3557	0.3454	-3.92	0.0078

$$SDG\ \% = 20.9973 - 19.87 \times SPE - 0.9426 \times Buffer - 9.1697 \times Enzyme - 10.2215 \times Time + 1.2532 \times SPE \times Buffer + 8.5952 \times SPE \times Enzyme + 9.6365 \times SPE \times Time - 0.2702 \times Buffer \times Time + 1.2920 \times Enzyme \times Time$$

Equation 7.4

Table 7.4. Parameter estimates for the predictive model for the minimization of SDG (2nd Screening design)

Term	Estimate	Std Error	t Ratio	Prob >|t|
Intercept	20.9973	0.5188	40.47	<0.0001
SPE[0, 1]	-19.87	0.7337	-27.08	<0.0001
Buffer concentration [(-1) 0.01, (1) 0.1; M)	-0.9426	0.5188	-1.82	0.1192
Enzyme [(-1) 50, (1) 100 ; U/mg DFM]	-9.1697	0.5188	-17.67	<0.0001
Time of incubation [(-1) 24, (1) 48; h]	-10.2215	0.5188	-19.70	<0.0001
SPE[0, 1]*Buffer concentration (M)	1.2532	0.7337	1.71	0.1385
SPE[0, 1]*Enzyme (U/mg DFM)	8.5952	0.7337	11.71	<0.0001
SPE[0, 1]*Time of incubation (h)	9.6365	0.7337	13.13	<0.0001
Buffer concentration (M)*Time of incubation (h)	-0.2702	0.3669	-0.74	0.4891
Enzyme (U/mg DFM)*Time of incubation (h)	1.2920	0.3669	3.52	0.0125

7.4.2.2. Similarities Between the 1st Screening Design and the Half Fraction of the 2nd Screening Designs not Using Prior Solid Phase Extraction Purification

If analysing separately the half of the 2nd screening design including only the 8 experimental combinations not using prior SPE purification, a predictive model can be obtained for the maximization of SECO that is similar to **Equation 7.2**. Therefore, similar results were achieved with 40 and 100 U of crude solution of β-glucuronidase from different batches, revealing that the hydrolysing capacities of enzymatic preparations can differ significantly between batches. When carrying out EH experiments, it is thus recommended to test each batch of enzyme by hydrolysing flaxseed extracts with known SDG contents using 50, 100 or more U of enzyme/mg sample if necessary.

From the point of view of reproducibility of results, at this stage, it can be concluded that recoveries of lignan aglycones of at least 86 % (or higher if more lignan aglycone standards are used for HPLC quantification) can be obtained by applying the experimental conditions identified with the 1st and the 2nd screening

designs (considering the ½ fraction of the 2nd design not using prior SPE). Briefly, aliquots of alkali hydrolysed extracts obtained from 50 mg sample are to be subjected to EH, without using prior SPE purification of extracts, in a total volume of 3 ml; the incubation is to be done at 37°C for 48 h in 0.01 M sodium acetate buffer of pH 5 using concentrations of enzymes (U/mg sample) of 40, 100 or higher depending on the capacities of different batches of crude solutions of β-glucuronidase to hydrolyse the SDG in flaxseed extracts with known SDG concentrations.

7.4.2.3. Effects of the Studied Factors on the Enzymatic Hydrolysis Yields Obtained With the 2nd Screening Design

When analysing the 2nd screening design using all 16 experimental combinations (including the SPE factor), it was found that using SPE purification prior to EH contributed significantly to the maximization of SECO through the depletion of SDG ($p < 0.0001$, **Table 7.3** and **Table 7.4**). This confirmed the hypothesis emitted above (section **7.4.1**) that compounds other than SDG, (e.g., PCouAG and FerAG, **Figure 7.2**) were hydrolysed preferentially thus decreasing the amounts of enzyme available for the hydrolysis of SDG. The purified extracts hydrolysed with 50 U enzyme/mg DFM for 48 h were free of SDG, PCouAG and FerAG, but the proportions of these compounds left un-hydrolysed in the un-purified extracts were similar with those obtained with the 1st screening design (**Figure 7.1** and **Figure 7.2**). It appeared that using SPE prior to EH contributed to the increased SECO formation by decreasing the amounts of PCouAG and FerAG in the purified microwave-assisted extracts.

The SPE also removed the salts formed from the NaOH used for MAE and the acid used for adjusting the pH of the extracts to 5. However, salts removal did not prove beneficial in terms of SECO formation, and had to be compensated for by using the more concentrated sodium acetate buffer (0.1 M) for EH. This was confirmed by the fact that the regression coefficients for the terms buffer

concentration and its interactions with the other factors affected the yields of SECO significantly for the 2nd screening design (**Equation 7.3**, **Table 7.3**), unlike for the 1st screening design (**Equation 7.2**, **Table 7.2**). This significance was due to the fact that 0.01 M buffer promoted the degradation of SECO to lignan artefacts in the purified extracts; degradation that was accentuated by the higher concentration of enzyme and longer time of incubation. For example, the proportions of SECO found in purified extracts incubated in 0.01 M buffer with 50 and 100 U enzyme, after the 1st and 2nd days of incubation were 73.1 and 73.2 %, and 75.9 and 72.1 %, respectively; which, resulted in 27 and 27.9 % lignans other than SECO (aglycone artefacts, and possibly SMG) after 48 h of incubation, respectively. In contrast, the % of lignans other than SECO after 48 h of incubation with 50 and 100 U enzyme in 0.1 M buffer were only 12.7 and 14.6, respectively.

For the extracts subjected to SPE purification prior to EH, the highest SECO recovery of 87.3 %, was achieved for 48 h of incubation at 37°C in 0.1 M sodium acetate buffer of pH 5 with 50 U enzyme/mg DFM. A gain of SECO formation of 1.2 % might not be substantial enough for including an expensive SPE purification step for each extract analysed, especially when hundreds of samples need to be processed for constructing databases of lignan contents in foods. Moreover, it is unlikely that a unique method of SPE purification is suitable for extracts from all kinds of food samples. Lignans occur naturally conjugated to a variety of compounds in plants, and once released by alkaline hydrolysis will be more or less polar, and will interact differently with the packed bed of the SPE cartridge and the purification solvents causing unknown losses. The SPE method used in this work, was developed previously for purifying microwave-assisted flaxseed extracts; and had known losses of SDG depending on extract's concentration in flaxseed solids [25 % in this case; (Nemes and Orsat, 2011c)]. This SPE method is not suitable for the general purification of plant extracts prior to EH; the purpose of using it in this work was solely for quantifying its benefits

in terms of efficiency of converting the SDG from purified flaxseed extracts to SECO.

The effects of the factors Enzyme, Time and their interaction contributed significantly to the maximization of SECO through the depletion of SDG (**Equation 7.3** and **Equation 7.4**). However, as mentioned above, when the ½ fraction of the design not using SPE was analysed separately, the regression coefficient of the Enzyme × Time interaction was not significant in terms of maximization of SECO, as was the case with the 1st screening design (section **7.4.1**). Thus, this interaction affected the formation of SECO significantly only when the EH was carried out with purified extracts.

In order to understand how the Enzyme × Time interaction affected the EH of purified and un-purified extracts, the differences between the yields of EH (% hydrolysed SDG, and % formed SECO) obtained for 48 and 24 h of hydrolysis with 50 vs. 100 U of enzyme were calculated. For un-purified extracts incubated in 0.01 M buffer, these differences were 22.5 vs. 17.3 % hydrolysed SDG and 14.8 vs. 9.4 % released SECO, respectively. As was the case with the 1st screening design, the hydrolysis proceeded at a higher rate during the 1st day and slowed down significantly during the 2nd day, with more accentuated hydrolysis rates being observed when using the higher concentration of enzyme (100 vs. 50 U/mg DFM). For example, 76.5 and 9.4 % SECO were formed, and 78.6 and 17.3 % SDG were hydrolysed during the 1st and the 2nd day of EH, respectively, when using 100 U enzyme/mg DFM. Since the proportions of hydrolysed SDG were always greater than the proportions of formed SECO, the numbers could not be linked to the formation of SECO from SMG during the 2nd day of EH, unlike for the 1st screening design. The proportions of lignans that were unaccounted for by the sum of SDG and SECO after 48 h were 7.7 and 10% for 50 and 100 U enzyme, respectively. For purified extracts incubated with 0.1 M buffer, the differences between 48 and 24 h of hydrolysis for 50 vs. 100 U of enzyme were 4.2 vs. 0 % hydrolysed SDG and 5.5 vs. % 0 released SECO, respectively. During

the 2nd day of EH, 5.5 % more SECO was formed (in addition to 81.8 % SECO formed during the 1st day) but only 4.2 % additional SDG were hydrolysed, indicating that the difference of 1.3 % SECO was released from the hydrolysis of SMG. When using 100 U enzyme/mg DFM, the SDG was depleted during the 1st day of EH but only 85.4 % SECO were released, which did not change during the 2nd day of EH, hence the 0 % differences. The proportions of lignan that were unaccounted for by the sum of SDG and SECO after 48 h of EH were 12.7 % for 50 U enzyme and 14.6 % for 100 U enzyme; the difference of 1.9 % (14.6 % - 12.7 %) was assumed to be due to the conversion of SECO to lignan artefacts.

7.4.2.4. Conclusions for the 2nd Screening Design, and Directions for Further Comparative Studies

Using SPE purification prior to EH facilitated the complete hydrolysis of SDG from flaxseed extracts, but also increased the proportions of SECO degraded to lignan aglycones artefacts. However appealing such gains in hydrolysis yield appear, the extensive use of purification solvents and chromatographic cartridges would increase tremendously the costs of building databases of lignan contents in foods if SPE purification of extracts would be used prior to EH. From this perspective, it is more appealing to accept an 86 % lignan aglycone recovery (or more if all lignan artefacts are quantified) without using SPE purification of extracts prior to EH, while reducing the costs of lignan analysis in foods.

Overall, the 2nd screening design proved that the hydrolysis of SDG proceeded at a high rate during the 1st day and slowed down significantly during the 2nd day, with more accentuated hydrolysis rates being observed when using the higher concentration of enzyme (100 vs. 50 U/mg DFM), as was the case with the 1st screening design. The similarity between the two designs could be taken further by hypothesising that the SDG hydrolysis rate could be increased during the 1st day, by using higher concentrations of enzyme. However, this would be

unlikely to happen given that the higher enzyme concentrations resulted in 86 % recovery of SECO with both designs and batches of enzyme used.

At this point, the question arises – could there be a gain in hydrolysis yields by replacing the crude solution of β-glucuronidase with partially purified preparations of the same type of enzyme in powder form, and using even higher enzyme concentrations? This possibility is to be investigated below.

7.4.3. Comparative Study Using Purified β-Glucuronidase and Cellulase in Excess

The design using enzymes in excess comprised 12 combinations of the factors enzyme type (β-glucuronidase, cellulase, and a mixture of the two enzyme types) and the time of incubation (24 and 48 h). Each experimental combination was carried out twice using aliquots of un-purified microwave-assisted extracts containing 50 mg DFM (1 mg SDG) in 3 ml of 0.01 M sodium acetate buffer pH 5 with 300 U of β-glucuronidase or cellulase, or with 300 + 300 U of β-glucuronidase + cellulase, for 24 or 48 h. The mixture of enzymes was studied with 300 U of each enzyme, rather than 150 U each in order to be able to identify a possible additive effect. The concentration of buffer of 0.01 M was chosen based on the results of the two screening studies as it was preferred for hydrolysing un-purified extracts. The effects of these hydrolysis conditions were studied by analysing the proportions of conversion of SDG to SECO, and the proportions of PCouAG and FerAG left un-hydrolysed.

7.4.3.1. Enzyme Type-Time of Incubation Interaction Effects

Two-way ANOVA was carried out followed by pair-wise comparison of means using the Tukey's HSD test for each of the studied compounds. In terms of SECO production from SDG, the model was significant ($p = 0.0002$) and had $R^2 = 0.968$ and $R^2_{adj.} = 0.941$. The factors enzyme type and time of incubation did not affect

significantly the yields of SECO (p = 0.2008 and p = 0.0833, respectively), but their interaction had a significant effect (p = 0.0002). In order to help understand how this interaction affected the yields of released SECO, their classification in terms of significance is shown in **Table 7.5**, and their graphic representation is shown in **Figure 7.3**. The classification of the interactions according to their significance in terms of affecting the % SDG left un-hydrolysed is presented in **Table 7.6**.

Table 7.5. Classification of the interactions of enzyme type × time of incubation based on their capacities of significantly affecting the yields of SECO

Interaction of enzyme type with the time of incubation	* Classification based on Tukey's HSD comparison test	LS Means (% SECO)
β-glucuronidase (300 U, 48 h)	A	66.3
mixture (300+300 U, 48 h)	B	61.1
mixture (300+300U, 24 h)	B	61.0
cellulase (300 U, 24 h)	B	59.9
β-glucuronidase (300 U, 24 h)	B	59.4
cellulase (300 U, 48 h)	C	55.7

* Interaction terms not connected by same letter are significantly different.

Table 7.6. Classification of the interactions of enzyme type × time of incubation based on their capacities of significantly affecting the % of SDG left un-hydrolysed

Interaction of enzyme type with the time of incubation	*Classification based on Tukey's HSD comparison test	LS Means (% SDG)
mixture (300+300 U, 48 h)	A	30.3
mixture (300+300U, 24 h)	B	27.8
cellulase (300 U, 48 h)	B C	25.9
cellulase (300 U, 24 h)	C	23.8
β-glucuronidase (300 U, 24 h)	D	8.14
β-glucuronidase (300 U, 48 h)	E	1.27

* Interaction terms not connected by same letter are significantly different.

In terms of % SDG left un-hydrolysed, the model was highly significant ($p < 0.0001$) and had $R^2 = 0.998$ and $R^2_{adj.} = 0.992$; the effect of the factor time of incubation was not significant ($p = 0.0749$), but the effects of the factors enzyme type and its interaction with the time of incubation were both highly significant ($p < 0.0001$).

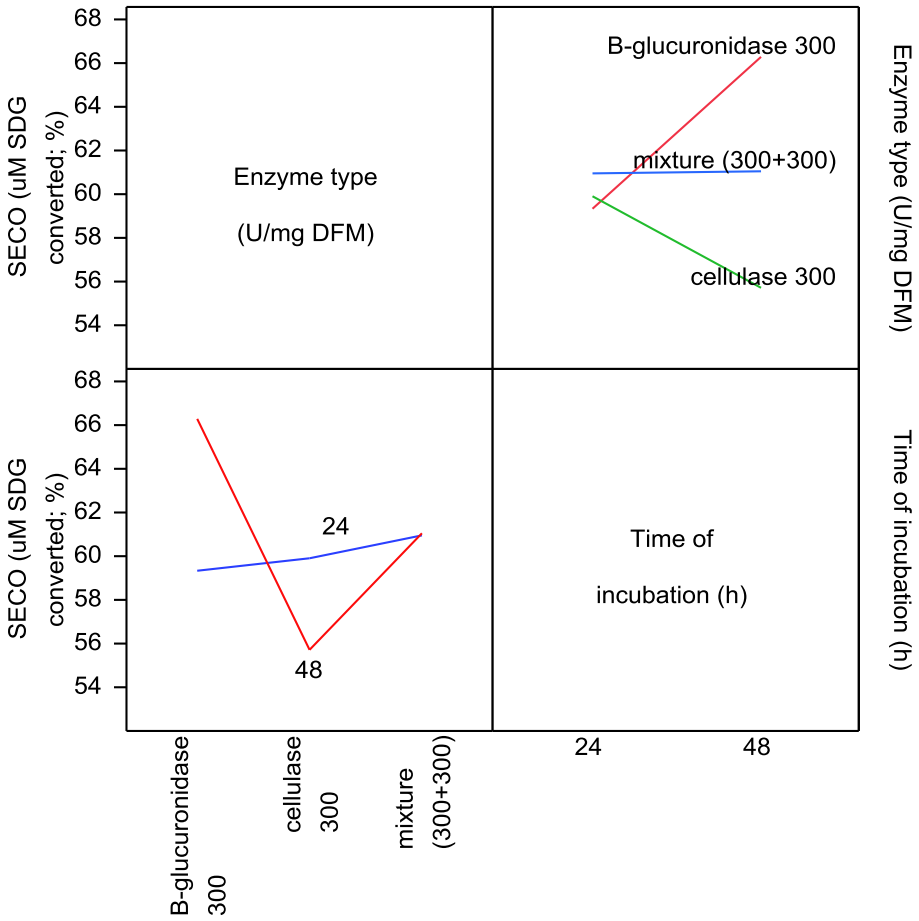

Figure 7.3. Enzyme type × time of incubation interaction profiler for SECO

The best hydrolysis condition using enzyme in excess was achieved with 300 U of β-glucuronidase/mg DFM for 48 h of incubation with 0.01 M buffer. The proportion of hydrolysed SDG recovered in the form of SECO was 66.3 % (**Table 7.5**). By comparison with the similar hydrolysis conditions studied with the 1st and the 2nd screening designs, except for the U of enzyme, the proportions

of SDG left un-hydrolysed were only 1.27 % (**Table 7.6**) for the present design as opposed to 4.43, and 4.41 % for the 1st and the 2nd screening designs respectively; and the proportions of hydrolysed SDG unaccounted for by the sum of SECO and SDG were much higher for the present design at 37.5 % as opposed to 9.17 and 10 % for the 1st and the 2nd screening designs, respectively.

These results support the hypothesis that the partially purified β-glucuronidase hydrolysed the SDG in a way that was significantly different from that achieved with the crude enzyme solutions. Although, the hydrolysis might seem almost complete (only 1.27 % SDG left), there is no way of assessing the reason why SECO was recovered in such low percentage. In order to determine what compounds contribute to the 37.5 % the chromatographic analysis has to include all the known lignan artefacts formed from SECO. However, it can be seen from **Figure 7.3** that the % of released SECO increased significantly from the 1st to the 2nd day of hydrolysis, indicating the possibility of having important amounts of SDG hydrolysed partially to SMG.

7.4.3.2. Conclusions for the Comparative Study Using Purified β-Glucuronidase and Cellulase in Excess

Based on the results obtained with the various hydrolysis conditions studied in this work, it can be concluded that using crude solutions of β-glucuronidase in order to hydrolyse un-purified lignan extracts remains the best option. The yields of EH did not benefit from incubating the extracts with the mixture of enzymes, meaning that there was no additive effect recorded. In general, cellulase produced the lowest SECO formation yields and the highest % of SDG left un-hydrolysed. Abnormal amounts of FerAG (3300 – 6700 % higher than the amounts subjected to EH) were detected in the extracts hydrolysed with cellulase or the mixture of enzymes. It is possible that cellulase released the FerAG from complex phenolic molecules found in flaxseed other than the lignan macromolecule.

The results obtained with the present design supported those recorded for the 1st and the 2nd screening design, which proved that 48 h of hydrolysis were necessary for obtaining increased yields of lignan aglycones. It is worth noting that most of the literature on EH reviewed for this paper reported overnight incubation of extracts with enzyme, with the exception of Kraushofer and Sontag (2002) who reported 22 h of incubation.

7.4.4. Study on the Degradation of Secoisolariciresinol Standard Using Purified β-Glucuronidase in Excess

The study on the degradation of SECO under EH conditions comprised four factorial combinations of the factors 0.01 and 0.1 M sodium acetate buffer (pH 5), with/without purified β-glucuronidase. Each experimental combination was replicated 3 times and used 20 µg SECO incubated in 2 ml buffer with/without 1500 U of enzyme for 24 h. The purpose of this design was to help understand to what extent the EH conditions can affect the degradation of readily available SECO to other lignan aglycones (artefacts).

7.4.4.1. Causes of Degradation of Secoisolariciresinol to Lignan Aglycone Artefacts

Overall, the EH conditions affected significantly the recovery of SECO (p = 0.0176, R^2 = 0.794, $R^2_{adj.}$ = 0.691); the factors buffer concentration and the presence or absence of enzyme had significant effects (p = 0.0308 and p = 0.0135, respectively), but not their interaction (p = 0.3482). As can be seen from **Figure 7.4**, the buffer alone affected the recovery of SECO significantly; when incubated in 0.01 and 0.1 M buffer without enzyme, 84.1 and 94.5 % SECO were recovered, respectively.

Based on the results of the current design, it can be concluded that both the buffer and the enzyme caused the degradation of SECO to lignan aglycone artefacts.

Thus, during the EH of plant extracts, once the SECO is released from conjugated lignan molecules it is not stable in the EH environment. The readily available SECO will be degraded by both the buffer and the enzyme. The use of excessive concentrations of enzyme is interesting for achieving increased lignan glucosides hydrolysis rates, but it also promotes increased rates of SECO degradation. The results presented in section **7.4.3** revealed that using excessive concentrations of enzyme (300 U/mg DFM) hydrolysed the SDG almost completely in 48 h, but the proportion of hydrolysed lignan recovered in the form of SECO was only 66.3 %.

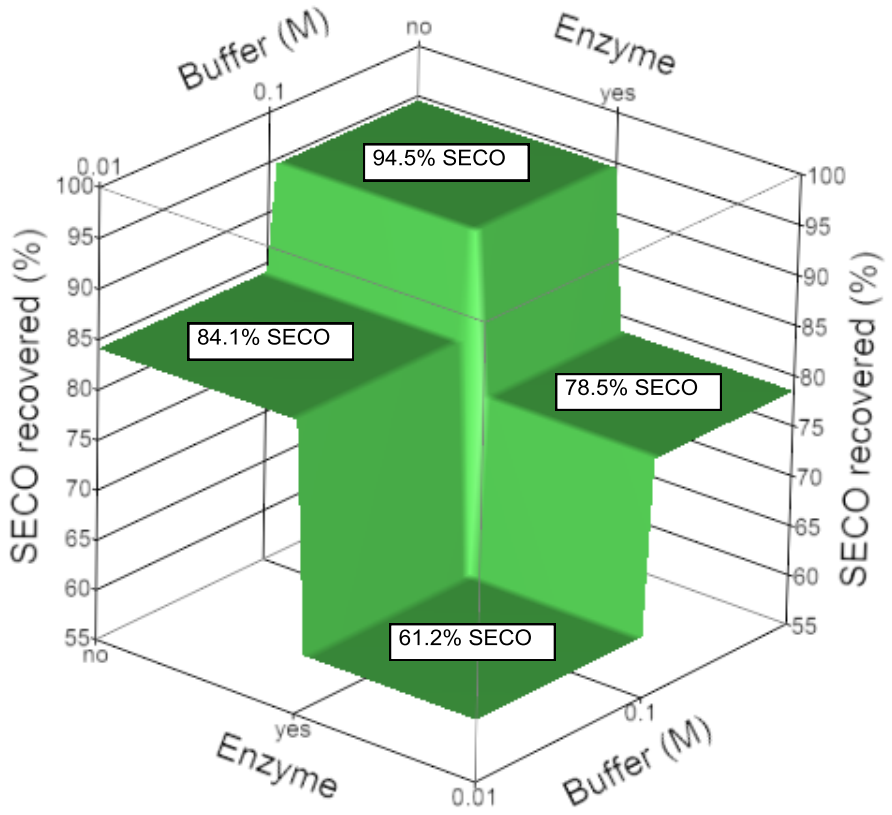

Figure 7.4. Surface profiler showing the recoveries of SECO as affected by the incubation with/without β-glucuronidase for 24 h in 0.01 and 0.1 M sodium acetate buffer

7.5. Summary of Results

The quantification of lignans in plant foods is complex as it requires the coupling of two types of hydrolysis methods. An initial alkaline hydrolysis is necessary in order to free the lignan glucosides from the plant matrices efficiently. For this purpose, the optimized MAE method has been proposed, which was developed using response surface optimization studies (Nemes and Orsat, 2010, 2011b); and was evaluated in terms of efficiency of extraction, repeatability of results, recovery of lignans, and for the extraction of lignans from a variety of flaxseed cultivars and oil seeds (Nemes and Orsat, 2011a). A second step is necessary for hydrolysing the lignan glucosides to aglycones before quantification by HPLC. For this purpose, microwave-assisted flaxseed extracts were used as reference model of plant lignan extracts, to study the effects of buffer concentration, enzyme concentration, purification, and the time of incubation on the results of EH.

The preferred enzymatic hydrolysis conditions were: 48 h of incubation with 40 or 100 U of crude solution of β-glucuronidase enzyme/mg sample, in 0.01M sodium acetate buffer pH 5, without using prior SPE purification. It was shown that the capacities of crude solutions of β-glucuronidase to hydrolyse SDG varied significantly from one batch of enzyme to another; thus, it is recommended to test the enzymes by using 50, 100, and 150 U/mg sample. The recovery of SECO for these enzymatic hydrolysis conditions was about 86 %, other hydrolysis products such as lignan artefacts and possibly partially hydrolysed SDG accounted for a total of about 10 %, and 4 % of the added SDG was left un-hydrolysed.

The hydrolysis of SDG from flaxseed extracts was almost complete (1.27 % SDG left un-hydrolysed) when excessive concentrations of 300 U of purified β-glucuronidase/mg sample were used; but only 66.3 % SECO was recovered possibly due to extensive degradation of SECO to lignan aglycone artefacts. The results indicated that once released from the lignan glucoside SDG, SECO was

not stable in the EH conditions, as both the buffer and the enzyme contributed to its degradation. The EH conditions suggested in this chapter can be used for hydrolysing plant extracts in view of quantifying their content in lignan aglycones. The lignan aglycone standards necessary for the chromatographic quantification are: SECO, pinoresinol, lariciresinol, isolariciresinol, matairesinol, medioresinol, syringaresinol, and demethoxy-secoisolariciresinol.

7.6. Conclusions

The coupling of MAE with enzymatic hydrolysis using the conditions developed in this work, is recommended for the general extraction of lignan aglycones from plant samples, and can be used for acquiring data for building databases of lignan contents in foods. Such databases are necessary for correlating the lignan dietary intakes with the outcomes of various health conditions. The novelty of this work consists in the approach of studying the various factors that affect the enzymatic hydrolysis yields, and the stability of released lignan aglycones. The results revealed that the formation of artefacts cannot be prevented but it can be minimised by avoiding using excessive concentrations of enzyme. However, the enzymatic hydrolysis is complex and cannot be entirely standardized, as the hydrolysing capacity of β-glucuronidase preparations can vary significantly from batch to batch.

Connecting Statement to Chapter 8

In **Chapter 7**, the factors affecting the enzymatic conversion of lignan glucosides to lignan aglycones were studied, and the enzymatic conditions that can generally be applied for the analysis of plant lignans in foods were identified. The enzymatic hydrolysis of lignan glucosides is problematic as the released lignan aglycones are not stable in the reaction conditions. In addition, the method could not be standardized, due to the variability in the hydrolysing capacity of different batches of enzyme. Recommendations were made in order to help overcome these problems, and to ensure that the enzymatic hydrolysis of plant lignans can be carried out accurately and efficiently.

The optimized microwave-assisted extraction (MAE) method for lignan analysis was updated in **Chapter 3**, was evaluated in **Chapter 5** for the general determination of lignans in flaxseed cultivars, flaxseed derivatives, and a selection of oil seeds, and was coupled in **Chapter 7** with an enzymatic hydrolysis method for the purpose of general quantification of lignan aglycones in foods. In **Chapter 6**, the MAE method was coupled with a developed solid phase extraction (SPE) method for producing purified flaxseed extracts that can be used for further experimentation. All these applications require the quantification of lignans by high performance liquid chromatography (HPLC).

In **Chapter 8**, an alternative is proposed to HPLC analysis for the quantification of lignan in flaxseed cultivars and flaxseed derivatives. Artificial neural network and partial least squares regression models were calibrated that can be used to accurately and inexpensively determine the content of lignan in flaxseed materials.

Chapter 8 is written in manuscript style and was published in edited form.

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Chapter 8 - Calibration of Artificial Neural Network and Partial Least Squares Regression Models for the Prediction of Secoisolariciresinol Diglucoside Contents in Microwave-Assisted Extracts of Various Flaxseed (*Linum usitatissimum* L*)* Samples

8.1. Abstract

Accurate and precise partial least squares (PLS) and artificial neural network (ANN) regression models were calibrated for the prediction of secoisolariciresinol diglucoside (SDG) contents in various flaxseed cultivars. ANN models were also calibrated for mixed datasets composed of defatted and non-defatted flaxseed cultivars, and flaxhull samples. The SDG was extracted from the flaxseed samples by microwave-assisted extraction (MAE) and quantified by high performance liquid chromatography (HPLC). The models were calibrated using the SDG chemical indexes of the microwave-assisted extracts and their respective UV-Vis absorptions at the wavelengths 289, 298, 343 and 765 nm following Folin-Ciocalteu assays. The accuracy and the precision of the calibrated models were evaluated using the following statistical indices: the mean of differences between the reference and the predicted values (bias), the root mean square difference (RMSD), the standard error of performance (SEP), the ratio of the standard deviation of the reference data to SEP (RPD), the accuracy factor (AF) and the bias factor (BF). The predictive qualities of the PLS and ANN models ranged from good to excellent (RPD = 5.0 – 13.7) with the predicted values falling within ± 0.7 – 4.9 % of the measured SDG values. These models can be used for quality and process control applications in the flaxseed processing industries; and they can be used for regulatory issues as well. Simple linear regression models using corrected gallic and ferulic acids equivalents were also generated for the estimation of SDG in flaxseed cultivars. These models being less accurate than the PLS and ANN ones, are recommended for screening applications only. The

PLS and ANN models presented in this chapter are useful for rapidly and accurately predicting the SDG contents of flaxseed extracts based on their UV-Vis absorptions without having to use advanced and expensive analytical techniques and equipments such as an HPLC.

Key words: ANN, PLS, SDG, UV-Vis, MAE, spectrophotometric calibration, Folin-Ciocalteu, polyphenol

8.2. Introduction

Flaxseed is the richest food source of secoisolariciresinol diglucoside (SDG) (Muir and Westcott, 2003; Peterson et al., 2010); which along with high contents of omega-3 fatty acids, and soluble fibre (mucilage) makes flaxseed a key raw material in the nutraceutical and functional food industries (Oomah, 2001). There has been increasing interest in the quantification of SDG in flaxseed and other plant sources over the past two decades due to its nutraceutical qualities. SDG is a phytoestrogen, which upon ingestion is transformed in the human digestive tract to enterodiol and enterolactone (also referred to as mammalian lignans or enterolignans). After absorption into the body, the enterolignans can be identified in blood and urine (Webb and McCullough, 2005). They protect humans against cardiovascular diseases by reducing: the concentration of lipids in blood, the blood pressure, the oxidative stress, and the inflammation in the body (Adolphe et al., 2010; Peterson et al., 2010). The enterolignans have the capacity to bind to the mammalian estrogen receptors, due to similarities between their chemical structures and those of estrogenic hormones, thus providing protection against breast cancer. They prevent pre-cancerous cellular changes and reduce the angiogenesis and metastasis (Adolphe et al., 2010).

In flaxseed, SDG is part of a lignan macromolecule; which is found mainly in the seed coat (also known as flax hull) (Attoumbré et al., 2011) and has the following composition: 62 % SDG, 11 % 3-hydroxy-3-methylglutaric acid, 12.2 % *p*-coumaric acid glucoside, 9 % ferulic acid glucoside, and 5.7 % herbacetin

diglucoside (Struijs et al., 2009). Recently, caffeic acid glucoside was reported to be part of the lignan macromolecule in defatted flaxseed meal in a ratio of 1 mol caffeic acid glucoside to 5 moles *p*-coumaric acid glucoside and 2 moles ferulic acid glucoside (Kosińska et al., 2011a).

The quantification of SDG in flaxseed samples is usually done by high performance liquid chromatography (HPLC) after extraction with aqueous or alcoholic mixtures followed by alkaline hydrolysis (Oomah and Hosseinian, 2008) or after direct alkaline hydrolysis (Eliasson et al., 2003; Nemes and Orsat, 2010, 2011a, b). The latter approach eliminates the alcoholic extraction step thus achieving double benefits: reduced extraction time and increased extraction yields. Nemes and Orsat (2010, 2011b) developed and optimized, by means of response surface studies, a MAE method for extracting SDG from flaxseed. The method was updated in section **3.5.5**., and it requires 3 min of direct hydrolysis with 0.5 M NaOH under intermittent (30 sec on/off) microwave power (156 W) application in order to achieve a final extract temperature of 67°C. The MAE method has an excellent repeatability and 97.5 % SDG recovery (Nemes and Orsat, 2011a). The gain in the SDG extraction yield achieved with MAE was 6 % higher than that achieved by direct alkaline hydrolysis [1h hydrolysis with 1M NaOH at room temperature, reported by Eliasson et al. (2003)] (Nemes and Orsat, 2011b); 21.4 % higher than that obtained by sequential hydrolysis [1h hydrolysis with 0.3 M NaOH in 70 % methanol at 60°C followed by 1 h hydrolysis with 0.01M HCl in 70 % methanol at 60°C; reported by Smeds et al. (2007)]; and 26.6 % higher than that obtained with alcoholic extraction followed by alkaline hydrolysis [2 h extraction two times with 70 % methanol at 60°C, followed by 3 h hydrolysis with 1 M NaOH; reported by Thompson et al. (2006)] (Nemes and Orsat, 2011a).

The Folin-Ciocalteu (FC) assay is often used for the spectrophotometric quantification of total phenolics in foods. It has the advantage of being simple and accessible but it has the disadvantage of being non-specific, as the FC reagent

reacts with all the phenolic compounds found in the extracts and other easily oxidizable compounds (Shahidi and Naczk, 2004a; Singleton et al., 1999). The colorimetric reaction takes place in alkaline solutions as electrons are transferred from the phenolic compounds and reduce the FC reagent, which is a mixture of phosphomolybdic/phosphotungstic acid complexes, to form blue pigments (Ainsworth and Gillespie, 2007; Singleton et al., 1999). The blue pigments exhibit a light absorption maximum in the visible range of the spectrum at 765 nm, measurable with a spectrophotometer, which is proportional to the concentration of phenols (Singleton et al., 1999; Wrolstad et al., 2005).

UV-Vis spectrophotometric assays are known to overestimate the contents of polyphenols in crude plant extracts due to the presence of interfering compounds that absorb light in the same spectral region as the target compounds. The problem of overestimation of results can be corrected by using chemometric techniques for analysing the spectral data. In general, the chemometric techniques use chemical indexes as dependent (response) variables and UV-Vis spectral data as predictive variables. The chemical indexes are the concentrations of the components of interest measured with techniques considered accurate such as high performance liquid chromatography (HPLC). The construction of a predictive model using chemical indexes and spectral data is known as calibration (Shahidi and Naczk, 2004a). Examples of chemometric methods used for the calibration of predictive models are partial least squares (PLS) regression (Andjelkovic et al., 2008; Davis et al., 2007; Dias et al., 2009; Hossain et al., 2011; Naczk et al., 2001; Naczk et al., 2002), and artificial neural network (ANN) analysis (Borggaard, 2001; Dias et al., 2009; Williams, 2001).

Andjelkovic et al. (2008) used PLS regression to predict the total phenolics in olive oils measured using the FC assay from the HPLC-mass spectrometry data of the phenolic compounds. Davis et al. (2007) established PLS regression models for the determination of capsaicinoids in alcoholic extracts of habanero peppers (*Capsicum chinese)* based on their UV absorption spectral data and their

concentrations in capsaicinoids determined by HPLC. These models allowed for the rapid determination of capsaicinoids by spectrophotometric procedures without requiring the purification of pepper extracts in order to remove the interfering compounds. Hossain et al. (2011) developed PLS regression models for predicting the antioxidant activities of spices (rosemary, oregano, marjoram, sage, basil, thyme, fennel, celery, cumin and parsley) based on their concentrations of polyphenolic compounds determined by HPLC and the total phenolics content measured using the FC assay. Naczk et al. (2001) calibrated a PLS regression model for predicting the soluble condensed tannins in alcoholic extracts of canola and rapeseed hulls. The model was developed using UV absorption spectral data as predictive variables, and Vis-spectrophotometric (vanillin and proanthocyanidin) assays data as chemical indexes. Naczk et al. (2002) calibrated a PLS regression model for predicting the total phenolic acids in alcoholic extracts of canola and rapeseed meals based on UV spectral data. The chemical indexes were obtained with Vis-spectrophotometric (FC) assay. Dias et al. (2009) calibrated PLS and ANN models for estimating the concentrations of substrate (lactose) and product (galacto-oligosaccharides) during the industrial production of galacto-oligosaccharides. The predictive variables were the UV-spectral data and the responses were the concentrations of the two compounds determined by HPLC. The ANN model achieved higher accuracy of predictions than the PLS model.

Based on these facts, it was hypothesised that there is a need for simplified, inexpensive, and accurate methods of analysis that do not require the use of an HPLC, for the quantification of SDG in flaxseed cultivars and flaxseed derivatives.

The objective of this work is to calibrate PLS and ANN models for accurately predicting the concentration of SDG in extracts obtained from various flaxseed cultivars, flax hulls and defatted flaxseed meal based on their respective UV-Vis

spectrophotometric data. The SDG chemical indexes used in the calibration of the predictive models were obtained by HPLC quantification.

8.3. Materials and Methods

8.3.1. Flaxseed Samples

All the flaxseed (*Linum usitatissiumum* L.*)* samples were brown, and were milled with a coffee grinder. Six flaxseed cultivars: CDC Bethune, McBeth, Prairie Blue, Flanders, 09LS01, and CRGL 8.2 were used for the calibration of the PLS and ANN predictive models. The cultivars were provided by Mr. Yves Dion from Centre de Recherche sur les Grains Inc. (CEROM); and were grown in Saint-Mathieu-de-Beloeil (QC, Canada) in 2009. The Omega-3 Flax Hull (FH) produced in May 2010, was provided by Dr. Nam Fong Han from Natunola Health Inc. (Winchester, ON, Canada). The defatted flaxseed meal (DFM) was obtained by oil removal with hexane from a commercial flaxseed sample grown in Guatemala purchased in 2009 from a local grocery store (Montreal, QC, Canada). The oil was extracted twice with hexane (sample to liquid ratio 1:6, g/ml) for 1 h under magnetic stirring at room temperature, followed by vacuum filtration and evaporation of residual hexane under the fume hood overnight (Nemes and Orsat, 2010).

8.3.2. Chemicals

The reference standards: secoisolariciresinol (SECO, molecular weight 362.4, purity ≥ 95 %, HPLC grade), ferulic acid (FA, molecular weight 194.2, purity ≥ 99 %, HPLC grade), gallic acid (GA, molecular weight 170.1, purity 97.5 %) were purchased from Sigma-Aldrich (Oakville, ON, Canada); and secoisolariciresinol diglucoside (SDG, molecular weight 686.7, purity 97.6 %, HPLC grade) was purchased from Chromadex (Santa Ana, CA, USA). The solvents: acetonitrile, methanol and hexane of HPLC grade were obtained from Fisher Scientific (Ottawa, ON, Canada). The reagents: sodium hydroxide ≥ 98 %,

sulphuric acid 95 – 98 % ACS, phosphoric acid ≥ 85 %, di-potassium hydrogen phosphate 98 %, Folin and Ciocalteu's 2 N phenol reagent, and anhydrous sodium carbonate 99 % were purchased from Sigma-Aldrich (Oakville, ON, Canada).

8.3.3. Microwave-Assisted Extraction

The microwave apparatus used for extraction was a mono-mode Star System 2 (CEM, Mathews, USA; nominal power declared by the manufacturer 800 W, microwave frequency 2.45 GHz) which delivers a maximum power of 710.5 W (section **4.4.1**). The system was equipped with built-in IR temperature sensors placed at the bottom of the extraction vessels (250 ml, borosilicate glass) and a Graham-type reflux condenser. The flaxseed extracts were obtained following our previously published optimized methodology (Nemes and Orsat, 2010, 2011b) which was updated in section **3.5.5**. Briefly, samples of 0.6 - 1g of flaxseed meal, FH and DFM were hydrolysed with 50 ml of 0.5 M NaOH at 22 % power level [156 W as calculated with the calibration in **Equation 4.2** = 7.105 × Power level (%), presented in section **4.4.1**]; the microwave power was applied intermittently (30 s on/off) for 3 min. A type K thermocouple (accuracy ± 0.5°C, Fisher Scientific) was used to measure the final temperature of the extracts after briefly stirring them. The temperature of the extracts rose from room temperature (22 – 23°C) to about 67°C over the 3 min span.

8.3.4. High Performance Liquid Chromatography Analysis of Secoisolariciresinol Diglucoside in Flaxseed Extracts

The extracts were analysed in triplicates with an Agilent 1100 series HPLC controlled by the Chemstation software for LC systems [Rev. B.01.03 (204), Agilent Technologies]. The chromatograms were recorded at 280 nm using a variable wavelength detector. The separation of SDG was done as previously reported by Nemes and Orsat (2011a) using a slightly modified version of the method reported by Johnsson et al. (2000). Briefly, SDG was eluted at 25°C on a

reversed-phase C18 column (particle size 5 µm, length 25 cm, internal diameter 4.6 mm; Discovery, Sigma-Aldrich) fitted with a guard cartridge (particle size 5 µm, length 2 cm, internal diameter 4 mm; Supelguard, Sigma-Aldrich) using a linear gradient of the solvents A - 0.01 M phosphate buffer with a pH of 2.8 containing 5 % acetonitrile, and - B acetonitrile. The solvent A decreased from 100 % to 50 %, at a rate of 1 %/min over a period of 50 min and flow rate of 1 ml/min. The elution time of SDG was 19.5 min. The standard curve (SDG = peak height/0.4494) was built using six levels of SDG concentration ranging from 5 to 200 µg SDG/ml methanol; the coefficient of determination (R^2) was > 0.999. In order to render the extracts suitable for HPLC analysis, the water soluble carbohydrates and proteins were precipitated under magnetic stirring (3000 rpm) for 15 min following acidification to pH 3 with 5.55 ml of 5 N H_2SO_4 (pH measured with Accumet 25, Fisher Scientific) and methanol addition in proportion of 2:1 (v/v, ml/ml). The solid and liquid phases were separated by centrifugation (10 min, 3000 rpm; Spinette centrifuge, International Equipment Company; Needham Heights, MA, USA). The flaxseed cultivar and FH extracts were concentrated to about 25 ml by vacuum evaporation (30 min, 65°C, 195 rpm; Buchi Rotavapor 205 equipped with a B490 heating bath) and then defatted by liquid/liquid extraction with hexane in proportion of 2:1 (v/v, ml/ml). Aliquots of extracts with concentrations of 10 mg sample/1 ml extract were filtered through 0.22 µm nylon syringe filters (13 mm diameter, Whatman Puradisc) and analysed by HPLC.

8.3.5. Folin-Ciocalteu's Assay and UV-Vis Spectrophotometric Measurements

The FC reactions were carried out according to a protocol adapted from Singleton et al. (1999) and Ainsworth and Gillespie (2007). Briefly, 5.5 ml of aqueous microwave-assisted flaxseed extracts (containing 2 - 6 mg flaxseed cultivars, 1.5 - 2.5 mg FH, or 2.4 - 3.6 mg DFM), and 0.5 ml FC reagent (2 N) were added into a 15 ml centrifuge tubes and vortexed; 4 ml Na_2CO_3 (0.7 M) were added after

5 min, then the tubes were capped, vortexed, wrapped in aluminum foil and incubated at room temperature for 2 h. The flaxseed extracts were replaced with blank microwave-assisted extracts for the preparation of blank FC reactions. The blue flaxseed extracts were transferred into 3 disposable cuvettes (1 cm path length) each, and the light absorption was measured against blank FC extracts at 765 nm with a spectrophotometer (Ultrospec 2100 Pro, Biochrom Ltd., Cambridge, England). The light absorbance of the extracts was also measured in the UV region (200 - 400 nm) of the spectrum. Wave scans were carried out from 190 to 800 nm every 2 nm for FC reactions with MAEs of flaxseed cultivars, FH, DFM and the reference standards SDG, FA and GA. In all cases, maximum UV light absorbance was exhibited in the range of 268 and 272 nm; but the repeatability of results was very poor for the interval of 190 to 288 nm with coefficients of variation (standard deviation*100/mean) ranging from 3 to 95 %. The spectral responses were reproducible (coefficients of variation < 3 %) from 288 to 800 nm. Three wavelengths were found to be representative for the UV-Vis light absorption behaviour of flaxseed extracts as all the FC extracts containing SDG exhibited peaks at 289, 298 and 343 nm. Moreover, the peak recorded at 343 nm wavelength was also representative for the FC assays with FA (FA is released in glucosidic form along with SDG during the MAE of flaxseed).

8.3.6. Experimental Design and Statistical Analyses

8.3.6.1. Ordinary Least Squares Regression, Pair-Wise Comparison and Multivariate Correlation Analyses

Microwave-assisted extracts replicated 2 or 3 times were obtained for the six flaxseed cultivars, FH and DFM. FC reactions were carried out in triplicates for each microwave-assisted flaxseed extract at three levels of dilution with known SDG concentrations; and the light absorption was measured at 289, 298, 343, and 765 nm. The data was used to obtain one ordinary least squares (OLS) regression model for each extract in order to assess the linearity of the relationship between the concentration of SDG and the intensity of the UV-Vis light absorption. The

FC reactions (FCR) had different concentrations of nM SDG/ml extract due to differences in the composition of flaxseed cultivars. The six flaxseed cultivars were previously shown by Nemes and Orsat (2011a) to differ in terms of SDG concentration. Therefore it was necessary to identify the range of SDG concentrations that was common to all the extracts from the six flaxseed cultivars. The spectral responses for each FC extract were predicted in the common SDG range (6.45 – 9.27 nM SDG/ml FC extract) in order to obtain a data set suitable for pair-wise comparison test, and PLS regression. According to Williams (2001), it is recommended to calibrate PLS regression models using data sets composed of samples distributed evenly among equally spaced levels of concentrations for the compound of interest. OLS regression equations were also established for the GA (15.7 - 35.4 nM/ml FCR), FA (11.7 - 23.4 nM/ml FCR), SDG (4.4 - 14.6 nM/ml FCR), and SECO (5.2 - 16.6 nM/ml FCR) standards using their absorbance at 765 nm following the FC assays. The statistical analyses: OLS regressions, two-way Analysis of Variance (ANOVA) with the Tukey HSD pair-wise comparison test, and multivariate correlations using the Residual Maximum Likelihood (REML) method were carried out with the JMP 8.0.2 software (SAS Institute Inc., Cary, NC, USA); the significance of the tests was considered at $p < 0.05$.

8.3.6.2. Partial Least Squares Regression

Partial least squares (PLS) regression was used to calibrate a model for predicting the SDG contents of extracts from six flaxseed cultivars based on their respective UV-Vis light absorption data. PLS is a multivariate regression technique widely used for constructing empirical predictive models; it correlates the spectral data with chemical indexes and attributes weightings to the spectral data accordingly (Dias et al., 2009; Shahidi and Naczk, 2004a). The calibration of models through PLS assumes that the chemical indexes are correct (Shahidi and Naczk, 2004a) and that the relationship between the concentration of the component of interest and its UV-Vis light absorption is linear (Gowda et al., 2009). The chemical

indexes are stored in a $n \times k$ matrix denoted **Y,** where n is the number of observations and k is the number of responses. The values of the predictors are stored in a $n \times q$ matrix denoted **X,** where n is the number of observations and q is the number of predictors. The values of the predictors are highly autocorrelated (Dias et al., 2009; Mutihac and Mutihac, 2008) hence eliminating the possibility of using multiple linear (OLS) regression which requires independent, uncorrelated predictors with covariance equal to zero. PLS uses features from principal component analysis (the principle component scores are uncorrelated thus overcoming the problem of autocorrelation between predictor variables) and multiple linear regression. It predicts the response variables by decomposing simultaneously both the predictor (**X)** and the response matrices (**Y**) in order to find the number of latent vectors that explain as much as possible the covariance between **X** and **Y**. Then, the decomposition of **X** is used in a regression step to predict the **Y** (Abdi, 2007). The number of the latent vectors is selected through cross-validation (Abdi, 2007; Dias et al., 2009); a technique that fits the model repeatedly for all data points except one (one-at-a-time method), until each data point is left out once, while minimizing the prediction error for the data point that was left out. The number of latent vectors associated with the lowest prediction root mean square error is chosen. The evaluation of predictive models built with sample sizes ≤ 60 is done by cross-validation (Williams, 2001). The PLS regression was carried out with the JMP 8.0.2 software (SAS Institute Inc., Cary, NC, USA).

8.3.6.3. Artificial Neural Network Analysis

Feed-forward artificial neural network (ANN) models were trained for predicting the SDG content of extracts from six flaxseed cultivars, FH and DFM based on their respective UV-Vis light absorption data. ANNs are nonlinear parametric regression models with architectures inspired by the organization of the central nervous system. They can be used to analyse any set of data (Tirozzi et al., 2006) and can be used to model any spectroscopic and chromatographic data (Mutihac

and Mutihac, 2008). ANN uses nodes as artificial neurons, which are organized in input, hidden, and output layers. The information is passed from one node to the neighbouring nodes through weights. The information is scaled in the nodes using the sigmoid (logistic) function in order to have the mean equal to zero and the standard deviation equal to one. The node is activated if the value of the information surpasses a certain threshold, then the information is passed to the neighbouring nodes. The most commonly used type of ANN technique is the feed-forward ANN trained by back-propagation of the error (Borggaard, 2001). For calibrating ANN models, the training is supervised and it starts by presenting the ANN with a set of spectral data and their corresponding chemical indexes (Dias et al., 2009). The information flows forward from the input layers, through the hidden, to the output layer of nodes. The strengths of the weights are adjusted repeatedly until the differences between the predicted and the reference (chemical index) values are small enough. This tuning of the weights is called the back-propagation of the error (Borggaard, 2001; Mendivil, 2007). The training process is carried out until the sum of squared differences between the predicted and the reference values is minimized (Borggaard, 2001). For small data sets, *K*-fold cross-validation is used to find the ANN model that generalizes well to new data sets. The cross-validation starts by fitting the model to the whole data set to obtain initial statistical values. Then the data is divided in *K* subsets, and the model is fitted repeatedly by leaving one *K* subset out at a time. The cross-validation averaged R^2 (CV- R^2) which is obtained is lower than the R^2 of the initial model (Anonymous, 2009). The ANN analysis was carried out with the JMP 8.0.2 software (SAS Institute Inc., Cary, NC, USA).

8.3.6.4. Evaluation of the Accuracy and the Precision of the Partial Least Squares and Artificial Neural Network Calibrated Models

A series of statistical calculations are necessary for evaluating the accuracy and the precision of the calibrated models. The difference between the reference (chemical index) and the predicted values (*d,* **Equation 8.1**) is the basis of the

calculation of the bias ($\bar{d}$, **Equation 8.2**), root mean square difference (RMSD, **Equation 8.3**), the standard error of performance (SEP, **Equation 8.4**), and the ratio of the standard deviation of the reference data (SD_x) to the SEP (RPD = SD_x / SEP). These are used in conjunction with the coefficient of determination (R^2) in order to compare the performance of different calibration models (Borggaard, 2001; Williams, 2001). The equations for the validation statistics are adapted from Williams (2001) in order to present them in a simplified form below. Where, *i = 1, ..., n;* and *n* is the number of experimental samples; *x* represents the reference values measured by HPLC (chemical indexes) and *y* represents the predicted values. Other statistical indices used for the evaluation and the comparison of predictive models are the accuracy factor (AF, **Equation 8.5**) and the bias factor (BF,

Equation 8.6) (Hossain et al., 2011); these equations were developed by Ross (1996).

$$d_i = x_i - y_i \qquad \textbf{Equation 8.1}$$

$$\bar{d} = \frac{\sum_{i=1}^{n} d_i}{n} \qquad \textbf{Equation 8.2}$$

$$SEP = \sqrt{\frac{\sum_{i=1}^{n}(d_i - \bar{d})^2}{n-1}} \qquad \textbf{Equation 8.3}$$

$$RMSD = \sqrt{\frac{\sum_{i=1}^{n} d_i^2}{n-1}} \qquad \textbf{Equation 8.4}$$

$$AF = 10^{\frac{\sum_{i=1}^{n}\left|\log\left(\frac{y_i}{x_i}\right)\right|}{n}} \qquad \textbf{Equation 8.5}$$

$$BF = 10^{\frac{\sum_{i=1}^{n}\log\left(\frac{y_i}{x_i}\right)}{n}} \qquad \textbf{Equation 8.6}$$

8.4. Results and Discussion

8.4.1. Ordinary Least Squares Regression and Comparison of the UV-Vis Light Absorption Data of Six Flaxseed Cultivars

One OLS linear regression line was obtained for each microwave-assisted extract of flaxseed cultivar using the UV-Vis light absorption data recorded for FC reactions replicated three times at three levels of SDG concentrations (nM SDG/ml FCR). The regression equations, with $R^2 > 0.995$, were used to predict the absorption data for 6.45, 7.86 and 9.27 nM SDG/ml FCR concentrations, which correspond to the SDG range common to all the FC reactions. This step was necessary as the flaxseed cultivars used in this experiment have been previously classified in three distinctive groups of SDG concentration per gram of seed, based on the HPLC analysis of their respective microwave-assisted extracts (Nemes and Orsat, 2011a). The new data set contained 42 samples, and was used for comparing the flaxseed cultivars in terms of their light absorption at 289, 298, 343 and 765 nm. Two-way ANOVA with pair-wise comparison of means using the Tukey HDS test were used in order to establish the significance of differences among the six cultivars in terms of their UV-Vis light absorption capacity. The results are presented in **Table 8.1**.

The six flaxseed cultivars could be classified in three significantly different groups, with some degree of overlap, based on their light absorbance behaviour. The intensity of light absorption decreased significantly with the increasing of the wavelength. It is interesting that the highest light absorption intensity was not observed for the cultivar with the highest SDG content (09LS01) but for a cultivar classified in the low SDG group (McBeth). This indicated that there were significant interferences from other compounds that absorbed UV light at 289, 298 and 343 nm, and also had the capacity to interact with the FC reagent.

Table 8.1. Classification of the flaxseed cultivars according to their UV-Vis light absorption behaviour and their SDG content

Flaxseed cultivar	[1]Classification of mean UV-Vis light absorbance for 7.86 nM SDG/ml FCR				[2]Classification of mean SDG content (mg/g seed)
	289 nm	298 nm	343 nm	765 nm	
McBeth	1.450 A	1.225 A	0.325 A	0.292 A	14.9 C
Flanders	1.389 AB	1.170 AB	0.302 B	0.274 B	16.6 B
Prairie Blue	1.357 B	1.148 B	0.300 B	0.273 B	14.6 C
CRGL 8.2	1.353 B	1.148 B	0.307 AB	0.280 AB	16.5 B
09LS01	1.320 BC	1.116 BC	0.295 B	0.269 B	18.9 A
CDC Bethune	1.227 C	1.040 C	0.273 C	0.252 C	16.7 B

[1]The grouping letters were assigned based on significant differences between the means at $p < 0.05$.

[2]The data is from Nemes and Orsat (2011a).

Compounds that could exhibit such UV-Vis behaviour, and not be part of the lignan macromolecule, include: trans-ferulic acid, trans-sinapic acid, trans-*p*-coumaric acid, trans-caffeic acid, trans-hydroxybenzoic, and syringic acid. These phenolic acids were reported to vary in flaxseed from 800 to 1000 mg/100g of seeds, depending on the cultivar (Shahidi and Naczk, 2004b). Phenolic (hydroxycinnamic) acids are known to absorb maximum UV light in the ranges of 290 - 300 nm (Shahidi and Naczk, 2004a) and 320 - 355 nm (Naczk et al., 2002).

It was hypothesised that the same components caused the absorption interferences at all 4 wavelengths as the absorption intensities were highly autocorrelated among wavelengths. This was proved by the coefficients of correlation $r \geq 0.994$, which were highly significant ($p < 0.0001$) (**Figure 8.1**). No clear pattern could be observed among the classifications of flaxseed cultivars based on their light absorbance behaviour and SDG content; the group with medium light absorbance intensity included cultivars from the three categories of SDG content.

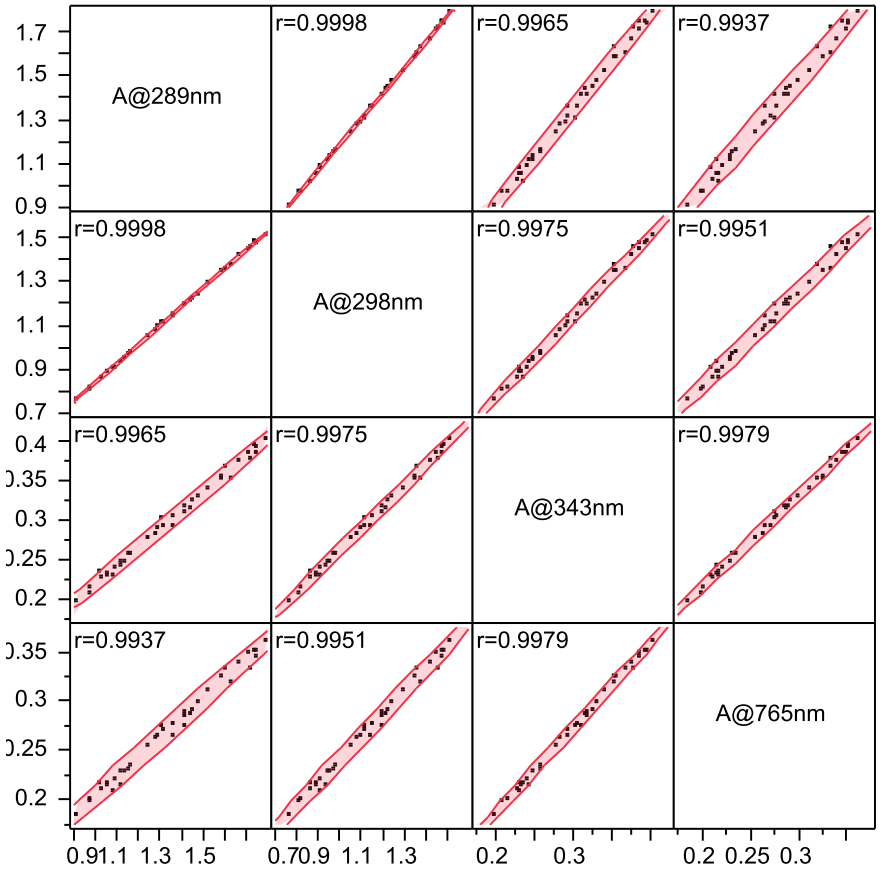

Figure 8.1. Scatter-plot matrix of 95 % bi-variate normal density ellipses of correlations between each pair of light absorption intensities

Example of interpretation – the 2nd cell on the 1st row shows the correlation between the absorption at 298 nm (A@298nm), with the values on the x axis, and the absorption at 289 nm (A@289nm), with the values on the y axis; the coefficient of correlation r = 0.9998 indicates a strong, positive, linear relationship between the two variables.

The cultivar McBeth was consistently classified in the highest light absorbance intensity group (A); and exhibited similar behaviour with the cultivar Flanders at 289 and 298 nm, and with the cultivar CRGL 8.2 at 343 and 765 nm. The cultivar CDC Bethune was consistently classified in the low light absorbance intensity group (C), and had similar behaviour with the cultivar 09LS01 at 289 and 298 nm. This dataset was composed of varied samples; it had at least two identifiable sources of variation, the SDG content and the phenolic acids composition, both being dependent on the cultivar; thus, it was considered as being a good candidate

for the fitting of calibration predictive models that could generalize to a larger population of flaxseed cultivars. For the calibration of predictive models it is required to use datasets composed of samples that have as much inherent variation as possible in order to account for all possible sources of variation (Davis et al., 2007) without having to quantify any other components except for the compounds of interest (Gowda et al., 2009). Datasets with strong autocorrelations among absorption intensities at different wavelengths are good candidates for the calibration of predictive models using PLS and ANN analyses (Dias et al., 2009; Mutihac and Mutihac, 2008).

8.4.2. Calibration of the Partial Least Squares Regression Model for Predicting the Secoisolariciresinol Diglucoside Content of Extracts from Six Flaxseed Cultivars

The data set containing 42 samples with SDG contents at three levels of concentrations (6.45, 7.86 and 9.27 nM SDG/ml FCR) was used for the calibration of the PLS model. The UV-Vis absorption data was centered (mean subtracted from each observation) and scaled (divided by the standard deviation). Initially, a model was fitted (4 latent vectors selected based on cross validation, R^2 = 0.942) and the data set was analysed for the presence of outliers using a residual by predicted variables plot. Three data points were detected as outliers; they originated from extracts of CDC Bethune at 7.86 and 9.27 nM SDG/ml FCR, and Prairie Blue at 9.27 nM SDG/ml FCR. After the outliers were eliminated, the data set was left with 39 samples and the PLS model was fitted again. The number of latent vectors was chosen based on cross-validation; the statistics are shown in **Table 8.2**. Only three latent vectors were necessary for explaining the totality of the variation in the predictor variables (UV-Vis absorption at the four wavelengths). However, by considering the 4^{th} latent vector significant gain was recorded for the explanation of the variation in the response variable. The choice of including the 4^{th} latent vector is supported by the lowest root mean square error

of 0.225 and the highest R^2 = 0.961. Thus, four latent vectors were used for the construction of the PLS model.

Table 8.2. Statistics of cross-validation of the PLS regression model

Number of latent vectors	Cumulative R^2 for the predictive variables	Cumulative R^2 for the response variable	Root mean square error of cross-validation
1	0.998	0.925	0.285
2	0.999	0.940	0.263
3	1.000	0.953	0.236
4	1.000	0.961	0.225

The variable importance for projection (VIP) statistic was used to assess if any of the four wavelengths was a candidate for deletion from the model (**Table 8.3**). VIP is the weighted sum of squares for the PLS weights calculated from the amount of variance of the response variable of each latent vector (Wold et al., 2001). Any variable with a VIP statistic value ≤ 0.8 (Wold's criterion) is a candidate for deletion (Anonymous, 2009). As it can be seen from **Table 8.3**, all predictor variables had a significant contribution to the predictive value of the model (VIP > 0.8). The value of the VIP statistic increased with the increasing of the wavelength.

Table 8.3. The VIP statistics for the absorbance at (A@) the 4 wavelengths data

Predictor variable	VIP statistic
A@289nm	0.992
A@298nm	0.996
A@343nm	1.025
A@765nm	1.028

The calibrated PLS regression model, shown in **Equation 8.7,** was used to predict the values of nM SDG/ml FCR for the 39 extracts of flaxseed cultivar samples. The relationship between the actual and the predicted SDG variables is presented

in **Figure 8.2** within a 95 % confidence ellipse. The two variables were significantly correlated ($p < 0.0001$) with r = 0.98.

$$nM\ SDG = 1.56279 - 29.7068 \times A@289nm + 47.58461 \times A@298nm - 73.46682 \times A@343nm + 51.49182 \times A@765nm$$

Equation 8.7

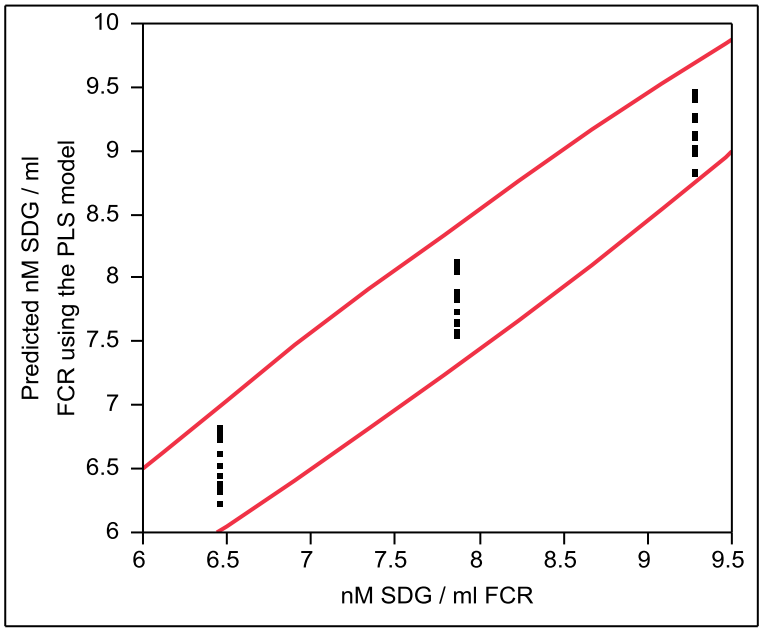

Figure 8.2. Plot of correlation of the actual vs. the predicted SDG variables for the PLS model, shown within a 95 % confidence ellipse

8.4.3. Calibration of Artificial Neural Network Model for Predicting the Secoisolariciresinol Diglucoside Content in Extracts from Six Flaxseed Cultivars

The predictor variables (A@ 289, A@298, A@ 343, A@765 nm; **Figure 8.3**) are scaled in the nodes (H1-H3, **Figure 8.3**) to have the mean equal to zero and the standard deviation equal to one, using the S-shaped (logistic) function from **Equation 8.8**. Where, S is the logistic function used to calculate the hidden

nodes; A denotes the linear combinations of the predictor variables (UV-Vis light absorption at the four wavelengths), and e ≈ 2.718.

$$S(A) = \frac{1}{1+e^{-A}}$$ **Equation 8.8**

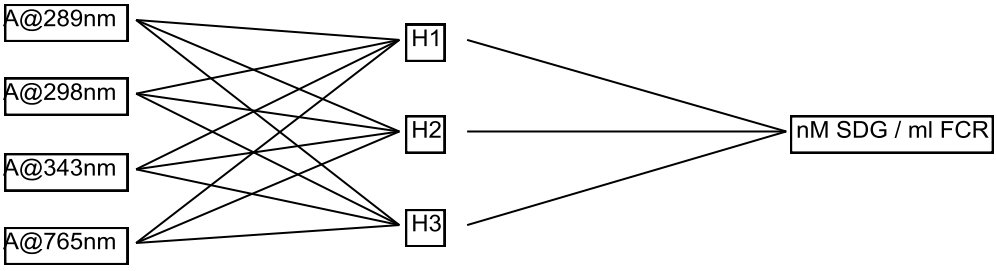

Figure 8.3. Diagram of the architecture of the ANN model

The 42 samples data set was used to fit an initial ANN model, using a sequence of fits with the number of hidden nodes ranging from 1 to 4, and 5-fold cross-validation, in order to search the data set for the presence of outliers. The initial ANN with the highest R^2 = 0.977 and CV-R^2 = 0.945 had 3 hidden nodes. Three outliers were identified, using a plot of the residual vs. predicted variables, which originated from the cultivar CDC Bethune with levels of SDG of 7.86 and 9.27 nM/ml FCR, and the cultivar McBeth with 7.86 nM SDG/ml FCR. After the elimination of outliers, the data set contained 39 samples. Another sequence of fits was carried out for a number of nodes ranging from 1 to 4 and using 5-fold cross-validation. The number of nodes was chosen to be three based on the model with the highest R^2 = 0.995 and CV-R^2 = 0.988. The three nodes H1, H2, and H3 from the predictive model (**Equation 8.12**) were calculated by replacing the A in **Equation 8.8** with the A1 from **Equation 8.9**, the A2 from **Equation 8.10** and the A3 from **Equation 8.11**, respectively.

$$A1 = 35.83448 - 5.13724 \times A@289nm - 7.28726 \times A@298nm - 21.34385 \times A@343nm - 40.69131 \times A@765nm$$

Equation 8.9

$$A2 = 29.33599 - 5.23391 \times A@289nm - 7.0961 \times A@298nm - 20.95087 \times A@343nm - 39.88199 \times A@765nm$$

Equation 8.10

$$A3 = -14.22387 + 3.07078 \times A@289nm + 2.16216 \times A@298nm + 18.90184 \times A@343nm + 7.17884 \times A@765nm$$

Equation 8.11

$$nM\ SDG = 11.89264 - 2.70105 \times H1 - 2.7077 \times H2 - 2.65284 \times H3$$

Equation 8.12

The ANN calibrated model from **Equation 8.12** was used to predict the SDG content (nM/ml FCR) of the six flaxseed cultivars. The correlation between the actual vs. predicted values (**Figure 8.4)** indicates that there is a strong linear relationship between the two variables with $r = 0.997$ and $p < 0.0001$.

8.4.4. Evaluation of the Accuracy and the Precision of the Partial Least Squares and the Artificial Neural Network Calibrated Models

A series of statistical indices were proposed by Williams (2001) and Ross (1996) to evaluate the performance of predictive models, and for comparing different calibrated models in terms of their predictive qualities. The bias ($\bar{d}$) is the mean error (the mean of differences between the reference and the predicted values). The SEP is the standard error of performance (the standard deviation of errors). The RMSD is the root mean square of differences (also known as RMSE). The lower are the values of the bias, SEP and RMSD, the better is the performance of a given model by comparison with other models.

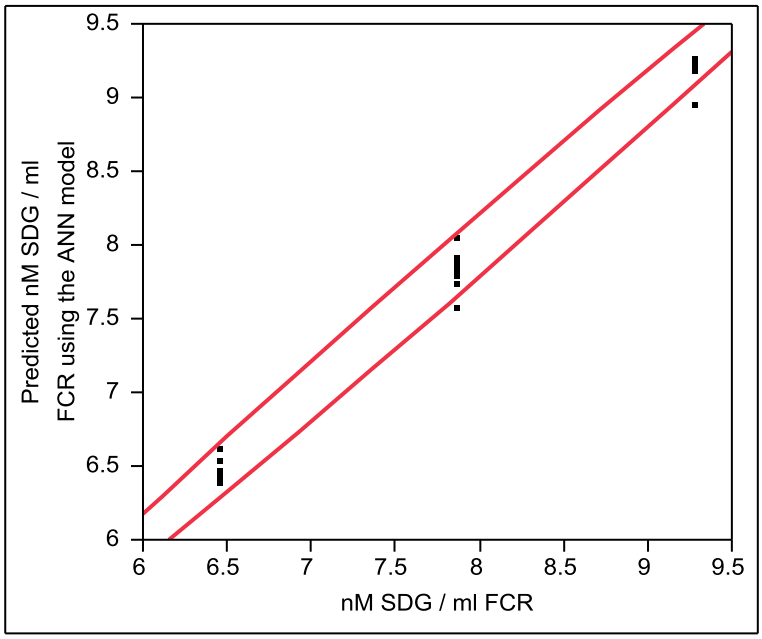

Figure 8.4. Plot of correlation of the actual vs. the predicted SDG variables for the ANN model, shown within a 95 % confidence ellipse

The RMSD estimates the standard deviation to be associated with future predictions; not being corrected for bias, the RMSD has to be presented alongside with the bias values, which show the average amount (the offset) by which the predictions differ from the mean reference values (Williams, 2001). If the bias is close to zero, the RMSD = SEP, which means the model does not have the tendency to over- or under-estimate the predicted values, as the positive and negative errors cancel each other out. Williams (2001) developed a scale of RPD indices for the classification of calibrated predictive models according to their performance. The RPD relates the standard deviation of the predictions to the standard deviation of the reference samples; the higher are the values of RPD, the better is the predictive quality of the model. RPD values of ≥ 5 are ideal, but values of ≥ 3 are acceptable for applications of the model for very rough screening applications. The recommended application of the calibrated predictive models based on their RPD values include: quality control applications, 5 - 6.4; process control, 6.5 - 8, and any applications for RPD values ≥ 8.1. Ross (1996)

developed the formulas for the accuracy (AF) and the bias (BF) factors in order to provide the possibility of using simple multiplicative factors that indicate the spread of the predicted values about the reference values, and the overall trend of over- or under-estimation of predictions, respectively. Ideally, the AF and the BF would be equal to 1. The statistical indices for the PLS and ANN calibrated models for predicting the content of SDG in flaxseed cultivars extracts are presented in **Table 8.4**.

Table 8.4. Statistical indices for the evaluation and the comparison of performances of the PLS and ANN calibrated models for predicting the SDG contents in flaxseed cultivar extracts

Model	Bias	RMSD	SEP	RPD	AF	BF	R^2
PLS	3.05E-15	0.231	0.231	5.03	1.026	1.000	0.961
ANN	4.22E-5	0.087	0.087	13.7	1.007	1.000	0.995

The bias was negligible for both models indicating that the fits were accurate, and that the results were predicted equally below and above the reference SDG values, as indicated by the BFs values of 1. It is difficult to estimate an interval of deviation of future predictions about the reference values using the RMSD and SEP. However, such an estimate was provided by the AF, which indicated that predicted SDG values for future samples will be contained within ± 2.6 % and ± 0.7 % of the reference SDG values for the PLS and ANN models, respectively. The smaller values for RMSD and SEP of the ANN model confirmed that the interval for predictions was narrower and more accurate than that of the PLS model. The coefficient of determination was higher for the ANN model. Overall, the ANN model was more accurate and precise than the PLS model. Based on the guidelines and recommendations from Williams (2001), the PLS model ($RPD \geq 5$, $R^2 \geq 0.96$) is recommended for most applications in the food industry and quality control requiring the quantification of SDG in flaxseed cultivars. The ANN predictive model has an excellent RPD value of 13.7, and $R^2 > 0.99$ which indicates that the model can be used for any type of applications. It was hypothesised that, whether the chemical index data and their respective UV-Vis

light absorptions exhibited linear relationships, the light absorption interferences were be better controlled by the non-linear functions of the ANN regression model as previously suggested by Borggaard (2001).

8.4.5. Possible Applications for the Partial Least Squares and Artificial Neural Network Calibrated Models for Predicting the Secoisolariciresinol Diglucoside Contents in Various Flaxseed Cultivars

Based on the results presented above, it can be concluded that the PLS and ANN calibrated models are very useful for rapidly and accurately predicting the SDG content of extracts from flaxseed cultivars without requiring oil removal and the use of an HPLC for quantification. However, the accuracy (closeness of prediction to the actual result) and the precision (degree of reproducibility of the result) depend on the accuracy and the precision of the extraction method. The MAE method used in this experiment was reported to be accurate (recovery of SDG 97.5 %), precise (coefficients of variation for different operators over time < 4.03 %), and repeatable (coefficients of variation for a single operator within a day < 1 %). In addition, it was recommended for routine SDG analysis in flaxseed cultivars, and possibly for generating lignan values to be included in the quality data tables for flaxseed (Nemes and Orsat, 2011a). Nonetheless, the MAE method requires a research-dedicated microwave extraction unit that might not be available in smaller laboratories. For such situations, it is recommended to extract the SDG from flaxseed cultivars using the direct hydrolysis method (1h hydrolysis with stirring at room temperature with 1 m NaOH) reported by Eliasson et al. (2003) coupled with the subsequent preparation steps reported in this paper. It is worth noting that the extraction yield achieved by the direct hydrolysis method was 6 % lower than that obtained with the MAE method (Nemes and Orsat, 2011b). It is hypothesised that both PLS and ANN models will generalize well for future UV-Vis light absorption data of various flaxseed cultivars, as the dataset used for the calibration of these models included at least two identifiable sources

of variation: different cultivars with significantly different SDG contents, and significantly different UV-Vis light absorption behaviour related to unidentified components (probably phenolic acids). It is possible that yellow-seeded flaxseed cultivars exhibit a UV-Vis light absorption behaviour similar to the brown-seeded cultivars studied here. Thus, PLS and ANN regression models could be calibrated using mixed data sets of yellow- and brown-seeded cultivars. Ideally, the data set would be periodically updated with chemical indexes for SDG and their corresponding UV-Vis light absorption data for extracts of flaxseed cultivars grown at various locations in different years, in order to recalibrate the PLS and ANN models for accounting for new sources of variation. Regularly, re-calibrated PLS and ANN models could be created in advanced research laboratories and made available for a cost to collaborators in the flaxseed processing industries.

8.4.6. Calibration of Artificial Neural Network Models Using Mixed Datasets Composed of Extracts from Flaxseed Cultivars, Defatted Flaxseed Meal and Flax Hull

It was hypothesised that both the flaxseed embryo and oil components contained compounds that interfered with the UV-Vis light absorbance of SDG. It would be interesting to know if defatted and non defatted flaxseed extracts can be predicted by the same model, or if the absence of the embryo requires separate calibration models for flax hulls. In order to investigate these aspects, UV-Vis light absorption data was acquired for FC reactions replicated three times per level of dilution for defatted microwave-assisted extracts of the six flaxseed cultivars, DFM and FH in the ranges of 16.5 - 23.5, 8 - 12, and 9 - 13.5 nM SDG/ml FCR, respectively.

A new mixed dataset was composed by combining all the samples of flaxseed extracts (total 103) and PLS and ANN analyses were carried out. No satisfactory PLS calibration model could be obtained (R^2 = 0.88) with the inclusion of FH. A very complex ANN model could be established for this data set; the model had 7

nodes, R^2 = 0.963, bias = 2.7E-4, RMSD = 0.751, SEP = 0.751, RPD = 5.22, BF = 1.005, and AF = 1.069. Although, the RPD value indicated that the model was good; the RMSD value was too high; the BF value showed a tendency for overestimation of results; and the AF value indicated that the future predictions would tend to fall within an interval of ± 6.9% around the measured SDG values. Due to the complexity of the model formula, and the unsatisfactory accuracy and precision statistical indices, the predictive model equation is not shown. Although, it is possible that with a bigger and more varied data set an acceptable model could be obtained, it is concluded that the model obtained here is not recommended for predictions of SDG content in mixed datasets.

From repeated tests it was observed that acceptable calibration models could be established for mixed datasets only if these were composed of samples that had only one source of UV-Vis light absorption interference in common, the oil or the embryo. For example, FH contains about 23 % oil (Oomah and Sitter, 2009) and only traces of embryos, so it can be combined with DFM and flaxseed cultivars defatted after the extraction which contain the embryos but are oil free. Also, flaxseed cultivars with an average oil content of 46 % (Barthet, 2009) can be combined with cultivars defatted before or after the extraction. In general better results were obtained with ANN models; the PLS models left as much as 20 % of the variation unexplained. This confirmed the hypothesis of Borggaard (2001) that spectral interferences are not necessarily described by linear models but rather by the combinations of linear with logistic functions of the ANN models.

An ANN model was calibrated for a mixed dataset of 48 samples of FC extracts of flaxseed cultivars defatted after the MAE, DFM and FH. The equations for calculating the predictions are given below. The A in **Equation 8.8** were replaced by those from **Equation 8.13**, **Equation 8.14** and **Equation 8.15** in order to calculate the nodes H1, H2 and H3 for using in the predictive model of **Equation 8.16.**

$$A1 = -13.92667 + 5.57382 \times A@289nm + 4.89803 \times A@298nm - 4.25382 \times A@343nm - 7.26985 \times A@765nm$$

Equation 8.13

$$A2 = 42.22948 - 10.23271 \times A@289nm - 4.43428 \times A@298nm - 4.66507 \times A@343nm - 33.69431 \times A@765nm$$

Equation 8.14

$$A3 = -4.6156 - 6.22817 \times A@289nm - 1.56764 \times A@298nm + 24.3082 \times A@343nm + 23.25835 \times A@765nm$$

Equation 8.15

$$nM\ SDG = 17.46868 - 22.98626 \times H1 - 12.07957 \times H2 + 18.85391 \times H3$$

Equation 8.16

An ANN model was calibrated for a mixed dataset of 63 samples of FC extracts from DFM and non-defatted flaxseed cultivars. The A in **Equation 8.8** were replaced by those from **Equation 8.17**, **Equation 8.18** and **Equation 8.19** in order to calculate the nodes H1, H2 and H3 for using in the predictive model of **Equation 8.20**.

$$A1 = -32.3043 + 5.46943 \times A@289nm + 5.81772 \times A@298nm + 6.70287 \times A@343nm + 32.10697 \times A@765nm$$

Equation 8.17

$$A2 = 6.16739 - 0.19852 \times A@289nm - 1.15636 \times A@298nm - 0.38401 \times A@343nm - 6.54275 \times A@765nm$$

Equation 8.18

$$A3 = 6.8619 + 0.292108 \times A@289nm - 1.57282 \times A@298nm - 5.94434 \times A@343nm - 9.85221 \times A@765nm$$

Equation 8.19

$$nM\ SDG = 16.89449 - 3.51572 \times H1 - 5.41468 \times H2 - 5.66866 \times H3$$

Equation 8.20

An ANN model was calibrated for a mixed dataset of 80 samples composed of FC extracts of defatted and non-defatted flaxseed cultivars, and DFM. The A in **Equation 8.8** were replaced by those from **Equation 8.21**, **Equation 8.22** and **Equation 8.23** in order to calculate the nodes H1, H2 and H3 for using in the predictive model of **Equation 8.24**.

$$A1 = 8.48941 - 9.92835 \times A@289nm + 3.77605 \times A@298nm + 22.57026 \times A@343nm - 18.92564 \times A@765nm$$

Equation 8.21

$$A2 = 4.39172 + 1.65512 \times A@289nm - 2.76991 \times A@298nm - 7.06012 \times A@343nm - 0.09243 \times A@765nm$$

Equation 8.22

$$A3 = 9.21193 + 0.14146 \times A@289nm - 5.55857 \times A@298nm - 8.36948 \times A@343nm + 0.64915 \times A@765nm$$

Equation 8.23

$$nM\ SDG = 13.17942 + 19.55285 \times H1 - 11.4104 \times H2 - 15.81038 \times H3$$

Equation 8.24

The statistical indices for evaluating the accuracy and the precision of the ANN models (**Equation 8.16**, **Equation 8.20** and **Equation 8.24**) for predicting the SDG content of flaxseed extracts from mixed datasets are presented in **Table 8.5**. The RPD values indicate that all three models are good for quality control applications. They had the tendency to overestimate the results by 0.1 - 0.2 % (BF); and the future predictions will tend to fall within intervals of ± 2.9 - 4.9 % (AF) around the measured SDG values. It is worth noting that the models were constructed with SDG/UV-Vis light absorption data obtained from batches of DFM and FH that were homogeneous and did not account for variations due to cultivar differences.

Table 8.5. Statistical indices for evaluating the accuracy and the precision of the ANN models to predict the SDG content in flaxseed extracts from mixed datasets

Model	Bias	RMSD	SEP	RPD	AF	BF	R^2
ANN, Equation 8.16	-2.79E-5	0.402	0.002	10.1	1.029	1.002	0.990
ANN, Equation 8.20	-1.98E-5	0.230	0.230	5.72	1.029	1.001	0.970
ANN, Equation 8.24	1.34E-5	0.568	0.568	7.25	1.049	1.002	0.981

The ANN model from **Equation 8.16** can be used for screening mixed samples of defatted flaxseed cultivars (oil removal before or after the MAE) and FH according to their SDG content. The ANN model from **Equation 8.20** can be used for screening mixed samples of DFM and non-defatted flaxseed cultivars. The ANN model from **Equation 8.24** can be used for screening samples of DFM and flaxseed cultivars with/without oil removal after the MAE. Based on these results, two rules were devised for creating mixed data sets of flaxseed samples in view of calibrating ANN models; (1) embryo-free samples, such as FH, could be combined only with oil-free embryo-containing samples; (2) embryo-containing samples could be mixed regardless of their oil content.

8.4.7. Possible Applications for the Artificial Neural Network Predictive Models Calibrated with Mixed Datasets of Flaxseed Extracts

The results presented above demonstrated that the SDG content could be predicted by the same model in flaxseed extracts with very different compositions, as long as they shared only one source of UV-Vis light absorption interference, e.g., the oil or the embryos. It was not necessary to know the exact oil content of the samples, as well, as it was not necessary to know the nature and the quantity of any other interfering compounds. Therefore, it can be extrapolated that ANN models could be calibrated for mixed datasets of brown and yellow flaxseed cultivars, and defatted meals and hulls derived from them. Such models would be applicable in the food industry, where flaxseed enters the composition of various functional food formulations; and in the natural health products industry, that uses flaxseed to extract SDG lignan – a nutraceutical. The models could be used to screen various sources of flaxseed in order to select the best raw materials from the point of view of lignan content/price, without having to use expensive analytical methodologies that require advanced chromatographic analysis. Moreover, the task could be performed by personnel without high level of analytical training. Such predictive models could have great many possibilities of applications even for advanced analytical laboratories, which could construct huge SDG/UV-Vis light absorption data bases and train ANN predictive models, which could be made available to business collaborators for a cost. The models would be updated regularly by inclusion of new data.

8.4.8. UV-Vis Light Absorption Guidelines for Applying the Partial Least Squares Regression and Artificial Neural Network Models for Predicting the Content of Secoisolariciresinol Diglucoside in Various Flaxseed Extracts

For future applications of the calibrated models for predicting the SDG content (nM/ml FCR) in various flaxseed extracts care must be taken to use only UV-Vis light absorption data that falls within the ranges shown in **Table 8.6**.

Table 8.6. UV-Vis light absorption ranges of the calibrated models for prediction of SDG contents in various flaxseed extracts

Model	A@289nm	A@298nm	A@343nm	A@765nm
PLS, Equation 8.7 ANN, Equation 8.12	0.911 - 1.787	0.767 - 1.511	0.198 - 0.401	0.186 - 0.360
ANN, Equation 8.16	0.927 - 2.945	0.782 - 2.536	0.202 - 0.673	0.192 - 0.590
ANN, Equation 8.20 ANN, Equation 8.24	0.911 - 2.945	0.767 - 2.536	0.198 - 0.673	0.186 - 0.590

8.4.9. Investigation of the Possibility of Using Standard Equivalents for Predicting the Secoisolariciresinol Diglucoside Content in Flaxseed Cultivar Extracts

OLS regression equations were established for the GA (15.7 - 35.4 nM/ml FCR), FA (11.7 - 23.4 nM/ml FCR), SDG (4.4 - 14.6 nM/ml FCR), and SECO (5.2 - 16.6 nM/ml FCR) standards using their light absorbance at 765 nm following the FC assays. The R^2 was greater than 0.99 in all cases. Since the standards were pure, there were no light absorbance interferences to be corrected; thus it was not necessary to carry out PLS and ANN regressions including absorbance data at 289, 298, and 343 nm in addition to 765 nm. It was observed that FA was a good predictor for SDG and SECO on an equimolar basis (1 nM FA = 1 nM SDG, 1 nM FA = 1 nM SECO) within the following ranges of concentrations: 11.5 - 14.2 nM SDG/ml FCR, and 10 - 13 nM SECO/ml FCR,

respectively. This suggests that the more expensive SDG and SECO standards can be replaced by the less expensive FA standard for expressing results on an FA equivalent basis. GA standard is recommended in general for quantifying the amounts of total phenolic compounds in foods in terms of GA equivalents (Ainsworth and Gillespie, 2007; Singleton et al., 1999). The GA and FA standard curves were used to calculate their respective equivalents in extracts of six flaxseed cultivars using the dataset from PLS analysis (39 samples). The AF and BF indices were calculated; the results showed that the GA and FA predictive models over estimated SDG in flaxseed cultivars 2.467- and 2.049-fold, respectively. The AF and BF are equal when there is constant overestimation of results. The GA and FA equivalents (GAE, FAE) regression equations were corrected by division with their corresponding AF values, as shown in **Equation 8.25** and **Equation 8.26**, respectively.

$$nM\ SDG_{GA} = \frac{1}{2.467} GAE = \frac{1}{2.467}(5.88959 + 48.71239 \times A@765nm)$$

Equation 8.25

$$nM\ SDG_{FA} = \frac{1}{2.049} FAE = \frac{1}{2.049}(3.46202 + 45.89305 \times A@765nm)$$

Equation 8.26

The statistical indices for evaluating the accuracy and the precision of **Equation 8.25** and **Equation 8.26** to predict the content of SDG in extracts of flaxseed cultivars based on their light absorbance at 765 nm following the FC assays were calculated and presented in **Table 8.7**. Overall, **Equation 8.26** was more appropriate for estimating the SDG contents of flaxseed cultivar extracts; the RPD = 3.93 indicates that the model is useful for screening applications; the future predictions will tend to fall within ± 3.3 % around the measured SDG value.

Table 8.7. Statistical indices for the evaluation and the comparison of performances of the corrected OLS regression models for predicting SDG in extracts of flaxseed cultivars

Model	Bias	RMSD	SEP	RPD	AF	BF	[1]R^2	[2]r
Equation 8.25	0.017	0.341	0.340	3.33	1.037	1.000	0.995	0.957
Equation 8.26	0.003	0.303	0.303	3.93	1.033	1.000	0.991	0.967

[1]The coefficients of determination were calculated for the uncorrected equations represented by the expressions in the brackets in the **Equation 8.25** and **Equation 8.26**.

[2]The coefficients of correlation were calculated for the corrected models.

8.4.10. Possible Applications of the Correction Factors for Ferulic Acid Equivalents and Gallic Acid Equivalent in Order to Estimate the Secoisolariciresinol Diglucoside Content of Extracts from Flaxseed Cultivars

The correction factors of 1/2.467 for GAE and 1/2.049 for FAE applied in **Equation 8.25** and **Equation 8.26**, respectively, should be used with caution. They might not generalise well to other data sets of flaxseed cultivars as they did not incorporate any corrections for light absorption interferences. As it was shown in **Table 8.1**, various flaxseed cultivars exhibited significantly different light absorption behaviours. Therefore, any future applications of these correction factors should consider comparing the results obtained with **Equation 8.25** and **Equation 8.26** with those obtained with **Equation 8.7** and/or **Equation 8.12**.

8.5. Conclusions

Accurate and precise PLS and ANN models were calibrated for predicting the SDG contents of various flaxseed cultivars. While the PLS model is useful for quality and process control applications in the flaxseed processing industry, the ANN model is suitable for any kind of applications due to its excellent statistical quality indices. More general ANN predictive models of interest for the functional

foods, nutraceutical and other flaxseed processing industries were calibrated for mixed datasets which allowed for SDG quantification in oil-free extracts of flaxseed meal and flaxseed cultivars, and non-defatted extracts of flaxseed cultivars and flax hulls samples. Two rules were coined for compiling mixed flaxseed datasets in view of ANN calibrations; (1) any flaxseed samples containing the embryo could be mixed regardless of their oil content; and (2) embryo-free flaxseed samples (flax hull) could be mixed with embryo-containing samples only if these were oil-free. By extrapolation, it is suggested that such predictive models could also be calibrated using mixed datasets of yellow and brown flaxseed cultivars, and their defatted meals and hulls products. Predictive models using corrected gallic acid and ferulic acid equivalents were generated for the estimation of the SDG content in various flaxseed cultivars. These simple models are recommended only for screening applications as they did not incorporate corrections for interfering compounds. In this chapter, it has been demonstrated that it was not necessary to know the nature and the contents of the compounds that interfered with the UV-Vis light absorption of SDG in various flaxseed extracts, as any interference could be overcome by the use of advanced chemometric techniques, and most specifically by the use of neural network modeling. The PLS and ANN models presented in this chapter allow for the rapid and accurate SDG quantification in various flaxseed samples without having to remove the oil or to use advanced analytical techniques or instruments such as HPLC.

Connecting Statement to Chapter 9

From **Chapter 3** to **Chapter 8**, recommendations were made for ensuring the successful replication of the optimized microwave-assisted extraction method in other laboratories, and complementary methods of extraction, purification and quantification of lignans were developed in order to fulfill the set objectives of this thesis (section **1.5**).

Chapter 9 concludes this thesis by summarizing the main results of the presented research, highlights the intended and possible applications of the developed methodologies, states the ways that the generated results contribute to the scientific knowledge, and enumerates the recommendations for future research.

Chapter 9 – Summary of Findings, General Conclusions, Contribution to Knowledge and Recommendations for Future Research

9.1. Actualization of the Microwave-Assisted Extraction Method for Lignans, and Recommendations for its Successful Replication

A previously developed optimized microwave-assisted extraction (MAE) method for flaxseed lignans (Nemes and Orsat, 2010, 2011b) was updated (**section 3.5.5**) in terms of translating the optimized power level (%), programmed in the control panel of the MAE instrument during method development experimentation, into actual microwave power (W) using the calibration in **Equation 4.2** presented in **Chapter 4**, section **4.4.1**. For ensuring the successful replication of the MAE method in other laboratories, recommendations were made in **Chapter 3** and **Chapter 4**.

Briefly, for replicating the MAE method, one needs to hydrolyse 0.5 – 1.6 g samples with 50 ml of 0.5 M NaOH at a programmed microwave power level of 156 W (corresponding to 22 % power level setting for the Star System 2 instrument in our laboratory), using an intermittent power delivery application that follows a pattern of 30 s on/off, for a total extraction time (encompassing the on/off time) of 3 min. These conditions cause the temperature of the extraction mixture to reach 67°C by the end of the extraction time (3 min). In the absence of accurate temperature monitoring during MAE (e.g., fiber optic in the presence of efficient stirring), the researcher should measure the final temperature of the extraction mixture with an accurate thermocouple (accuracy $\leq \pm$ 0.5°C) after briefly stirring the extract.

In order to ensure the success of the extraction, evaluated in terms of final temperature of the extraction mixture of 67°C, the researcher should work with

extraction mixture volumes that are within the range recommended by the manufacturer of the MAE system. In the eventuality that the volume of the extraction mixture has to be modified to comply with these recommendations, the researcher should use the microwave power density of 3.12 W/g extraction mixture (section **3.5.5**) for calculating the microwave power to be programmed into the MAE system. These recommendations are also valid for translating the optimized MAE method for flaxseed lignans to pilot scale for production purposes.

For a correct application of the microwave power level during extraction, one must consider calibrating the actual microwave power output as this is expected to differ significantly from the nominal power declared by the manufacturer of the MAE system. If, the MAE system is a mono-mode type, the calibration should be carried out according to the procedures developed for the mono-mode Star System 2 instrument (**Chapter 4**, section **4.3.1**). If the MAE system is a multi-mode type, the calibration could follow the procedures published by Swain et al. (2006) updated with guidelines from **Chapter 4**. Briefly, fresh loads of distilled water equilibrated at room temperature in the vessels should be used for each experiment. The heating time should be 45 s, the recorded temperature differences should be in the range of 3 to 25 °C, and the final water temperature should be ≤ 50°C. Only linear regression equations with non-significant lack of fit tests should be accepted.

9.2. Application of the Microwave-Assisted Method for the General Determination of Plant Lignans

The optimized and actualized MAE method can be applied for the general determination of lignans in plant matrices for analytical purposes, for generating lignan values for the flaxseed quality data tables, and for building databases of lignan contents in foods. As demonstrated in **Chapter 5**, the MAE method extracts the lignan from the plant matrix completely without formation of lignan

artefacts, is accurate (lignan recovery of 97.5 %), precise (coefficients of variation for different operators over time < 4.03 %), repeatable (coefficient of variation for a single operator within a day < 1%), and efficient (extraction yields 21.4 and 26.6 % higher than those obtained with two conventional reference extraction methods). For samples other than flaxseed, that require quantification in terms of lignan aglycones, the MAE method should be coupled with the enzymatic hydrolysis method presented in **Chapter 7**. For this purpose, 3 ml of buffered (0.01 M sodium acetate buffer, pH 5) microwave-assisted extract containing 100 mg sample, should be incubated for 48 h at 37°C with aliquots of crude solutions of β-glucuronidase in proportions of 40, 100 or more U enzyme/mg sample depending on the hydrolysing capacity of the enzyme, which should be tested for each new enzyme purchase (using 40, 100 or more U enzyme/mg sample for hydrolysing flaxseed extracts with known lignan content, section **7.4.2.2**). The released lignan aglycones are not stable in the enzymatic hydrolysis reaction conditions, thus, a variety of lignan aglycone standards are necessary for the chromatographic quantification (SECO, pinoresinol, lariciresinol, isolariciresinol, matairesinol, medioresinol, syringaresinol, and demethoxy-secoisolariciresinol, section **7.5**). The use of high performance liquid chromatography coupled with mass spectrometry is recommended when analysing samples other than flaxseed for the unambiguous identification of a variety of lignan aglycones.

The quantification of lignan in flaxseed cultivars, flax hull and defatted flaxseed meal can be done without using high performance liquid chromatography analysis by using the calibrated artificial neural network (ANN) and partial least squares (PLS) regression predictive models presented in **Chapter 8**. These models are useful for the food and functional food industries as accurate results (predicted values in the range of ± 0.67 to 4.85 % of the reference value; sections **8.4.4** and **8.4.6**) can be obtained fast, efficiently and at low cost. The required input values for using the ANN and PLS models presented in section **8.4** are the UV-Vis light absorption data of the microwave-assisted extracts recorded at 289, 298, 343, and 765 nm with a spectrophotometer, following the Folin-Ciocalteu's assay

(according to the methodology presented in section **8.3.5**). The UV-Vis light absorption data has to be within the ranges presented in **Table 8.6** in section **8.4.8** in order to correctly use the calibrated ANN and PLS models.

Two rules were coined for compiling datasets of UV-Vis and HPLC observations using mixed flaxseed samples (flaxseed cultivars, flax hull and defatted flaxseed meal) in view of calibrating general ANN predictive models (section **8.4.6**) for the benefit of the flaxseed processing, functional food and nutraceuticals industries. These rules are also useful for advanced research laboratories in order to calibrate and re-calibrate on a regular basis ANN predictive models for industrial customers.

Multiplication correction factors, to be used in conjunction with gallic acid equivalent (GAE) and ferulic acid equivalent (FAE) data recorded for microwave-assisted extracts following the Folin-Ciocalteu's assay, were computed (section **8.4.9**); these were 1/2.467 and 1/2.049 for GA and FAE, respectively. These correction factors are useful for transforming the much overestimated GAE and FAE data for flaxseed into more accurate lignan equivalents.

9.3. Purification of Microwave-Assisted Flaxseed Extracts by Solid Phase Extraction

A non-automated, affordable and accurate solid phase extraction (SPE) method was presented in **Chapter 6**, which can be used to obtain flaxseed lignan extracts free of the chemicals that were used for extraction. SPE purified extracts can be used for further experiments, such as testing the antioxidant activity and the stability of the lignan extracts during various storage conditions. Microwave-assisted extracts from 0.6 and 1.5 g defatted flaxseed meal can be used for SPE experiments in order to recover in the 10, 20 and 30 % ethanol pooled eluates 71.2 % (**Table 6.3**) and 60.6 % (section **6.4.5**), respectively, of the amount of lignan subjected to purification.

The extracts have to be prepared for SPE using the methodology presented in section **6.3.4.** It is important to adjust the pH of the extracts in two stages, 1st to pH 3 with sulphuric acid for facilitating the removal of water soluble proteins and carbohydrates by precipitation in order to prolong the life of the SPE funnel, and 2nd to pH 5 with sodium hydroxide in order to facilitate the retention of lignan by the packed SPE phase and reduce the lignan losses in the wash-water eluate.

The modeling of the recovery yields pooled according to three SPE elution groups was accurate, saved HPLC work time, and reduced the consumption of HPLC grade solvents (section **6.4.2** and **6.4.5**). These groups were defined as follows: the SPE elution group 1 includes the wash-water eluate and reflects the major lignan loss; the SPE elution group 2 pools the 10, 20 and 30 % ethanol eluates and reflects the major lignan recovery; and the SPE elution group 3 pools the 40, 50, and 100 % ethanol eluates and reflects the minor lignan loss.

The methodology of preparation of extracts in view of purification by SPE is useful for designing scale-up SPE purification processes in order to produce microwave-assisted flaxseed lignan extracts free of the chemicals that are used for extraction. Such extracts could be used for formulating functional foods enriched in lignan and for studying the health benefits of microwave-assisted flaxseed extracts.

9.4. General Conclusions

The novelty of the work presented in this thesis consists in the unique collection of accurate and efficient analytical methods for lignan determination [microwave-assisted extraction (MAE), enzymatic hydrolysis (EH), artificial neural network (ANN) and partial least squares (PLS) regression calibrated predictive models], and the affordable and reproducible methodology of purification of microwave-assisted flaxseed extracts by solid phase extraction (SPE). The optimized and actualized MAE method is proposed for the general quantification of

secoisolariciresinol diglucoside (SDG) in flaxseed cultivars, flax hull and defatted flaxseed meal. The MAE method can be used for adding lignan values to the flaxseed quality data tables, and for quantifying the lignan levels in flaxseed materials used in epidemiological and supplementation studies. For the general quantification of lignan aglycones in plants, the MAE method should be coupled with the EH method. The MAE-EH methodology can be used to construct databases of lignan contents in foods which are useful for correlating the intake of dietary lignans with observed health benefits such as protection against breast, prostate and colon cancer, risk reduction of developing cardiovascular diseases and diabetes. The ANN and PLS calibrated predictive models are accurate, easy to use and offer the alternative of quantifying the SDG content in flaxseed materials without having to use expensive chromatographic instruments and solvents as is the case with high performance liquid chromatography (HPLC). These predictive models are useful for researchers and for the flaxseed processing industry. The SPE purification method was developed to require minimum post-elution manipulation steps and to save HPLC work time and solvent. The SPE method can be used for producing purified flaxseed lignan extracts for further analytical experimentation and for designing simplified and efficient pilot scale applications for production purposes.

9.5. Contribution to Knowledge

This thesis was designed to contribute to the scientific knowledge by proposing solutions to specific needs and problems in the domains of extraction, quantification and purification of lignans nutraceuticals, of microwave-assisted extraction of natural compounds, and of value addition for the flax crop which is of great economical importance for Canada and the world.

Based on the literature reviewed in sections **2.4**, **2.5**, **2.6, 5.2** and **3.5.5** the following problem statement was formulated. There is a need for fast, efficient and accurate extraction and quantification methods that can be used for the

general determination of lignans in flaxseed materials and other plant samples. To fulfill these requirements, the following solutions were developed and proposed:

- Use the optimized and actualized microwave-assisted extraction (MAE) method described in section **3.5.5** and evaluated in **Chapter 5** as a generally applicable standardized method for the rapid, accurate and efficient extraction of lignans from flaxseed samples in view of quantification.

- Couple the standardized MAE method (using non-defatted samples) with the enzymatic hydrolysis method developed in **Chapter 7** for efficiently extracting lignan aglycones from plant samples in view of quantification.

- Couple the standardized MAE method with the Folin-Ciocalteu's assay, spectrophotometric measurements (section **8.3.5**), and the calibrated artificial neural network (ANN) and partial least squares (PLS) regression predictive models presented in sections **8.4.2**, **8.4.3** and **8.4.6** for the accurate, rapid and inexpensive quantification of lignan in flaxseed cultivars, flax hull and defatted flaxseed meal.

- Couple the standardized MAE method with the Folin-Ciocalteu's assay, spectrophotometric quantification in terms of gallic acid equivalents or ferulic acid equivalents (section **8.3.5**), and the multiplication correction factors presented in section **8.4.9** for the rapid and inexpensive screening of flaxseed cultivars according to their lignan content.

Based on the literature reviewed in section **6.2** the following problem statement was formulated. The efficiencies of the published solid phase extraction (SPE) methods for the purification of crude flaxseed extracts are not known. The following solution was developed and proposed:

- The SPE method developed in **Chapter 6** is simple, affordable, has known recoveries of flaxseed lignan, and is reproducible as proven by the results of the modeling of recovery patterns of purified compounds.

The collection of developed methodologies presented in this thesis offer possibilities for adding value to the flax crop and for developing profitable research projects:

- The MAE and SPE methods can be scaled up for producing value-added flaxseed ingredients rich in lignans that can be used as natural health products and as specialty ingredients for incorporation in functional food formulations.

- The MAE method in conjunction with the guidelines (section **8.4.6)** established for constructing sets of chemical indexes (HPLC data) and UV-Vis absorption data for mixed flaxseed materials, and ANN and/or PLS procedures, can be used by advanced research laboratories for calibrating and re-calibrating on a regular basis ANN and/or PLS predictive models for the benefit of customers in the flaxseed processing industry.

- The MAE method coupled with the EH method can be used by advanced research laboratories for building databases of lignan contents in foods for the benefit of nutritionists and medical researchers.

Based on the literature reviewed in **Chapter 3** and **Chapter 4** the following problem statement was formulated. It is often difficult to replicate the MAE methods reported in the literature due to missing information or to the ambiguity of the ways process parameters were controlled during MAE. In response to these problems, solutions were developed and proposed as follows:

- For designing efficient MAE processes, that do not use excessive microwave power densities, the power densities of 1.34, 3.35 and 5.36 W/g extraction mixture for processes that use solvents with good, medium and low microwave heating abilities, respectively, were proposed in section **3.7**.

- The successful development of new methods and the successful replication of published methods depend on the correct application of microwave power levels during MAE processes, which in turn depend on using calibration. An improved calibration method, which is specifically adapted for mono-mode MAE systems, was presented in section **4.3.1**, along with recommendation (section **4.4.3**) for the correct calibration of actual microwave power levels.

9.6. Recommendations for Future Research

The knowledge accumulated during the preparation of this thesis allows for making the following recommendations in view of further advancing the science of lignans extraction and analysis:

- General ANN models can be calibrated using datasets of HPLC and UV-Vis light absorption at 289, 298, 343, and 765 nm following the Folin Ciocalteu's assay, generated for microwave-assisted extracts from brown and yellow flaxseed varieties and their respective flax hulls and defatted meals. Thus, a single ANN model can be used to quantify the lignan contents of a great variety of flaxseed materials.

- For further reducing the costs and for obtaining more environmentally friendly calibrated predictive models, it is worth studying the possibility of obtaining ANN models for datasets of UV-Vis light absorption of crude

microwave-assisted extracts from a variety of flaxseed materials without using the Folin-Ciocalteu's assay.

- Value can be added to the flax crop by developing industrial processes that are fast, efficient, economical and environmentally friendly, by using microwave-assisted extraction to produce lignan-rich mucilage extracts. Such processes would use water as a solvent. By developing and optimizing such extraction processes using microwave heating, the extraction yields would be maximised and the extraction duration would be minimized, thus ensuring maximum productivity. The lignan-rich mucilage would confer health benefits such as nutritional dietary fibre, protection against colo-rectal cancer and decrease in the incidence of obesity [health benefits attributed to the mucilage component (Oomah, 2001)], in addition to the health benefits attributed to the lignan component which were mentioned in section **2.3** (e.g., risk reduction of developing breast cancer in post menopausal women, diabetes, cardiovascular diseases and colon cancer; reduced body mass index and total body fat in post menopausal women; antioxidant capacity and anti-inflammatory effects). The costs of producing the lignan rich mucilage would be low, as after extraction only filtration and spray drying would be required to obtain the final product (Oomah and Mazza, 2001). The purposes of producing lignan-rich mucilage by microwave-assisted extraction with water could encompass the development of nutraceutical dietary supplements and the production of specialty ingredients for formulating functional foods.

References

Abdi, H., 2007. Partial Least Square Regression, in: Salkind, N.J. (Ed.), Encyclopedia of Measurement and Statistics. Sage Reference, Thousand Oaks, CA, pp. 740-744.

Abe, K., Iino, T., Fujii, W., Suwa, Y., 2005. Process for producing sdg and foods and drinks containing the same, United States Patent and Trademark Office Pre-Grant Publication.

Adolphe, J.L., Whiting, S.J., Juurlink, B.H.J., Thorpe, L.U., Alcorn, J., 2010. Health effects with consumption of the flax lignan secoisolariciresinol diglucoside. Br J Nutr 103, 929-938.

Ainsworth, E.A., Gillespie, K.M., 2007. Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent. Nat. Protocols 2, 875-877.

Andjelkovic, M., Van Camp, J., Pedra, M., Renders, K., Socaciu, C., Verhé, R., 2008. Correlations of the phenolic compounds and the phenolic content in some Spanish and French olive oils. Journal of Agricultural and Food Chemistry 56, 5181-5187.

Anonymous, 2003. Acatris Develops Men's Health Product, www.nutraingredients-usa.com Breaking News on Supplements & Nutrition - North America. 2000-2009 Decision News Media SAS - All rights reserved.

Anonymous, 2008. Flaxseed Quality in Canada - Canadian Flax Quality Showns Long Term Consistency and Improvement Canadian Grain Commission, available on line at http://www.grainscanada.gc.ca/flax-lin/trend-tendance/qfc-qlc-eng.htm.

Anonymous, 2009. JMP 8 Statistical and Graphics Guide, 2nd ed. SAS Institue Inc., Cary, NC, USA.

Anonymous, 2011. Appendix C - Operating Procedures for the Registration of a Flax Cultivar for Production in Western Canada. Prairie Recommending Committee for Oil Seeds (PRCO), available on line at http://www.pgdc.ca/pdfs/oc/2011%20PRCO%20Operating%20Procedures%20Appendix%20C%20%28Flax%29.pdf March 2011, 1-18.

Attoumbré, J., Mahamane Laoualy, A.B., Bienaimé, C., Dubois, F., Baltora-Rosset, S., 2011. Investigation of lignan accumulation in developing Linum usitatissimum seeds by immunolocalization and HPLC. Phytochemistry Letters 4, 194-198.

Barthet, V.J., 2009. Quality of Western Canadian Flaxseed 2009 (available on line at www.grainscanada.gc.ca). Canadian Grain Commission, Winnipeg, MB, p. 14.

Bassett, C.M., Rodriguez-Leyva, D., Pierce, G.N., 2009. Experimental and clinical research findings on the cardiovascular benefits of consuming flaxseed. Applied Physiology, Nutrition and Metabolism 34, 965-974.

Beejmohun, V., Fliniaux, O., Grand, E., Lamblin, F., Bensaddek, L., Christen, P., Kovensky, J., Fliniaux, M.A., Mesnard, F., 2007. Microwave-assisted extraction of the main phenolic compounds in flaxseed. Phytochemical Analysis 18, 275-282.

Bélanger, J.M.R., Paré, J.R.J., 2006. Applications of microwave-assisted processes (MAP™) to environmental analysis. Analytical and bioanalytical chemistry 386, 1049-1058.

Bezerra, M.A., Santelli, R.E., Oliveira, E.P., Villar, L.S., Escaleira, L.A., 2008. Response surface methodology (RSM) as a tool for optimization in analytical chemistry. Talanta 76, 965-977.

Borggaard, C., 2001. Neural Networks in Near-Infrared Spectroscopy, in: Williams, P.C., Norris, K. (Eds.), Near-Infrared Technology in the Agricultural and Food Industries, 2nd ed. American Association of Cereal Chemists, Inc., St. Paul, Minnesota, USA, pp. 101-107.

Buck, K., Zaineddin, A.K., Vrieling, A., Linseisen, J., Chang-Claude, J., 2010. Meta-analyses of lignans and enterolignans in relation to breast cancer risk. Am J Clin Nutr 92, 141-153.

Buffler, C.R., 1993. Microwave cooking and processing : engineering fundamentals for the food scientist. Van Nostrand Reinhold, New York.

Cacace, J.E., Mazza, G., 2006. Pressurized low polarity water extraction of lignans from whole flaxseed. J Food Eng 77, 1087-1095.

Cheng, W.M., Raghavan, G.S.V., Ngadi, M., Wang, N., 2006. Microwave power control strategies on the drying process I. Development and evaluation of new microwave drying system. J Food Eng 76, 188-194.

Clark, W.F., Muir, A.D., Westcott, N.D., Parbtani, A., 2000. A novel treatment for lupus nephritis: lignan precursor derived from flax. Lupus 9, 429-436.

Collins, J.M., Leadbeater, N., 2007. Microwave energy: a versatile tool for the biosciences. Organic & biomolecular chemistry 5, 1141-1150.

Costa, L.M., Silva, F.V., Gouveia, S.T., Nogueira, A.R.A., Nóbrega, J.A., 2001. Focused microwave-assisted acid digestion of oils: an evaluation of the residual carbon content. Spectrochimica Acta Part B: Atomic Spectroscopy 56, 1981-1985.

Cui, W., Han, N.F., 2006. Process and apparatus for flaxseed component separation, Unites States Patent and Trademark Office Granted Patent.

Dai, J., 2006. Microwave-assisted extraction and synthesis studies and the scale-up study with the aid of FDTD simulation. PhD Thesis, Bioresource Engineering Department, McGill University, Ste-Anne-de-Bellevue, QC, Canada. Retreived from http://webpages.mcgill.ca/staff/deptshare/FAES/066-Bioresource/Theses/index.htm, p. 281.

Dai, J., Yaylayan, V.A., Raghavan, G.S.V., 2001. Influence of operating parameters on the use of the microwave-assisted process (MAP) for the extraction of azadirachtin-related limonoids from neem (Azadirachta indica) under atmospheric pressure conditions. Journal of Agricultural and Food Chemistry 49, 4584-4588.

Daniells, S., 2007. BioGin Confirms Water Solubility for 40pc SDG Flax Lignans, www.nutraingredients.com Breaking News on Supplements & Nutrition - Europe. 2000/2009 Decision News Media SAS - All rights reserved.

Daun, J.K., Barthet, V.J., Chornick, T.L., Duguid, S.D., 2003. Structure, Composition, and Variety Development of Flaxseed, in: Thompson, L.U., Cunnane, S.C. (Eds.), Flaxseed in Human Nutrition, Second Edition. AOCS Press, Champaingn, Illinois, pp. 1-40.

Davis, C.B., Markey, C.E., Busch, M.A., Busch, K.W., 2007. Determination of capsaicinoids in habanero peppers by chemometric analysis of UV spectral data. Journal of Agricultural and Food Chemistry 55, 5925-5933.

Decareau, R.V., Mudgett, R.E., 1985. Microwaves in the food processing industry. Academic Press, Orlando, Fla.

Desai, M., Parikh, J., Parikh, P.A., 2010. Extraction of natural products using microwaves as a heat source. Separation and Purification Reviews 39, 1-32.

Dias, L.G., Veloso, A.C.A., Correia, D.M., Rocha, O., Torres, D., Rocha, I., Rodrigues, L.R., Peres, A.M., 2009. UV spectrophotometry method for the monitoring of galacto-oligosaccharides production. Food Chemistry. 113, 246-252.

Diprose, M., 2001. Some considerations when using a microwave oven as a laboratory research tool. Plant and soil 229, 271-280.

Douaud, C., 2007a. ADM Enters Flax Lignan Market with NDI-Notified Beneflax, Www.nutraingredients-usa.com Breaking News on Supplements & Nutrition - North America. 2000/2009 Decision News Media SAS - All rights reserved.

Douaud, C., 2007b. BioGin Increases Flax Lignan Production Based on Demand, Www.nutraingredients-usa.com Breaking News on Supplements & Nutrition - North America. 2000/2009 Decision News Media SAS - All rights reserved.

Dweck, A.C., 2000. The folklore and cosmetic use of various Salvia species, in: Kintzisos, S.E. (Ed.), Sage the Genus Salvia. Harwood Academic Publishers, Amsterdam, pp. 1-26.

Eliasson, C., Kamal-Eldin, A., Andersson, R., Aman, P., 2003. High-performance liquid chromatographic analysis of secoisolariciresinol diglucoside and hydroxycinnamic acid glucosides in flaxseed by alkaline extraction. J Chromatogr A 1012, 151-159.

Eskilsson, C.S., Björklund, E., 2000. Analytical-scale microwave-assisted extraction. J Chromatogr A 902, 227-250.

FAOSTAT, 2012. FAO Satistics Division, Food and Agriculture Organization of the United Nations. http://faostat.fao.org/site/567/default.aspx#ancor.

Ford, J.D., Huang, K.S., Wang, H.B., Davin, L.B., Lewis, N.G., 2001. Biosynthetic Pathway to the Cancer Chemopreventive Secoisolariciresinol Diglucoside-Hydroxymethyl Glutaryl Ester-Linked Lignan Oligomers in Flax (Linum usitatissimum) Seed. J. Nat. Prod. 64, 1388-1397.

Frank, J., Eliasson, C., Leroy-Nivard, D., Budek, A., Lundh, T., Vessby, B., Aman, P., Kamal-Eldin, A., 2004. Dietary secoisolariciresinol diglucoside and its oligomers with 3-hydroxy-3-methyl glutaric acid decrease vitamin E levels in rats. Br J Nutr 92, 169-176.

Ganzler, K., Salgo, A., 1987. Microwave extraction: a new method superseding traditional Soxhlet extraction. Zeitschrift fur Lebensmittel-Untersuchung und -Forschung 84, 274-276.

Ganzler, K., Salgo, A., Valko, K., 1986. Microwave extraction : A novel sample preparation method for chromatography. J Chromatogr A 371, 299-306.

Gowda, N., Panghal, S., Vipul, K., Rajshree, M., 2009. Validation and application of high-performance liquid chromatographic and spectrophotometric methods for simultaneous estimation of nebivolol and hydrochlorothiazide: A novel approach to multivariate calibrations by R-Software Environment. J Aoac Int 92, 1356-1365.

Halliday, J., 2005. Acatris and ADM Form Flax Lignan Association, Www.nutraingredients-usa.com Breaking News on Supplements & Nutrition - North America. 2000/2009 Decision News Media SAS - All rights reserved.

Herrero, M.A., Kremsner, J.M., Kappe, C.O., 2008. Nonthermal Microwave Effects Revisited: On the Importance of Internal Temperature Monitoring and Agitation in Microwave Chemistry. The Journal of Organic Chemistry 73, 36-47.

Ho, C.H.L., Cacace, J.E., Mazza, G., 2007. Extraction of lignans, proteins and carbohydrates from flaxseed meal with pressurized low polarity water. LWT - Food Science and Technology 40, 1637-1647.

Hossain, M.B., Patras, A., Barry-Ryan, C., Martin-Diana, A.B., Brunton, N.P., 2011. Application of principal component and hierarchical cluster analysis to classify different spices based on in vitro antioxidant activity and individual polyphenolic antioxidant compounds. Journal of Functional Foods 3, 179-189.

Hosseinian, F.S., Beta, T., 2009. Patented techniques for the extraction and isolation of secoisolariciresinol diglucoside from flaxseed. Recent patents on food, nutrition & agriculture 1, 25-31.

Huie, C.W., 2002. A review of modern sample-preparation techniques for the extraction and analysis of medicinal plants. Analytical and Bioanalytical Chemistry 373, 23-30.

Hyvarinen, H.K., Pihlava, J.M., Hiidenhovi, J.A., Hietaniemi, V., Korhonen, H.J.T., Ryhanen, E.L., 2006. Effect of Processing and Storage on the Stability of Flaxseed Lignan Added to Bakery Products. J. Agric. Food Chem. 54, 48-53.

Jhala, A.J., Hall, L.M., 2010. Flax (Linum usitatissimum L.): Current Uses and Future Applications. Australian Journal of Basic & Applied Sciences 4, 4304-4312.

Johnsson, P., Kamal-Eldin, A., Lundgren, L.N., Aman, P., 2000. HPLC method for analysis of secoisolariciresinol diglucoside in flaxseeds. Journal of agricultural and food chemistry 48, 5216-5219.

Johnsson, P., Peerlkamp, N., Kamal-Eldin, A., Andersson, R.E., Andersson, R., Lundgren, L.N., Aman, P., 2002. Polymeric fractions containing phenol glucosides in flaxseed. Food Chemistry. 76, 207-212.

Kabza, K.G., Chapados, B.R., Gestwicki, J.E., McGrath, J.L., 2000. Microwave-Induced Esterification Using Heterogeneous Acid Catalyst in a Low Dielectric Constant Medium. The Journal of Organic Chemistry 65, 1210-1214.

Kamal-Eldin, A., Peerlkamp, N., Johnsson, P., Andersson, R., Andersson, R.E., Lundgren, L.N., Amar, P., 2001. An oligomer from flaxseed composed of secoisolariciresinoldiglucoside and 3-hydroxy-3-methyl glutaric acid residues. Phytochemistry 58, 587-590.

Kappe, C.O., 2004. Controlled Microwave Heating in Modern Organic Synthesis. Angewandte Chemie International Edition 43, 6250-6284.

Kappe, C.O., Dallinger, D., Murphree, S.S., 2009a. Chapter 2 Microwave Theory, in: Practical Microwave Synthesis for Organic Chemists. Wiley-VCH Verlag GmbH & Co. KGaA, pp. 11-44.

Kappe, C.O., Dallinger, D., Murphree, S.S., 2009b. Chapter 3 Equipment Review, in: Practical Microwave Synthesis for Organic Chemists. Wiley-VCH Verlag GmbH & Co. KGaA, pp. 45-85.

Kappe, C.O., Dallinger, D., Murphree, S.S., 2009c. Chapter 5 Starting With Microwave Chemistry, in: Practical Microwave Synthesis for Organic Chemists. Wiley-VCH Verlag GmbH & Co. KGaA, pp. 161-201.

Kaufmann, B., Christen, P., 2002. Recent extraction techniques for natural products: microwave-assisted extraction and pressurised solvent extraction. Phytochemical Analysis 13, 105-113.

Kingston, H.M., Jassie, L.B., 1986. Microwave energy for acid decomposition at elevated temperatures and pressures using biological and botanical samples. Analytical chemistry 58, 2534-2541.

Kitts, D.D., Yuan, Y.V., Wijewickreme, A.N., Thompson, L.U., 1999. Antioxidant activity of the flaxseed lignan secoisolariciresinol diglycoside and its mammalian lignan metabolites enterodiol and enterolactone. Molecular and Cellular Biochemistry 202, 91-100.

Klosterman, H.J., Smith, F., 1954. The Isolation of β-Hydroxy–β-Methylglutaric Acid from the Seed of Flax (*Linum usitatissimum)*. Journal of the American Chemical Society 76, 1229-1230.

Kosińska, A., Penkacik, K., Wiczkowski, W., Amarowicz, R., 2011a. Presence of Caffeic Acid in Flaxseed Lignan Macromolecule. Plant Foods for Human Nutrition (Formerly Qualitas Plantarum) 66, 270-274.

Kosińska, A., Urbalewicz, A., Penkacik, K., Karamać, M., Amarowicz, R., 2011b. SE-HPLC-DAD analysis of flaxseed lignan macromolecule and its hydrolysates. Polish Journal of Food and Nutrition Sciences 61, 263-271.

Kraushofer, T., Sontag, G., 2002. Determination of some phenolic compounds in flax seed and nettle roots by HPLC with coulometric electrode array detection. European Food Research and Technology 215, 529-533.

Krishnaswamy, K., Orsat, V., Gariépy, Y., Thangavel, K., 2012. Optimization of Microwave-Assisted Extraction of Phenolic Antioxidants from Grape Seeds (*Vitis vinifera*). Food and Bioprocess Technology. DOI:10.1007/s11947-012-0800-2.

Kubrakova, I.V., Toropchenova, E.S., 2008. Microwave heating for enhancing efficiency of analytical operations (review). Inorganic materials 44, 1509-1519.

Kuhnle, G.G.C., Dell'Aquila, C., Aspinall, S.M., Runswick, S.A., Joosen, A.M.C.P., Mulligan, A.A., Bingham, S.A., 2009. Phytoestrogen content of fruits and vegetables commonly consumed in the UK based on LC–MS and 13C-labelled standards. Food Chemistry. 116, 542-554.

Kuhnle, G.G.C., Dell'Aquila, C., Aspinall, S.M., Runswick, S.A., Mulligan, A.A., Bingham, S.A., 2008. Phytoestrogen Content of Beverages, Nuts, Seeds, and Oils. Journal of Agricultural and Food Chemistry 56, 7311-7315.

Lamblin, F., Hano, C., Fliniaux, O., Mesnard, F., Fliniaux, M.A., Lainé, É., 2008. Interest of lignans in prevention and treatment of cancers. Intérêt des lignanes dans la prévention et le traitement de cancers. Medicine/Sciences 24, 511-519.

Leadbeater, N.E., 2011. Microwave heating as a tool for sustainable chemistry. CRC Press, Boca Raton, FL.

Leardi, R., 2009. Experimental design in chemistry: A tutorial. Analytica Chimica Acta 652, 161-172.

Liao, X., Raghavan, G.S.V., Yaylayan, V.A., 2001. Dielectric properties of alcohols (C1-C5) at 2450 MHz and 915 MHz. Journal of Molecular Liquids 94, 51-60.

Lidström, P., Tierney, J., Wathey, B., Westman, J., 2001. Microwave assisted organic synthesis--a review. Tetrahedron 57, 9225-9283.

Liggins, J., Grimwood, R., Bingham, S.A., 2000. Extraction and Quantification of Lignan Phytoestrogens in Food and Human Samples. Analytical Biochemistry 287, 102-109.

Lundstedt, T., Seifert, E., Abramo, L., Thelin, B., Nystrom, A., Pettersen, J., Bergman, R., 1998. Experimental design and optimization. Chemometrics and Intelligent Laboratory Systems 42, 3-40.

Madhusudhan, B., Wiesenborn, D., Schwarz, J., Tostenson, K., Gillespie, J., 2000. A dry mechanical method for concentrating the lignan secoisolariciresinol diglucoside in flaxseed. Lebensmittel-Wissenschaft and Technologie. Food Science and Technology 33, 268-275.

Meda, V., Orsat, V., Raghavan, G.S.V., 2005. Microwave heating and the dielectric properties of foods, in: Regier, M., Schubert, H. (Eds.), The microwave processing of foods. Woodhead Publishing Ltd., Cambridge, England, pp. 61-75.

Mendivil, F., 2007. Artificial Neural Network, in: Salkind, N.J. (Ed.), Encyclopedia of Measurement and Statistics. Sage Reference, Thousand Oaks, CA, pp. 46-47.

Milder, I.E.J., Arts, I.C.W., Putte, B.v.d., Venema, D.P., Hollman, P.C.H., 2005. Lignan contents of Dutch plant foods: a database including lariciresinol, pinoresinol, secoisolariciresinol and matairesinol. Br J Nutr 93, 393-402.

Milder, I.E.J., Arts, I.C.W., Venema, D.P., Lasaroms, J.J.P., Wahala, K., Hollman, P.C.H., 2004. Optimization of a liquid chromatography-tandem mass spectrometry method for quantification of the plant lignans secoisolariciresinol, matairesinol, lariciresinol, and pinoresinol in foods. Journal of Agricultural and Food Chemistry 52, 4643-4651.

Mingos, D.M.P., 2005. Theoretical aspects of microwave dielectric heating, in: Tierney, J.P., Lidström, P. (Eds.), Microwave Assisted Organic Synthesis. Blackwell Publishing, pp. 1-21.

Moazzami, A., Kamal-Eldin, A., 2006. Sesame seed is a rich source of dietary lignans. Journal of the American Oil Chemists' Society 83, 719-723.

Moazzami, A.A., Andersson, R.E., Kamal-Eldin, A., 2006. HPLC Analysis of Sesaminol Glucosides in Sesame Seeds. Journal of Agricultural and Food Chemistry 54, 633-638.

Morisset, A.-S., Lemieux, S., Veilleux, A., Bergeron, J., John Weisnagel, S., Tchernof, A., 2009. Impact of a lignan-rich diet on adiposity and insulin sensitivity in post-menopausal women. Br J Nutr 102, 195-200.

Moses, M., Cousins, R., 1997. Advances in Nutrient Metals Digestion using Open and Closed Vessel Microwave Systems, in: Othen, L. (Ed.), Proceedings of the Second Conference of the Nutrition Advisory Group (NAG) of the American Zoo and Aquarium Association (AZA) on Zoo and Wildlife Nutrition, Ft. Worth, TX.

Mudgett, R.E., 1989. Microwave food processing. Food Technol 43, 117-126.

Muir, A.D., 2006a. Flax Lignans - Analytical Methods and How They Influence Our Understanding of Biological Activity. J Aoac Int 89, 1147-1157.

Muir, A.D., 2006b. Flax lignans - analytical methods and how they influence our understanding of biological activity. J Aoac Int 89, 1147-1157.

Muir, A.D., Westcott, N.D., 2000. Quantitation of the lignan secoisolariciresinol diglucoside in baked goods containing flax seed or flax meal. Journal of Agricultural and Food Chemistry 48, 4048-4052.

Muir, A.D., Westcott, N.D., 2003. Flax : the genus linum. Routledge, London ; New York.

Mutihac, L., Mutihac, R., 2008. Mining in chemometrics. Analytica Chimica Acta 612, 1-18.

Naczk, M., Pink, J., Amarowicz, R., Pink, D., Shahidi, F., 2001. Multivariate model for the prediction of soluble condensed tannins in crude extracts of polyphenols from canola and rapeseed hulls. J. Am. Oil Chem. Soc. 78, 411-414.

Naczk, M., Pink, J., Zadernowski, R., Pink, D., 2002. Multivariate model for the prediction of total phenolic acids in crude extracts of polyphenols from canola and rapeseed meals. A preliminary study. J. Am. Oil Chem. Soc. 79, 759-762.

Nemes, S.M., Orsat, V., 2010. Screening the Experimental Domain for the Microwave-Assisted Extraction of Secoisolariciresinol Diglucoside from Flaxseed Prior to Optimization Procedures. Food and Bioprocess Technology 3, 300-307.

Nemes, S.M., Orsat, V., 2011a. Evaluation of a Microwave-Assisted Extraction Method for Lignan Quantification in Flaxseed Cultivars and Selected Oil Seeds. Food Analytical Methods 5, 551-563.

Nemes, S.M., Orsat, V., 2011b. Microwave-Assisted Extraction of Secoisolariciresinol Diglucoside—Method Development. Food and Bioprocess Technology 4, 1219-1227.

Nemes, S.M., Orsat, V., 2011c. Modeling the recovery patterns from solid phase extraction purification of secoisolariciresinol diglucoside, p-coumaric acid glucoside, and ferulic acid glucoside from microwave-assisted flaxseed extracts. Food and Bioproducts Processing 90, 453-465.

Nemes, S.M., Orsat, V., Raghavan, G.S.V., 2012. Calibration of Artificial Neural Network and Partial Least Squares Regression Models for the Prediction of Secoisolariciresinol Diglucoside Contents in Microwave-Assisted Extracts of Various Flaxseed (Linum usitatissimum) Samples. Food Chemistry 133, 1588-1595.

Nóbrega, J.A., Trevizan, L.C., Araújo, G.C.L., Nogueira, A.R.A., 2002. Focused-microwave-assisted strategies for sample preparation. Spectrochimica Acta Part B: Atomic Spectroscopy 57, 1855-1876.

Nollet, L.M.L., 2000. Food analysis by HPLC, 2nd ed. Marcel Dekker, New York.

Oomah, B.D., 2001. Flaxseed as a functional food source. Journal of the science of food and agriculture 81, 889-894.

Oomah, B.D., Hosseinian, F.S., 2008. Phytoestrogens, in: Hurst, W.J. (Ed.), Methods of analysis for functional foods and nutraceuticals, 2nd ed. CRC Press, Boca Raton, pp. 1-84.

Oomah, B.D., Mazza, G., 2001. Optimization of a spray drying process for flaxseed gum. Int. j. food sci. technol 36, 135-143.

Oomah, B.D., Sitter, L., 2009. Characteristics of flaxseed hull oil. Food Chemistry. 114, 623-628.

Orsat, V., Raghavan, G.S.V., Meda, V., 2005. Microwave technology for food processing: an overview, in: Regier, M., Schubert, H. (Eds.), The microwave processing of foods. Woodhead Publishing Ltd., Cambridge, England, pp. 105-118.

Paré, J.R.J., Bélanger, J.M.R., 1997. Microwave-assisted process (MAP^{TM}): Principles and applications, in: Pare, J.R.J., Belanger, J.M.R. (Eds.), Instrumental methods in food analysis. Elsevier, Amsterdam; New York, pp. 395-420.

Paré, J.R.J., Bélanger, J.M.R., Stafford, S.S., 1994. Microwave-assisted process (MAP^{TM}): a new tool for the analytical laboratory. TrAC Trends in Analytical Chemistry 13, 176-184.

Penalvo, J.L., Adlercreutz, H., Uehara, M., Ristimaki, A., Watanabe, S., 2008. Lignan Content of Selected Foods from Japan. J. Agric. Food Chem. 56, 401-409.

Penalvo, J.L., Haajanen, K.M., Botting, N., Adlercreutz, H., 2005. Quantification of Lignans in Food Using Isotope Dilution Gas Chromatography/Mass Spectrometry. Journal of agricultural and food chemistry 53, 9342-9347.

Peterson, J., Dwyer, J., Adlercreutz, H., Scalbert, A., Jacques, P., McCullough, M.L., 2010. Dietary lignans: physiology and potential for cardiovascular disease risk reduction. Nutrition reviews 68, 571-603.

Pihlava, J.-M., Hyvarinen, H., Ryhanen, E.-L., Hietantemi, V., 2004. Process for isolating and purifying secoisolariciresinol diglucoside (SDG) from flaxseed, United States Patent and Trademark Office Pre-Grant Publication.

Plattner, R.D., Powell, R.G., 1978. A secoisolariciresinol branched fatty diester from Salvia plebeia seed. Phytochemistry 17, 149-150.

Powell, R.G., Plattner, R.D., 1976. Structure of a secoisolariciresinol diester from Salvia plebeia seed. Phytochemistry 15, 1963-1965.

Prasad, K., 1997. Hydroxyl radical-scavenging property of secoisolariciresinol diglucoside (SDG) isolated from flax-seed. Molecular and cellular biochemistry 168, 117-123.

Prasad, K., 1999. Reduction of serum cholesterol and hypercholesterolemic atherosclerosis in rabbits by secoisolariciresinol diglucoside isolated from flaxseed. Circulation 99, 1355-1362.

Prasad, K., 2000a. Antioxidant activity of secoisolariciresinol diglucoside-derived metabolites, secoisolariciresinol, enterodiol, and enterolactone. International Journal of Angiology 9, 220-225.

Prasad, K., 2000b. Oxidative stress as a mechanism of diabetes in diabetic BB prone rats: Effect of secoisolariciresinol diglucoside (SDG). Molecular and Cellular Biochemistry 209, 89-96.

Prasad, K., 2002. Suppression of Phosphoenolpyruvate Carboxykinase Gene Expression by Secoisolariciresinol Diglucoside (SDG), a New Antidiabetic Agent. International Journal of Angiology 11, 107-109.

Prasad, K., Mantha, S., Muir, A., Westcott, N., 2000. Protective effect of secoisolariciresinol diglucoside against streptozotocin-induced diabetes and its mechanism. Molecular and Cellular Biochemistry 206, 141-150.

Puvirajah, A.S., 2011. Quality of Western Canadian Flaxseed 2010. Canadian grain commission www.grainscanada.gc.ca, 1-14.

Puvirajah, A.S., 2012. Quality of Western Canadian Flaxseed 2011. Canadian grain commission www.grainscanada.gc.ca, 1-14.

Qiu, S.X., Lu, Z.Z., Luyengi, L., Lee, S.K., Pezzuto, J.M., Farnsworth, N.R., Thompson, L.U., Fong, H.H.S., 1999. Isolation and characterization of flaxseed (Linum usitatissimum) constituents. Pharmaceutical biology. Jan 37, 1-7.

Raynie, D.E., 2006. Modern extraction techniques. Analytical chemistry 78, 3997-4003.

Robinson, J., Kingman, S., Irvine, D., Licence, P., Smith, A., Dimitrakis, G., Obermayer, D., Kappe, C.O., 2010. Understanding microwave heating

effects in single mode type cavities-theory and experiment. Physical Chemistry Chemical Physics 12.

Ross, T., 1996. Indices for performance evaluation of predictive models in food microbiology. Journal of Applied Microbiology 81, 501-508.

Routray, W., Orsat, V., 2011. Microwave-Assisted Extraction of Flavonoids: A Review. Food and Bioprocess Technology, 5, 409-424.

Schwartz, H., Sontag, G., Plumb, J., 2009. Inventory of phytoestrogen databases. Food Chemistry. 113, 736-747.

Shahidi, F., Naczk, M., 2004a. Methods of Analysis and Quantification of Phenolic Compounds, Phenolics in Food and Nutraceuticals. CRC Press.

Shahidi, F., Naczk, M., 2004b. Phenolic Compounds of Major Oilseeds and Plant Oils, Phenolics in Food and Nutraceuticals. CRC Press.

Shimizu, Y., 1998. Purification of Water-Soluble Natural Products, in: Cannell, R.J.P. (Ed.), Methods in Biotechnology. Humana Press Inc, Totowa, NJ, pp. 329-341.

Shukla, R., Hilaly, A.K., Moore, K.M., 2004. Process for obtaining lignan, U. S. Patent 6,767,565.

Sicilia, T., Niemeyer, H.B., Honig, D.M., Metzler, M., 2003. Identification and stereochemical characterization of lignans in flaxseed and pumpkin seeds. Journal of agricultural and food chemistry 51, 1181.

Singh, A., Sabally, K., Kubow, S., Donnelly, D.J., Gariepy, Y., Orsat, V., Raghavan, G.S.V., 2011a. Microwave-Assisted Extraction of Phenolic Antioxidants from Potato Peels. Molecules 16, 2218-2232.

Singh, K.K., Mridula, D., Rehal, J., Barnwal, P., 2011b. Flaxseed: A potential source of food, feed and fiber. Critical Reviews in Food Science and Nutrition 51, 210-222.

Singleton, V.L., Orthofer, R., Lamuela-Raventós, R.M., 1999. [14] Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent, in: Lester, P. (Ed.), Methods in Enzymology. Academic Press, pp. 152-178.

Smeds, A.I., Eklund, P.C., Sjoholm, R.E., Willfor, S.M., Nishibe, S., Deyama, T., Holmbom, B.R., 2007. Quantification of a Broad Spectrum of Lignans in Cereals, Oilseeds, and Nuts. J. Agric. Food Chem. 55, 1337-1346.

Smeds, A.I., Jauhiainen, L., Tuomola, E., Peltonen-Sainio, P., 2009. Characterization of Variation in the Lignan Content and Composition of Winter Rye, Spring Wheat, and Spring Oat. Journal of Agricultural and Food Chemistry 57, 5837-5842.

Spence, J.D., Thornton, T., Muir, A.D., Westcott, N.D., 2003. The Effect of Flax Seed Cultivars with Differing Content of α-Linolenic Acid and Lignans on Responses to Mental Stress. J Am Coll Nutr 22, 494-501.

Stead, P., 1998. Isolation by Preparative HPLC, in: Cannell, R.J.P. (Ed.), Methods in Biotechnology. Humana Press Inc, Totowa, NJ, pp. 165-208.

Strandås, C., Kamal-Eldin, A., Andersson, R., Aman, P., 2008. Composition and properties of flaxseed phenolic oligomers. Food Chemistry. 110, 106-112.

Struijs, K., Vincken, J.-P., Doeswijk, T.G., Voragen, A.G.J., Gruppen, H., 2009. The chain length of lignan macromolecule from flaxseed hulls is determined by the incorporation of coumaric acid glucosides and ferulic acid glucosides. Phytochemistry 70, 262-269.

Struijs, K., Vincken, J.-P., Verhoef, R., van Oostveen-van Casteren, W.H.M., Voragen, A.G.J., Gruppen, H., 2007. The flavonoid herbacetin diglucoside as a constituent of the lignan macromolecule from flaxseed hulls. Phytochemistry 68, 1227-1235.

Struijs, K., Vincken, J.P., Verhoef, R., Voragen, A.G.J., Gruppen, H., 2008. Hydroxycinnamic acids are ester-linked directly to glucosyl moieties within the lignan macromolecule from flaxseed hulls. Phytochemistry 69, 1250-1260.

Stuerga, D., Delmotte, M., 2002. Wave-Material Interaction, Microwave Technology and Equipment, in: Loupy, A. (Ed.), Microwaves in Organic Synthesis. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, pp. 1-32.

Swain, M.J., Ferron, S., Coelho, A.I.P., Swain, M.V.L., 2006. Effect of continuous (intermittent) use on the power output of domestic microwave ovens. Int. j. food sci. technol 41, 652-656.

Thompson, L.U., Boucher, B.A., Cotterchio, M., Kreiger, N., Liu, Z., 2007. Dietary phytoestrogens, including isoflavones, lignans, and coumestrol, in nonvitamin, nonmineral supplements commonly consumed by women in Canada. Nutrition and cancer 59, 176-184.

Thompson, L.U., Boucher, B.A., Liu, Z., Cotterchio, M., Kreiger, N., 2006. Phytoestrogen Content of Foods Consumed in Canada, Including Isoflavones, Lignans, and Coumestan. Nutr Cancer 54, 184-201.

Thompson, L.U., Rickard, S.E., Cheung, F., Kenaschuk, E.O., Obermeyer, W.R., 1997. Variability in anticancer lignan levels in flaxseed. Nutrition and cancer 27, 26-30.

Tirozzi, B., Puca, S., Pittalis, S., Bruschi, A., Morucci, S., Ferraro, E., Corsini, S., 2006. Artificial Neural Networks, Neural Networks and Sea Time Series: Reconstruction and Extreme-Event Analysis. Modeling and Simulation in Science, Engineering and Technology. Springer, pp. 35-40.

Touré, A., Xueming, X., 2010. Flaxseed lignans: source, biosynthesis, metabolism, antioxidant activity, bio-active components, and health benefits. Comprehensive Reviews in Food Science and Food Safety 9, 261-269.

Venkatesh, M.S., Raghavan, G.S.V., 2004. An overview of microwave processing and dielectric properties of agri-food materials. Biosystems Engineering 88, 1-18.

Wang, J.X., Xiao, X.H., Li, G.K., 2008. Study of vacuum microwave-assisted extraction of polyphenolic compounds and pigment from Chinese herbs. J Chromatogr A 1198-1199, 45-53.

Wang, L., Weller, C.L., 2006. Recent advances in extraction of nutraceuticals from plants. Trends in Food Science & Technology 17, 300-312.

Wang, L.Q., 2002. Mammalian phytoestrogens: Enterodiol and enterolactone. Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences 777, 289-309.

Webb, A.L., McCullough, M.L., 2005. Dietary lignans: potential role in cancer prevention. Nutrition and cancer 51, 117-131.

Westcott, N.D., Muir, A.D., 1998. A process for extracting lignans from flaxseed, U. S. Patent 5,705,618.

Westcott, N.D., Muir, A.D., 2003. Chemical studies on the constituens of *Linum* spp., in: Muir, A.D., Westcott, N.D. (Eds.), Flax: the genus Linum. Routledge, London ; New York, pp. 55-73.

Westcott, N.D., Muir, A.D., Lafond, G., McAndrew, D.W., May, W., Shirtliffe, S., Bruulsema, T.W., 2002. Factors affecting the concentration of a nutraceutical lignan in flaxseed, in: Bruulsema, T.W. (Ed.), Proceedings of the Symposium on Fertilizing Crops for Functional Foods. Potash & Phosphate Institute/ Potash & Phosphate Institute of Canada (PPI/PPIC), Indianapolis, Indiana, USA, pp. 1-4.

Westcott, N.D., Paton, D., 2001. Complex containing lignan, phenolic and aliphatic substances from flax and process for preparing, U. S. Patent 6,264,853.

Wiesenborn, D., Tostenson, K., Kangas, N., 2003. Continuous abrasive method for mechanically fractionating flaxseed. JAOCS, Journal of the American Oil Chemists' Society 80, 295-300.

Will, H., Scholz, P., Ondruschka, B., 2004. Microwave-assisted heterogeneous gas-phase catalysis. Chemical Engineering and Technology 27, 113-122.

Williams, P.C., 2001. Implementation of Near-Infrared Technology, in: Williams, P.C., Norris, K. (Eds.), Near-Infrared Technology in the Agricultural and Food Industries, 2nd ed. American Association of Cereal Chemists, Inc., St. Paul, Minnesota, USA, pp. 145-169.

Wold, S., Sjöström, M., Eriksson, L., 2001. PLS-regression: a basic tool of chemometrics. Chemometrics and Intelligent Laboratory Systems 58, 109-130.

Wrolstad, R.E., Acree, T.E., Decker, E.A., Penner, M.H., Reid, D.S., Schwartz, S.J., Shoemaker, C.F., Smith, D., Sporns, P., 2005. Polyphenolics, Handbook of Food Analytical Chemistry: Pigments, Colorants, Flavors, Texture, and Bioactive Food Components. John Wiley & Sons, Inc., pp. 461-535.

Wu, T., Yan, J., Liu, R., Marcone, M.F., Aisa, H.A., Tsao, R., 2011. Optimization of microwave-assisted extraction of phenolics from potato and its downstream waste using orthogonal array design. Food Chemistry.

Wu, X.-H., Sun, D.-H., Zhuang, Z.-X., Wang, X.-R., Gong, H.-F., Hong, J.-X., Lee, F.S.C., 2002. Analysis and leaching characteristics of mercury and arsenic in Chinese medicinal material. Analytica Chimica Acta 453, 311-323.

Yuan, J.P., Li, X., Xu, S.P., Wang, J.H., Liu, X., 2008. Hydrolysis kinetics of secoisolariciresinol diglucoside oligomers from flaxseed. J Agric Food Chem 56, 10041-10047.

Zhang, H.-F., Yang, X.-H., Wang, Y., 2011. Microwave assisted extraction of secondary metabolites from plants: Current status and future directions. Trends in Food Science and Technology 22, 672-688.

Zhang, W., Xu, S., 2007. Microwave-assisted extraction of secoisolariciresinol diglucoside from flaxseed hull. Journal of the Science of Food and Agriculture 87, 1455-1462.

Zhang, Z., Lv, G., Pan, H., Fan, L., 2012. Optimisation of the microwave-assisted extraction process for six phenolic compounds in Agaricus blazei murrill. Int. j. food sci. technol 47, 24-31.