Fabrication d'extraits bioactifs bénéfiques pour la santé et riches en glucoraphanine à partir de rejets industriels de Brassica oleracea (brocoli) en utilisant la technologie verte

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Résumé

Le brocoli est une excellente source de composés nutraceutiques ayant de nombreux effets sur la santé tels que les propriétés anticancéreuses, anti-diabétiques, antioxydantes et antimicrobiennes. Les glucosinolates, les polyphénols, les vitamines, les minéraux et les fibres alimentaires sont les principales molécules présentes dans le brocoli. La production annuelle mondiale de brocoli est de 21 millions de tonnes. On estime que 35 à 40% des cultures horticoles sont perdues en raison de pratiques agricoles inadéquates, générant d'énormes quantités de déchets agricoles. Ces cultures perdues pourraient être utilisées comme matières premières pour l'extraction et la purification d'ingrédients bioactifs destinés à l'industrie nutraceutique et alimentaire.

L'objectif principal de ce projet était de développer une technique économique et respectueuse de l'environnement pour la fabrication d'un extrait riche en glucoraphanine à partir de rejets industriels de brocoli, en fournissant une voie alternative pour sa valorisation. Ce travail se concentre principalement sur l'identification, la caractérisation et la quantification des glucosinolates et des polyphénols présents dans 10 lots rejetés de graines de brocoli et de résidus industriels de brocoli tels que les fleurons, les tiges et le mélange de fleurons et de tiges. De plus, le procédé d'extraction de la glucoraphanine a été optimisé en utilisant des solvants verts tels que l'éthanol et l'eau. En outre, la glucoraphanine provenant d'extraits de brocoli bruts a été purifiée en utilisant des résines échangeuses d'ions par une Méthodologie de Surface de Réponse, basé sur le Box-Behnken Design (BBD) et l'Analyse des Composants Principaux. Enfin, des expériences pilotes ont été réalisées en utilisant les paramètres optimisés pour vérifier leur adéquation pour une application industrielle.

La caractérisation et la quantification simultanées par UPLC MS/MS ont indiqué la présence de 12 glucosinolates (principalement de la glucoraphanine) et de 5 polyphénols dans les sous-produits du brocoli. La teneur en glucosinolates variait de 0,2 à 2% de matière sèche (MS), tandis que les polyphénols étaient inférieurs à 0,02% de MS. L'abondance relative de la glucoraphanine dans les sous-produits du brocoli a fait un

matériau de départ prometteur pour la fabrication de compléments alimentaires fonctionnels.

De plus, un procédé d'extraction de la glucoraphanine écologique et à base de solvant a été optimisé pour les sous-produits de graines de brocoli et de fleurons. Un extracteur à agitation magnétique unique a maximisé l'extractibilité de la glucoraphanine. Les paramètres d'extraction optimisés étaient de 50% et 70% d'éthanol aqueux extraits pendant 60 et 30 minutes à 60 et 23°C pour les sous-produits de graines et de fleurons, respectivement, en utilisant un rapport matière/solvant de 1:20. Le procédé vert optimisé a donné un rendement de glucoraphanine de 55,5 g/kg MS de graines et de 4,3 g/kg MS de fleurons. Le procédé vert développé dans cette étude a fourni 37 et 81 fois plus d'extractibilité de la glucoraphanine que la technique analytique standard basée sur le méthanol.

Enfin, un procédé de purification de la glucoraphanine respectueux de l'environnement et industriellement réalisable a été développé en utilisant des résines échangeuses d'ions par approche de surface de réponse pour les sous-produits de graines de brocoli et de fleurons. Un ensemble de 27 essais, 3 niveaux dans le BBD ont été proposés pour les résines cationiques et anioniques en série, pour maximiser les réponses du processus. La purification de la glucoraphanine à partir de l'extrait de graines de brocoli en utilisant une résine cationique a permis une récupération maximale de 94% et une pureté de 14% en utilisant 1:5 du rapport matière/résine pendant 91 min à 80 rpm/min. Dans le cas de la résine anionique, les variables expérimentales de 1:5, 140 min, 160 rpm/min et 7% d'hydroxyde d'ammonium dans de l'éthanol à 70% ont donné un rendement de 72% et une pureté de 37%. Alors que pour les rejets industriels de fleurons de brocoli, les paramètres optimisés pour la purification de la glucoraphanine étaient un ratio matière/résine de 1:1.87, un temps de contact de 30 min, une vitesse d'agitation de 80 rpm/min et un solvant d'élution de 100% eau. La purification subséquente de l'extrait cationique en utilisant la résine anionique a été réalisée en utilisant les paramètres expérimentaux optimisés du rapport matière/résine de 1:1.3 pendant 170 min à 140 rpm/min et éluée en utilisant 7% d'hydroxyde d'ammonium dans 70% d'éthanol, fournissant une récupération de 78% et pureté de 5%. Enfin, les paramètres du processus d'extraction et de purification optimisés à

l'échelle du laboratoire ont été extrapolés à l'échelle pilote pour la fabrication d'extraits en poudre, indiquant que le procédé optimisé était très efficace pour récupérer la glucoraphanine avec une grande pureté même à grande échelle.

Par conséquent, la présente étude a mis au point un procédé écologique efficace et industriellement viable pour la fabrication d'extraits de rejets industriels de brocoli. Le processus optimisé a fourni une voie alternative économiquement viable pour la valorisation de la récolte perdue qui nous rapproche de la sécurité alimentaire et la durabilité environnementale.

Abstract

Broccoli is an excellent source of nutraceutical compounds with many health effects such as anticancerous, anti-diabetic, antioxidant and anti-microbial properties. Glucosinolates, polyphenols, vitamins, minerals, dietary fibers are the most important molecules present in broccoli. The global annual production of broccoli is 21 million tons. It is estimated that 35-40% of the horticultural crops are lost due to inadequate agricultural practices, generating huge quantities of agro-waste. These lost crops, could be used as raw materials for the extraction and purification of bioactive ingredients for the nutraceutical and food industry.

The main objective of this project was to develop an economical and environmental friendly technique for the fabrication of an extract rich in glucoraphanin from broccoli industrial discards, providing an alternative route for its valorization. This work predominantly focuses on the identification, characterization and quantification of glucosinolates and polyphenols present in 10 rejected lots of broccoli seeds and broccoli industrial residues such as florets, stalks and the mixture of florets and stalks. Additionally, the glucoraphanin extraction process was optimized using green solvents such as ethanol and water. Further, the glucoraphanin from crude broccoli extracts were purified using ion exchange resins by Response Surface Methodology, based on Box-Behnken Design (BBD) and Principle component analysis. Finally, pilot experiments were performed using the optimized parameters to verify their industrial applicability.

The simultaneous characterization and quantification by UPLC MS/MS indicated the presence of 12 glucosinolates (predominantly glucoraphanin) and 5 polyphenols in broccoli by-products. The glucosinolates content varied from 0.2 to 2% dry weight (DW), whereas, the polyphenols were less than 0.02% DW. The relative abundance of glucoraphanin in broccoli by-products makes it a promising starting material for the fabrication of functional food supplements.

Further, an eco-friendly, solvent based glucoraphanin extraction process was optimized for broccoli seeds and florets by-products. A single batch magnetically stirred extractor was found to maximize glucoraphanin extractability. The optimized extraction parameters were

50% and 70% aqueous ethanol extracted for 60 and 30 minutes at 60 and 23°C for seeds and florets by-products, respectively, using a feed to solvent ratio of 1:20. The optimized green process provided a glucoraphanin yield of 55.5 g/Kg DW seeds and 4.3 g/kg DW florets by-products. The green process developed in this study provided 37 and 81 times more glucoraphanin extractability than the standardized methanol based analytical technique.

Finally, an environmental friendly and industrially feasible glucoraphanin purification process was developed using ion exchange resins by response surface approach for broccoli seeds and florets by-products. A 27 run, 3 level BBD, were proposed for cationic and anionic resins in series, to maximize the process responses. Glucoraphanin purification from broccoli seeds extract using cationic resin provided a maximal recovery of 94% and purity of 14% using 1:5 of feed to resin ratio for 30 min, at 80 rpm agitation speed and eluting solvent concentration of 100% water. For anionic resin, the experimental variables of 1:5, 140 min, 160 rpm and 7% ammonium hydroxide in 70% ethanol provided a process efficiency of 72% and a purity of 37%. Whereas, for broccoli florets industrial discards, the optimized process parameters for the purification of glucoraphanin were a feed to resin ratio of 1:1.87, contact time of 30 min, agitation speed of 80 rpm and eluting solvent of 100% water. Subsequent purification of the cationic extract using the anionic resin was performed using the optimized experimental parameters of feed to resin ratio of 1:1.3 for 170 min at 140 rpm and eluted using 7% ammonium hydroxide in 70% ethanol, providing a recovery of 78% and purity of 5%. Finally, the laboratory scale optimized extraction and purification process parameters was extrapolated onto the pilot scale for the fabrication of powdered extracts, indicated that the optimized process was highly efficient in recovering glucoraphanin with high purity even on large scale operation.

Hence, the present study developed an efficient, industrially viable green process, for the fabrication of extracts from broccoli industrial discards. The optimized process provided an economically feasible alternative route for the valorization of the lost crop bringing us closer to food security and environmental sustainability.

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List of abbreviations

^{1}H-NMR	: Proton nuclear magnetic resonance
AD	: Alzheimer's disease
AOAC	: Association of official chemist
BBD	: Box-Behnken design
CCD	: Central Composite Design
CHS	: Chalcone synthase
CID	: Collision induced dissociation
DAD	: Diode array detection
DEAE	: Diethylaminoethyl
DW	: Dry Weight
ESI	: Electron spray ionisation
ESP	: Epithiospecifier protein
FAO	: Food and Agricultural Organisation
GC-MS	: Gas chromatography- mass spectrometry
GLS	: Glucosinolates
HPLC	: High Performance Liquid Chromatography
IER	: Ion Exchange Resin
LDL	: Low Density Lipoprotein
MRM	: Multiple Reaction Monitoring
MS	: Matière Sèche
MS/MS	: Tandem mass spectrometry
OECD	: Organisation for economic co-operation and development
PA	: Parkinson's
PAL	: Phenylalanine ammonia lyase
PCA	: Principle Component Analysis
ROS	: Reactive oxygen species
RP-HPLC	: Reverse phase- High performance chromatography
RSD	: Response Surface Design
SFE	: Supercritical fluid extraction
TLC	: Thin layer chromatography

TQD : Triple Quadrupole Mass Spectrometer

UPLC : Ultra Performance Liquid Chromatography

UV : Ultraviolet

WHO : World Health Organisation

Acknowledgement

First of all, I would like to thank my guide and "philosopher" Dr. Paul Angers for his aspiring guidance and support during my doctoral studies. He has been a great mentor, guiding me to be an improved scientist. Also, I wish to thank him for the freedom of decisions, allowing me to become autonomous. I wish to thank him for having faith in me, when I was lost, allowing me to learn and to be a better researcher. Secondly, I would like to thank my co-supervisor Dr. André Gosselin for all the support and industrial exposure, given to our team over the years. His vision has enabled me to take this project to a whole new level, providing an industrial perspective for my studies. His promptness is highly inspiring and something I wish to imbibe in my life. I would also like to thank my second co-supervisor Dr. Yves Desjadrins for providing me the insight and expertise that greatly assisted my research. His valuable corrections and constructive criticism during my work have enabled me to improve my scientific writing skills.

I'm also grateful to our post-doctoral fellow Ashraf Badr for his advice, encouragement and support throughout my studies. A heartfelt thanks to my previous supervisor Dr. Joseph Arul for introducing me to the world of research.

I wish to express my gratitude to our industrial partners Diana Food Canada Inc., Productions Maraîchères Mailhot Inc., and Norseco Inc., Québec, Canada. I would also like to thank Natural Science and Engineering Research Council of Canada (NSERC) for their financial support.

I wish to extend my appreciation to the analytical platform team, Pascal Dubé, Véronique Richard and Perrine Feutry for their support, advice, encouragement and help to explore the world of molecules throughout my studies. Heartfelt thanks to all my team members Maelle, Marlene, Aurelie, Ines, Mathilde, Ana Sofia, Rafik and Ichrak for the help and fun times in the lab. I am extremely lucky to have such excellent people as colleagues.

I would like to thank my friends Pallavi, Priyanka Patel, Sai Sampath, Ramesh, Siddarth, Mansa, Kapil, Kallol Dutta and all my Indian friends, who made me feel at home in a land far away from home.

I'm indebted to my parents (K.J Tomy and Rose Marie), my in-laws, siblings, my beloved husband (Dr. Jithin Davies) and our baby Isabelle for encouraging me to dream high and pursue them against all odds.

Last but not least, I thank god for all the grace and blessing bestowed upon me.

Foreword

Broccoli (*Brassica oleracea* var. italica) is an economically important crop known for its disease preventive and health beneficial properties. The health beneficial properties of broccoli are mainly due to its abundance in glucosinolates, polyphenols, carotenoids, vitamins and minerals, but, most distinctively glucoraphanin. Increasing awareness among consumers about the positive health effects of fresh fruits and vegetables have led to its increased consumption which have steered to its increased production. Lack of good agricultural practices, postharvest facilities and stringent market requirements, generated huge quantities of agricultural wastes. Worldwide, the annual production of broccoli is 21 million tons and in broccoli close to 25% of the total produce is discarded. The abundance of glucoraphanin in the industrial discards of broccoli, make them a promising starting material for the fabrication of health beneficial bioactive molecules. This provides an alternative route towards industrial discard valorization. Despite technological advancements, valorisation of industrial discards is subjected to various limitations due to stringent market and consumer requirements. There is a deficit of published literature on valorisation of broccoli by-products using green solvents as compared to the normally consumed plant parts.

In this study, the industrial discards of broccoli were characterised and quantified for their relative abundance in glucosinolates and polyphenols, and most distinctively glucoraphanin. Further, an industrially viable green process for the fabrication of extract was developed. Finally, a glucoraphanin purification technique for the fabrication of extract rich in glucoraphanin using Ion Exchange Resin by green technology.

Chapter 1

Introduction

Changing lifestyles in the recent decades, have led to the development of food with long shelf lives, high nutritive value and easy to prepare. The U.S Food and Drug Administration and The British Food Standard Agency has generated an increased awareness about the negative impact of artificial food additives on health especially children enabling the substitution of artificial compounds with natural ingredients (Buka, Osornio-Vargas, & Clark, 2011; Kobylewski & Jacobson, 2010). The lethal effects of synthetic preservatives and the increased awareness about the health promoting impact of vegetables, has led to the exploration on plant based bioactive molecules (Krishnakumar & Gordon, 1996; Peschel, Sánchez-Rabaneda, Diekmann, Plescher, Gartzía, Jiménez, et al., 2006). Plant and plant products that constitute a major share of people's diet, offer more diverse group of plants bioactive than any drugs and many drugs are derived from the compounds originally present in plants (Moreno, Carvajal, López-Berenguer, & García-Viguera, 2006).

Over the past few decades, compelling evidences have been obtained linking to the increased consumption of fruits and vegetables, with reduced indices of numerous diseases such as cancer (Fresco, Borges, Diniz, & Marques, 2006), age-related chronic diseases (Fahey, Wehage, Holtzclaw, Kensler, Egner, Shapiro, et al., 2012), coronary heart diseases etc. (Terry, 2011). This is because of the high levels of nutraceutical and bioactive compounds (glucosinolates, phenolic acids, flavonoids, saponins) and nutrients (vitamin C and B-group vitamins, minerals (Se, Ca, Mg, Na, K, Cl and P), trace elements and sugars) (Domínguez-Perles, Martínez-Ballesta, Carvajal, García-Viguera, & Moreno, 2010), lipids (fats, fatty acids, waxes, sterols, carotenoids, fat-soluble vitamins, phospholipids, mono and di-glycerides), proteins, peptides and amino acids (Ares, Nozal, & Bernal, 2013), along with phytosterols, sulphides, protease inhibitors and terpenes (Schreiner & Huyskens-Keil, 2006). Once consumed, these compounds have different effects on health: antioxidant, anti-carcinogenic, antibacterial, antifungal, antiviral, antithrombotic and anti-inflammatory

effects. They also act by influencing blood pressure, sugar and cholesterol levels in the blood (Schreiner & Huyskens-Keil, 2006) as opposed to the refined sugar and high fat diet.

According to WHO World Health Report 2002, low intake of fruits and vegetables is associated with increased rates of chronic diseases. The influence of plant phytochemicals on health not only depends on the nature and abundance of these compounds but also on their metabolism, bioavailability and intake rate (Terry, 2011). Recently it has been found that, the current dietary intake of various fruits and vegetables are inefficient to provide health benefits, which leads to the need for dietary supplements (Fresco, Borges, Diniz, & Marques, 2006).

In today's society, where there is decreased availability of fresh food materials, with the escalating cost and increased demand for appropriate nutritional standards, together with great concern on environmental pollution; great emphasis is given on the recovery, recycling and upgradation of vegetable residues (Laufenberg, Kunz, & Nystroem, 2003). Residues from the horticulture and food industry can act as the source for bioactive compounds, leading to the production of potential, health promoting, value added products. Less than 25% of the total yield of a vegetable is consumed (Fink, Feller, Scharpf, Weier, Maync, Ziegler, et al., 1999) thereby, generating huge amounts of by-products, creating a negative impact on the agricultural environment (Domínguez-Perles, Martínez-Ballesta, Carvajal, García-Viguera, & Moreno, 2010). The possible reuse of these downgraded residues to generate value added products is getting the attention of the scientific community (Domínguez-Perles, Martínez-Ballesta, Carvajal, García-Viguera, & Moreno, 2010; Mahro & Timm, 2007).

Currently, these residues are used as animal feed or as green manure. Some developmental paths for obtaining value-added products are being researched as biomass for solid-state fermentation for microbial cultures (Laufenberg, Kunz, & Nystroem, 2003; Mahro & Timm, 2007) dietary fibres and finally functional food or value added food development (Domínguez-Perles, Martínez-Ballesta, Carvajal, García-Viguera, & Moreno, 2010). Developing value added products from downgraded vegetable residues, improves environmental performance by waste management, enhancing economic benefits to

stakeholders and developing leading products and extracts rich in bioactive compounds beneficial for health (Domínguez-Perles, Martínez-Ballesta, Carvajal, García-Viguera, & Moreno, 2010; Montgomery, 2004). Diet based disease preventive strategies can be beneficial and cost effective as no added cost for the health sector is involved (Moreno, Carvajal, López-Berenguer, & García-Viguera, 2006). The high variability in the quality of vegetables and bioavailability of bioactive compounds has led to the development of bioactive extracts, with beneficial health impact (Terry, 2011).

Vegetables belonging to *Brassica* species, most predominantly broccoli (*Brassica oleracea* var. *italica*) are potent source of plant secondary metabolites such as glucosinolates (GLS), its conversion product sulphoraphane and isothiocynates (Moreno, Carvajal, López-Berenguer, & García-Viguera, 2006) and polyphenolic compounds (Cartea, Francisco, Soengas, & Velasco, 2010) vitamins (vitamin C, K1) and minerals (Se, Ca, Mg, Na, K, Fe etc.) (Finley, Ip, Lisk, Davis, Hintze, & Whanger, 2001; Jones, Faragher, & Winkler, 2006). These compounds have received considerable attention for being potentially protective against cancer (Fresco, Borges, Diniz, & Marques, 2006), diabetes (Axelsson, Tubbs, Mecham, Chacko, Nenonen, Tang, et al., 2017), neurodegenerative diseases, coronary heart diseases (Sesso, Gaziano, Liu, & Buring, 2003) anti-microbial (Ganin, Rayo, Amara, Levy, Krief, & Meijler, 2013) potent anti-oxidant and anti-inflammatory capacity (Cartea, Francisco, Soengas, & Velasco, 2010). Scarce information is available regarding the by-products, which are good sources of bioactive compounds (Llorach, Gil-Izquierdo, Ferreres, & Tomás-Barberán, 2003).

1.1 Bioactive Molecules and Functional Food

Horticulture industry generates large amounts of by-products with little or low value in the vegetable market creating a negative impact on the agricultural environment. These downgraded vegetable residues which are rich in bioactive phytochemicals can be utilised for the development of functional food, which in turn can replace the synthetic additives used in the food sector (Domínguez-Perles, Martínez-Ballesta, Carvajal, García-Viguera, & Moreno, 2010; Mahro & Timm, 2007).

'Bioactive compounds' in plants can be defined as secondary plant metabolites eliciting pharmacological or toxicological effects in man and animals (Bernhoft, 2010). The identification and extraction of bioactive molecules from downgraded vegetable residues can lead to the development of functional foods, which are rich in physiologically active compounds that can provide health benefits beyond human nutrition (Clydesdale, 1999; Moreno, Carvajal, López-Berenguer, & García-Viguera, 2006).

1.2 Secondary Metabolites

Plants produce diverse range of phytochemicals that are involved in its growth, development and defence. Primary metabolites are produced by plants to ensure its growth and development. Unlike animals, plants cannot flee from pathogen, herbivores attack or various biotic and abiotic stresses. Hence, they produce chemical molecules that are not directly involved in growth and development, but harmful to other species. These plant phytochemicals are called secondary metabolites (Halkier & Gershenzon, 2006). Plant secondary metabolites are involved in the plant defence and they play a key role in pharmaceuticals and nutraceutical industries. These bioactive molecules are used in traditional (Bourgaud, Gravot, Milesi, & Gontier, 2001) and modern medicine (Ulbricht & Chao, 2010). **Figure 1** presents an overview of their classification according to their biosynthetic pathway.

Fruits and vegetables rich in bioactive molecules have won increasing attention. Efforts have been taken by governmental organisations to educate the public about the benefits of active lifestyle and healthy eating, as there have been indications regarding the correlation between the incidence of chronic disease and lifestyle (Schreiner & Huyskens-Keil, 2006).

Cancer and cardiovascular diseases are the two major ailments that have affected most of the population around the world. Various treatments are available to fight against these diseases such as surgery, radiotherapy and chemotherapy but they are subjected to various side effects. A person's diet can offer a great and diverse group of plant bioactive substances that are free of side effects and provide health promoting and disease preventive properties. For example, plant bioactives such as sulphoraphane and isothiocynates have anti-cancerous property, anti-microbial, anti-inflammatory and prevents cardiovascular

diseases (Moreno, Carvajal, López-Berenguer, & García-Viguera, 2006). These compounds not only delay but also prevent the incidence of diseases.

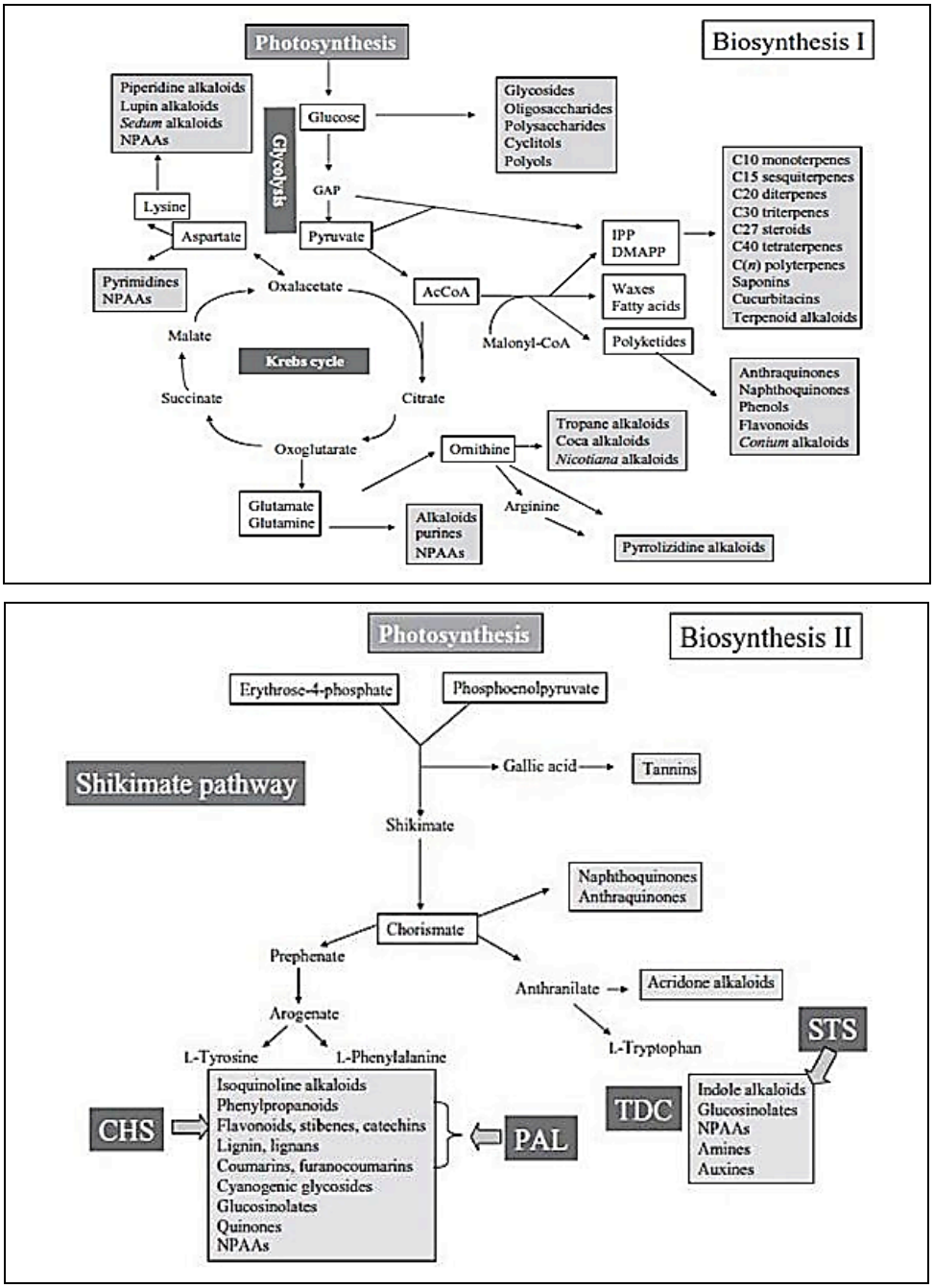


Figure 1: Overview of major metabolic pathways leading to the production of plant secondary (Wink, 2011).

Abbreviations: Biosynthesis 1: IPP (isopentenyl diphosphate), **DMAPP** (dimethyl allyl diphosphate), **GAP** (glyceraldehyde-3-phosphate), **NPAAs** (non-protein amino acids), **Acco** (acetyl coenzyme A).

Biosynthesis II: NPAAs (non-protein amino acids), **PAL** (phenylalanine ammonia lyase), **TDC** (tryptophan decarboxylase**), STS** (strictosidine synthase), **CHS** (chalcone synthase).

1.2.1 Glucosinolates

Glucosinolates are sulfur and nitrogen containing secondary metabolites derived from amino acids initially identified in mustard seeds in 1840 (Bones & Rossiter, 1996). The production of these molecules seems to be limited to certain families of dicotyledonous angiosperms of the order *Capparales: Capparaceae*, *Brassicaceae*, *Moringaceae* and *Tovariaceae* (Fahey, Zalcmann, & Talalay, 2001). Glucosinolates are however prominent in the brassicaceae family (Moreno, Carvajal, López-Berenguer, & García-Viguera, 2006).

1.2.1.1 Structure of glucosinolate

Glucosinolates are water soluble, anionic molecules with sulphate isothiocynate group as its core structure linked to a *β*-D-thioglucose group and a variable side chain (R) derived from amino acids **(Figure 2)**. The variations in the glucosinolate structure is obtained by modifying the central carbon of the isothiocynates and the glucose moiety (Clarke, 2010).

Glucosinolates are structurally based on the amino acid precursors: 1. aliphatic glucosinolates derived from methionine, isoleucine, leucine or valine, 2. aromatic glucosinolates are derived from phenylalanine or tyrosine, and 3. indole glucosinolates are derived from tryptophan (Kurilich, Tsau, Brown, Howard, Klein, Jeffery, et al., 1999). Currently over 200 glucosinolates have been identified (Clarke, 2010). The aglycone side chain of the glucosinolates are extensively modified via elongation, hydroxylation, O-methylation, desaturation, glycosylation, oxidation and acylation (Blažević, Montaut, Burčul, & Rollin, 2015). The possible structural modifications in the glucosinolates depend on the R-group, that is being restricted to C_1-C_{12} alkyl side chains. The glucosinolates content is highly variable within and across the plant species. A number of plant species contain single glucosinolate, whereas, the seeds and leaves of the ecotype Arabidopsis thaliana have been reported to have 34 individual glucosinolates. Majority of the plants contain 2-5 varieties of glucosinolates (Clarke, 2010). Glucosinolates commonly found in broccoli are presented in **Table 1**

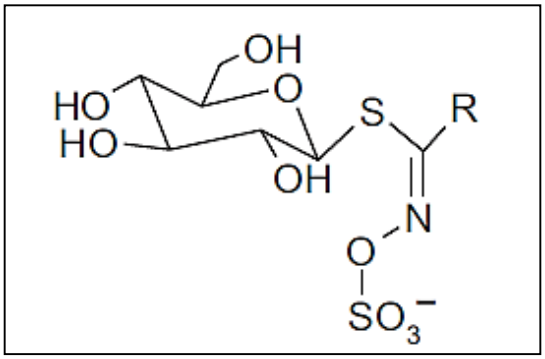
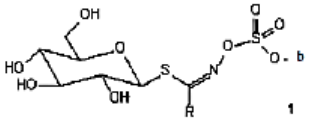
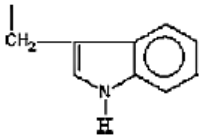
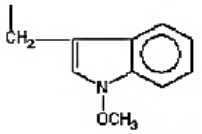
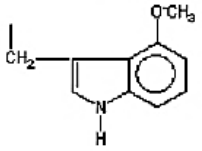


Figure 2: General structure of glucosinolates (Radojčić Redovniković, Glivetić, Delonga, & Vorkapić-Furač, 2008).

Table 1: Glucosinolates commonly found in broccoli (Kurilich, et al., 1999).

Chemical class	Chemical name	Trivial name	Radical
Sulphur-containing GLS	4-Mehylsulphinyl-3-butenyl	Glucoraphenin	$CH_2CH_2CH{=}CH\text{-}S(=O)\text{-}CH_3$
	4-(Methylsulphinyl)butyl	Glucoraphanin[a]	$CH_2(CH_2)_3\text{-}S(=O)\text{-}CH_3$
	5-(Methylsulphinyl)pentyl	Glucoalyssin[a]	$CH_2(CH_2)_4\text{-}S(=O)\text{-}CH_3$
	3-(Methylsulphinyl)propyl	Glucoiberin[a]	$CH_2CH_2CH_2\text{-}S(=O)\text{-}CH_3$
	4-(Methylthio)butyl	Glucoerucin	$CH_2(CH_2)_2CH_2\text{-}S\text{-}CH_3$
	5-(Methylthio)pentyl	Glucoberteroin	$CH_2(CH_2)_3CH_2\text{-}S\text{-}CH_3$
	3-(Methylthio)propyl	Glucoiberverin	$CH_2CH_2CH_2\text{-}S\text{-}CH_3$
Aliphatic olefins	3-Butenyl	Gluconapin[a]	$CH_2CH_2CH{=}CH_2$
	2(*R*)-2-Hydroxy-3-butenyl	Progoitrin[a]	$CH_2CH(OH)CH{=}CH_2$
	2-Hydroxy-4-pentenyl	[Gluco]napoleiferin	$CH_2CH(OH)CH_2CH{=}CH_2$
	4-Pentenyl	Glucobrassicanapin[a]	$CH_2CH_2CH_2CH{=}CH_2$
	1-Pentenyl		
	2-Propenyl	Allyl, sinigrin	$CH_2CH{=}CH_2$

Aromatic indole GLS	Indol-3-ylmethyl 4-Hydroxy-3-indolylmethyl	Glucobrassicin[a] 4-Hydroxyglucobrassicin[a]	
	1-Methoxyindol-3-ylmethyl	Neoglucobrassicin[a]	
	4-Methoxyindol-3-ylmethyl	4-Methoxyglucobrassicin[a]	

1.2.1.2 Biosynthesis of glucosinolate

Glucosinolates are derived from amino acids and based on their amino acid precursors, they can be divided into (1) aliphatic glucosinolates, derived from alanine, leucine, isoleucine, valine, and methionine; (2) aromatic glucosinolates, derived from phenylalanine or tyrosine and (3) indolic glucosinolates, derived from tryptophan. The glucosinolates biosynthesis proceeds through 3 phases: (1) Amino acid side chain elongation (2) Biosynthesis of core glucosinolate structure and (3) Secondary modifications (Radojčić Redovniković, Glivetić, Delonga, & Vorkapić-Furač, 2008).

1.2.1.2.1 Amino acid side chain elongation: The side chain elongation process involves the sequential addition of 1-9 methylene group to the side chain of methionine **(Figure 3)**. The process is initiated by transamination of methionine to its corresponding 2-oxo acids. The 2-oxo acid enters a cycle of three successive transformation steps: condensation with acetyl-CoA by methylthioalkylmalate synthase (MAM); isomerization by isopropylmalate isomerase (IPMI) and oxidative decarboxylation by isopropylmalate dehydrogenase (IPM-DH). The 2-oxo acid condenses with acetyl-CoA to form a substituted 2-malate derivative (2-alkylmalic acid), which then isomerizes with a 1,2-hydroxyl shift to a 3-malate derivative (3-alkylmalic acid), that undergoes oxidative dexarboxylation to yield a 2-oxo acid, with one more methylene group ($-CH_2$) than the starting compound. Upto nine cycles are known to occur in plants. The chain elongated methionine can either enter another round of chain elongation or proceed towards the biosynthesis of a core

glucosinolate structure (Velasco, Rodríguez, Francisco, Cartea, & Soengas, 2016).

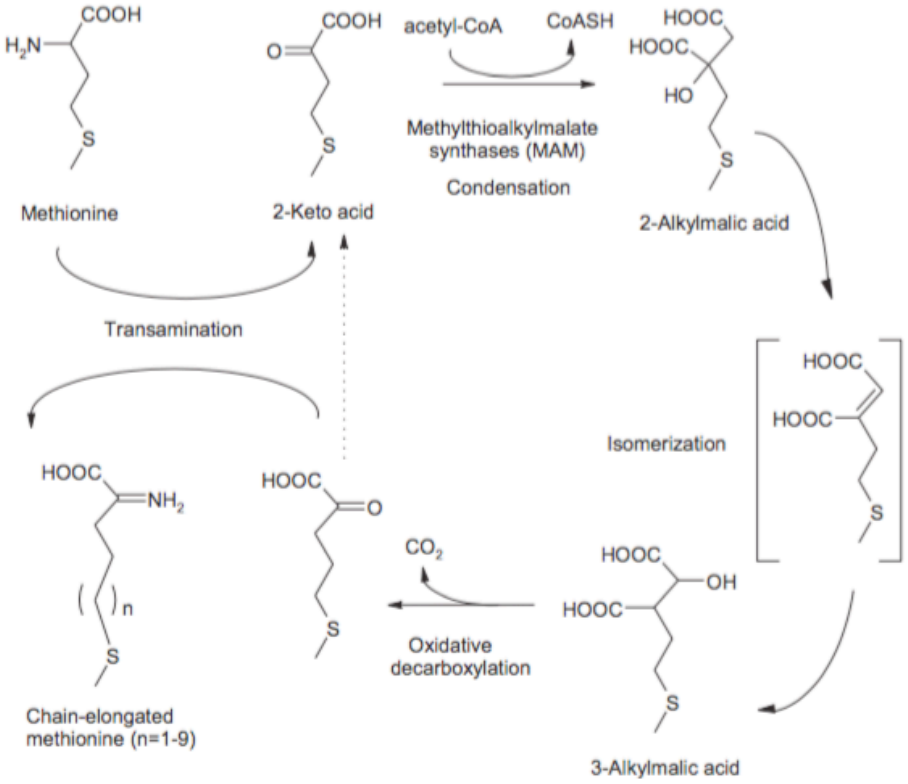


Figure 3: The chain elongation pathway of methionine in glucosinolates biosynthesis (Radojčić Redovniković, Glivetić, Delonga, & Vorkapić-Furač, 2008).

***1.2.1.2.2 Biosynthesis of core glucosinolate structure*:** The conversion of precursor amino acid to the final glucosinolates involves intermediates common to all glucosinolates. The core structure of the glucosinolates is accomplished in 5 steps via oxidation catalysed by cytochrome P450 of CYP79 and CYP83 families, followed by C-S-lyase, s-glycosyltransferase and sulfotransferase **(Figure 4)**. The intermediates involved in this pathway include N-hydroxy amino acids, aldoximes, *aci*-nitro or nitrile oxide compounds, S-alkyl thiohydroximates, thiohydroximic acids and desulfo glucosinolates. The cytochrome P450 enzymes of CYP79 families catalyse the conversion of amino acids to aldoximes. Five of the seven CYP79 homologues have been identified. Phenylalanine is the substrate for CYP79A2. The conversion of tryptophan to indole-3-acetaldoxime is catalysed by CYP79B2 and CYP79B3. The chain elongated methionine derivatives are metabolized by CYP79F1 and CYP79F2. All chain elongated methionine derivatives are metabolized by CYP79F1, whereas the catalytic activity of CYP79F2 is restricted to

pentahomo- and hexahomomethionine. The second step of the glucosinolate core formation is catalysed by the members of the CYP83 family, where an unstable aci-nitro intermediate is formed, that conjugates with the thiol group of cysteine. The two recombinants of CYP83 family are CYP83A1 that has high affinity for aliphatic aldoximes and CYP83B1 that prefers indole-3-acetaldoxime and aromatic aldoxime as substrates. The remaining steps of the glucosinolate core biosynthesis are common to all glucosinolates, regardless of the side chains involved. Where, C-S lyase catalyse the conversion of S-alkyl thiohydroximates to thiohydroximic acids. The enzymes s-glycosyltransferase and sulfotransferase catalyses the glycosylation and sulfation reactions, that finally forms the core structure of the glucosinolates (Velasco, Rodríguez, Francisco, Cartea, & Soengas, 2016).

1.2.1.2.3 Secondary modifications: The core glucosinolates structures are subjected to a wide range of modifications of the R-group as well as the glucose moiety. The modifications include oxidation, hydroxylation, methoxylation, desaturation and sulfation. For example, the R-group of glucosinolates derived from methionine and its chain elongated homologue are subjected modifications such as stepwise oxidation of the sulfur atom in the methylthioalkyl side chain leading to methylsulfinylalkyl and methylsulfonylalkyl moieties. Methylsulfinylalkyl side chain can be modified by oxidative cleavage to form alkenyl or hydroxyalkyl chains. The indolic glucosinolates usually undergo hydroxylation and methoxylation reactions catalysed by the CYP family of enzymes (Radojčić Redovniković, Glivetić, Delonga, & Vorkapić-Furač, 2008).

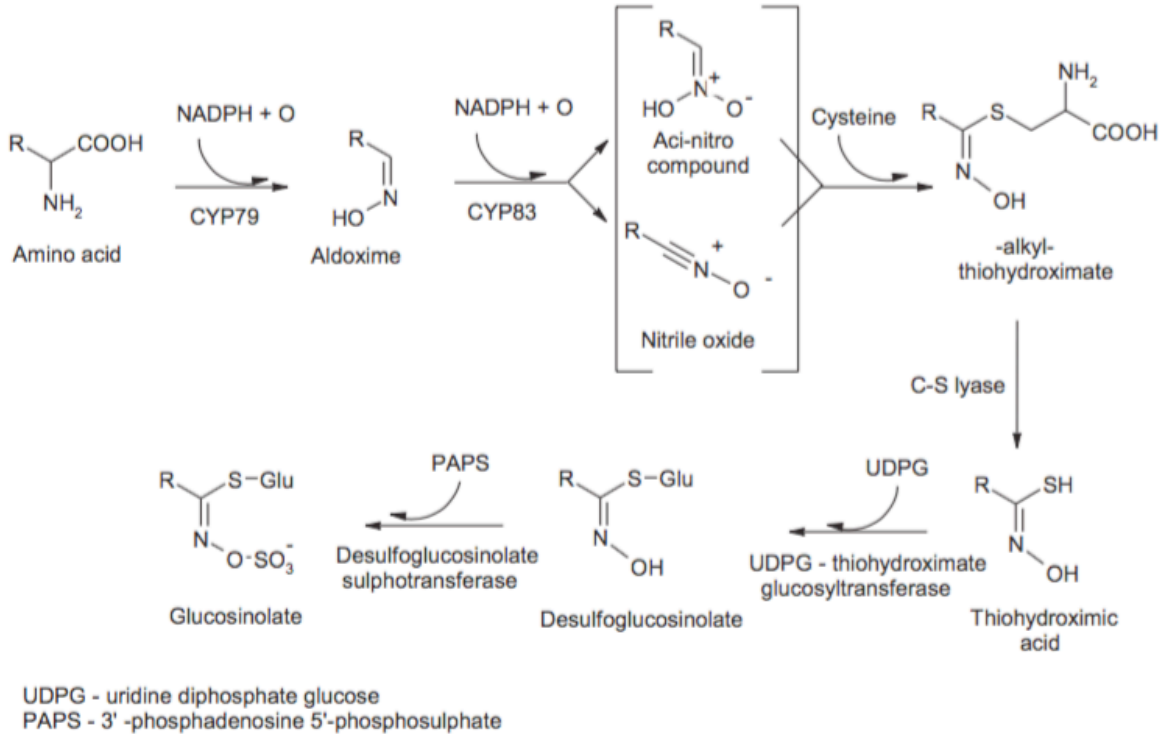


Figure 4: Biosynthesis of glucosinolate core structure (Radojčić Redovniković, Glivetić, Delonga, & Vorkapić-Furač, 2008).

1.2.1.3 Glucosinolate content in plants

Glucosinolates and their conversion molecules are widely studied for its pest resistance, health and nutritional value of crop plants. Historically, the organoleptic and goitrogenic properties of glucosinolates prevented its consumption and selection of vegetables with low glucosinolate content (Possenti, Baima, Raffo, Durazzo, Giusti, & Natella, 2017). Whereas, condiments such as mustard seeds (*Sinapis alba*) are bred for its piquancy. In recent times, the health benefits of broccoli surpass its flavour leading to its acceptability. Hence, hybrid varieties of broccoli have been bred that contains 3-4 times higher glucoraphanin content than the wild cultivars, providing superior health benefits (Blažević, Montaut, Burčul, & Rollin, 2015).

Glucosinolates are considered as the storage form of the biologically active isothiocynate and its concentration in brassica vegetables are highly variable. In fresh vegetables, the glucosinolates content is around 1% dry weight and in seeds it can exceed more than 10% in some species, for example seeds of *Moringa oleifera* have glucosinolate content as high

as 26%. The reproductive tissues (florets, flowers and seeds) have 10-40 times higher glucosinolate content than the vegetative segments. Seeds are considered the best source for the isolation of glucosinolates due to its abundance in the bioactive molecules (Possenti, Baima, Raffo, Durazzo, Giusti, & Natella, 2017). They are highly stable, easily stored and the low moisture content inside seeds helps retain the stability of the molecule preventing the conversion of glucosinolates to isothiocynates and its conversion molecules (Clarke, 2010).

The occurrence of glucosinolates in plants depend on varied biotic and abiotic factors such as variation within and between cultivars, plant parts and factors such as genetic, environmental and plant nutrients. The glucosinolate content in plants also vary depending on the developmental stages and plant cultivars (Villarreal-García & Jacobo-Velázquez, 2016). There is a high variability in the distribution of glucosinolates across the plant parts such as roots, stem, leaf and seeds, while the youngest tissue contains the highest amount. The abundance of glucosinolates in plant organs such as sprouts and seeds, provide a thorough insight on the use of these plant sources for its extraction and possible utilisation in agriculture as potential pesticides and in animal and human diets as functional nutraceuticals (Blažević, Montaut, Burčul, & Rollin, 2015).

1.2.1.4 Enzymatic degradation of glucosinolate

Glucosinolates are hydrophilic compounds, which are normally sequestered in the vacuoles. Endogenous enzyme called myrosinase (EC 3:2:3:1 thioglucosidase), is responsible for the hydrolysis of glucosinolates which are stored in the idioblastic cell (Clarke, 2010; Radojčić Redovniković, Glivetić, Delonga, & Vorkapić-Furač, 2008). Glucosinolates and myrosinase are stored separately inside the cell and once in contact initiates the hydrolysis of β-thioglucoside link leading to release of D-glucose and the formation of an unstable intermediate, or an aglycone thiohydroxamate-O-sulfonate, which spontaneously rearranges to its degradation products such as isothiocynates, thiocynates, nitriles, epithionitriles and oxazolidine-2-thiones (Moreno, Carvajal, López-Berenguer, & García-Viguera, 2006). **Figure 5** shows the different products of hydrolysis of glucosinolates. Various situations can cause the hydrolysis of glucosinolates: the wounding of the plant, chewing the fresh plant or injury caused by mechanical harvesting, freezing, transport or handling (Radojčić

Redovniković, Glivetić, Delonga, & Vorkapić-Furač, 2008). Parameters that influence the hydrolysis product of glucosinolates are: the structure of the parent glucosinolate, pH, temperature, presence of Fe^{2+} ions and protein interfering with myrosinase, such epithiospecifier protein (ESP) (Radojčić Redovniković, Glivetić, Delonga, & Vorkapić-Furač, 2008). The glucosinolate degradation products have varied health benefits such as anti-cancer, anti-inflammatory, anti-bacterial and prevention of cardiovascular diseases.

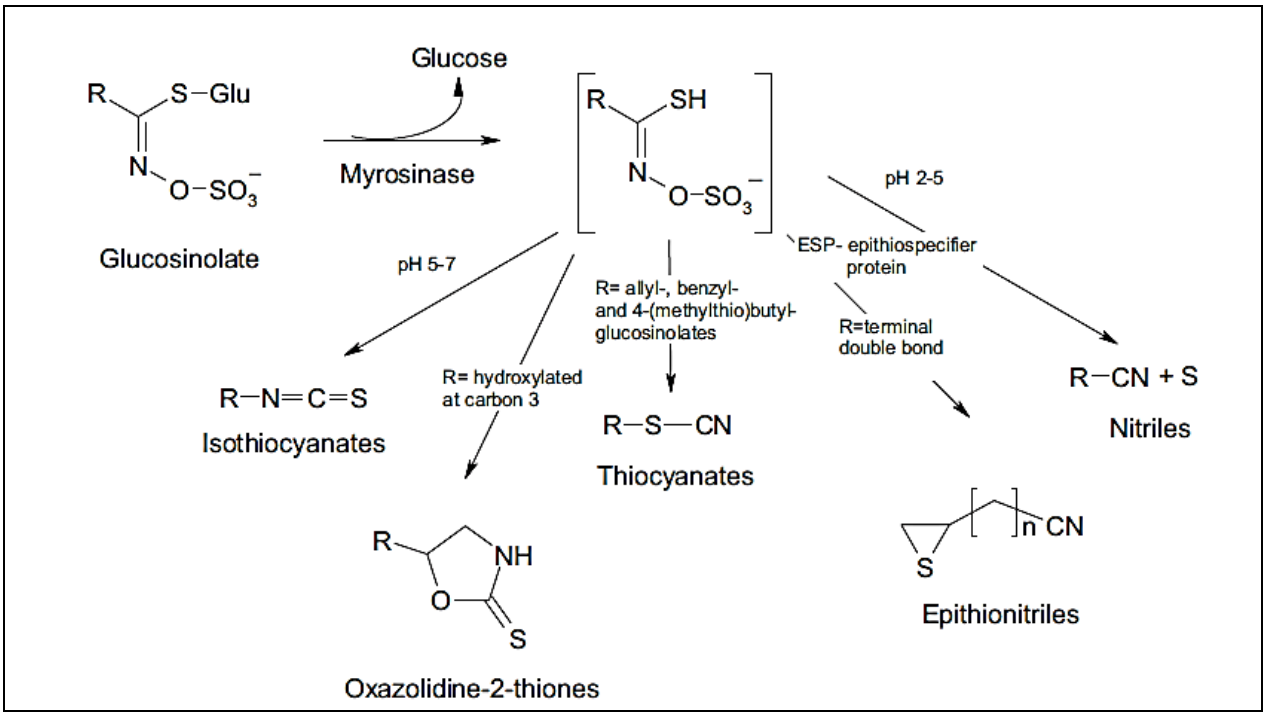


Figure 5: Structure of possible glucosinolate degradation production (Radojčić Redovniković, Glivetić, Delonga, & Vorkapić-Furač, 2008; Wittstock & Halkier, 2002).

1.2.2 Phenolic compounds

'Phenolic compounds' is a genetic term referring to a diverse group of compounds (more than 8000) in the plant kingdom, characterised by its hydroxylated aromatic ring **(Figure 6)**. They are plant secondary metabolites synthesised via the shikimic acid pathway. Phenylalanine ammonialyase (PAL) is the key enzyme in the shikimic acid pathway catalyses the biosynthesis of phenolic compounds from an aromatic amino acid phenylalanine (Cartea, Francisco, Soengas, & Velasco, 2010). Polyphenols play a prominent role as antioxidant compounds. Over the past few decades, extensive research has been done to identify and characterize plant polyphenols because of their health promoting and disease preventive properties (Jahangir, Kim, Choi, & Verpoorte, 2009).

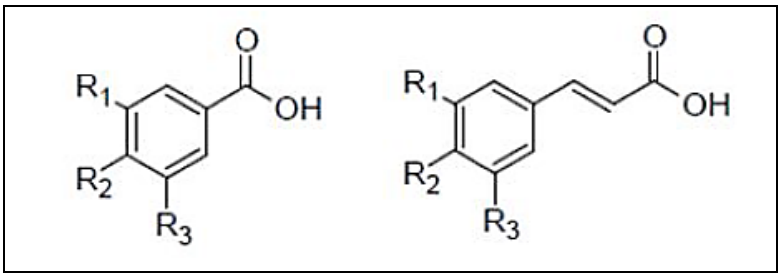


Figure 6: General structure of phenolic acids (A: hydroxybenzoic acid, B: hydroxycinnamic acid in which R1, R2, R3 = H, OH or OCH^3) (Crozier, Jaganath, & Clifford, 2006).

Phenolics range from simple, low molecular-weight, aromatic-ringed compounds to large complex tannins and derived polyphenols (Crozier, Jaganath, & Clifford, 2006; Pereira, Valentão, Pereir, & Andrade). Phenolic compounds are classified based on their arrangement and number of their carbon atom as flavonoids (flavonols, flavones, flavan-3-ols, anthocyanidins, flavanones, isoflavones etc.) (Crozier, Jaganath, & Clifford, 2006) and non-flavonoids (phenolic acids, hydroxycinnamates, stilbenes etc.) and they are commonly found conjugated to sugars and organic acids. In *Brassica* species, the most prevalent and diverse group of polyphenols are the flavonoids (flavonols and anthocyanins) and hydroxycinnamic acids (Cartea, Francisco, Soengas, & Velasco, 2010)**.**

1.2.2.1 Flavonoids

Flavonoid is the most predominant phenolic compound in the plant kingdom. They comprise of fifteen carbons, with two aromatic rings (A and B) connected by a three-carbon bridge (ring C) C6-C3-C6 **(**

Figure 7). Flavonols are the most widespread of the flavonoids. In *Brassica* species, the main flavonols are quercetin, kaempherol and isorhamnetin, commonly found as *O*-glycosides. In flavonoids conjugation occurs mostly in the 3rd position of the C-ring, however substitution can also occur at the 5, 7, 4′, 3′ and 5′ positions. In *Brassica* vegetables, there is a higher proportion of flavonoids conjugated to sugar moiety mainly glucose. They are also found acylated to different hydroxycinnamic acids. Flavonoids are localised in the epidermis of leaves and fruits and play an important role as a plant secondary metabolite. They are involved in UV protection, pigmentation, stimulation of nitrogen fixation and disease resistance (Crozier, Jaganath, & Clifford, 2006; Pereira, Valentão, Pereir, & Andrade).

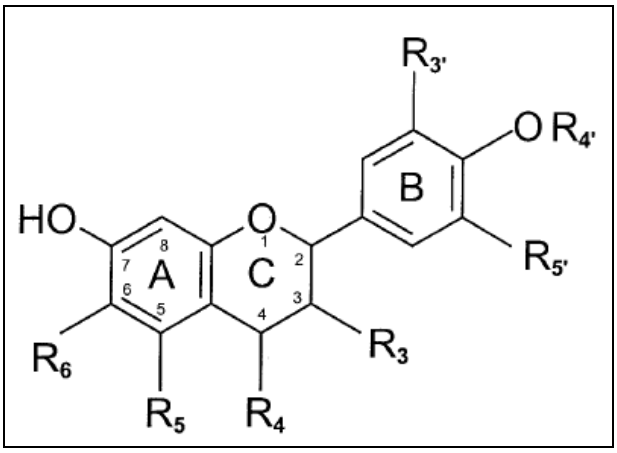


Figure 7: General structure and numbering pattern of food flavonoids in which R_4=H_5=OH and R_6=H, for majority of the food flavonoid (Beecher, 2003).

1.2.2.2 Hydroxycinnamic acid

Hydroxycinnamic acids are a kind of non-flavonoid phenolic characterized by a C6 - C3 structure. These compounds are found abundantly in plants and are involved in the structural and chemical defence strategies of plants. In the plant tissue, they are either present as insoluble polymers of the plant cell wall or compartmentalised in the plant cell vacuoles as soluble ester derivatives. In *Brassica* vegetables, the most common hydroxycinnamic acids are *p*-coumaric, sinapic and ferulic acids, often found in conjugation with sugars **(Figure 8)** (Cartea, Francisco, Soengas, & Velasco, 2010; Lin & Harnly, 2010; Olsen, Aaby, & Borge, 2010; Price, Casuscelli, Colquhoun, & Rhodes, 1997; Vallejo, Tomás-Barberán, & García-Viguera, 2002). Hydroxycinnamic acid and its derivatives are involved in the protection against UV-B and they are produced in response to plant injury.

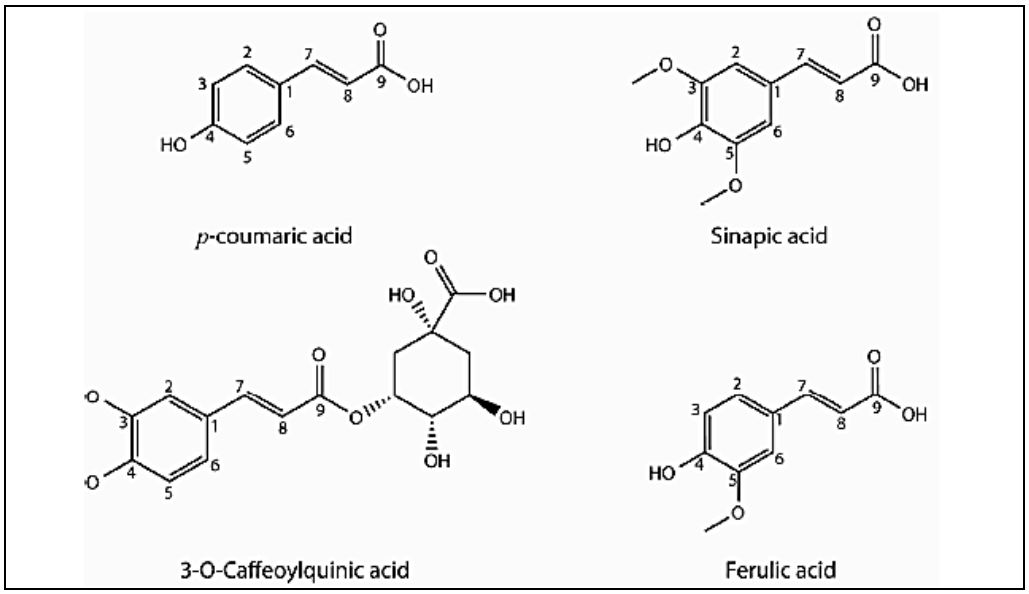


Figure 8: Hydroxycinnamic acid derivatives found in vegetable Brassica crops (Cartea, Francisco, Soengas, & Velasco, 2010).

1.3 Detection, Identification and Quantification

1.3.1 Glucosinolates, polyphenols and their degradation products

There are several methods for the determination of glucosinolates, polyphenols and their degradation products. One of the simplest methods for the determination of glucosinolates utilises their ability to form a coloured complex with palladium which can be detected using a colorimeter (Cartea, Francisco, Soengas, & Velasco, 2010). One of the earliest methods for the identification of glucosinolates and their hydrolysis products involve paper and thin layer chromatographic techniques (Moreno, Carvajal, López-Berenguer, & García-Viguera, 2006). West *et al*., (2002) devised a column based technique to resolve polar and non-polar glucosinolates. Similar techniques can be used to separate glucosinolates from polyphenols, using a reverse-phase C-18 column and 50mM ammonium acetate-methanol gradient mobile phase. Detection of glucosinolate conversion product sulphoraphane is achieved by HPLC techniques using a RP C-18 column with a gradient elution using 20% acetonitrile and water (Liang, Yuan, Dong, & Liu, 2006). A major breakthrough in the analysis of glucosinolates is achieved by an on-column enzymatic desulphation using sulphatase and quantitatively analysed by RP-HPLC **(Figure 9)** (Fenwick & Heaney, 1983).

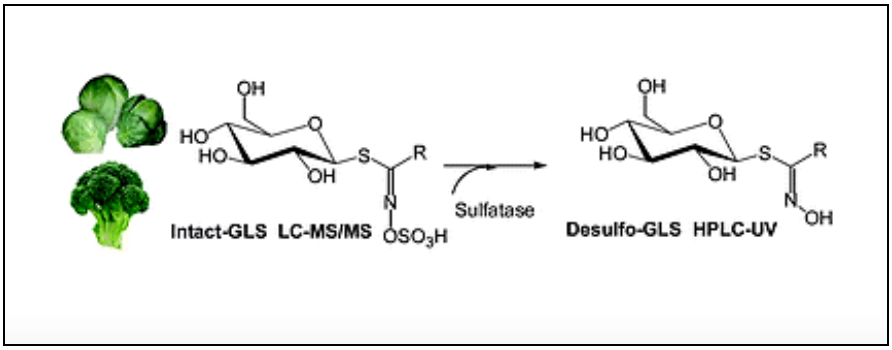


Figure 9*:* Desulfation of glucosinolates (Fenwick & Heaney, 1983; Moreno, Carvajal, López-Berenguer, & García-Viguera, 2006; Radojčić Redovniković, Glivetić, Delonga, & Vorkapić-Furač, 2008)**.**

Adsorption spectrometry UV/Visible and mass spectrometry (MS) are the most universal detection methods for glucosinolates and its degradation products. UV/Visible spectroscopy offers good sensitivity, short response time and versatility because many substances absorb in this spectral range. Mass spectrometry (MS) is a powerful method for detecting complex mixtures (Björkqvist & Hase, 1988). It requires the formation of gaseous molecular ions, separating them according to their mass-ratio of charge (m/z) and detection of emerging ions (De Hoffmann & Stroobant, 2007). Generally, intact glucosinolates are detected in negative ion mode, since they have a negative charge on their structure, whereas desulfoglucosinolates are detected in positive mode.

Most recent studies suggest the use of Electron Spray Ionization (ESI) technique for the detection of intact glucosinolates in the negative ion mode (Radojčić Redovniković, Glivetić, Delonga, & Vorkapić-Furač, 2008). MS/MS is a powerful detection technique that can be used to determine the structure of the molecule. It utilises collision-induced dissociation (CID) to fragment parent ion into daughter ions that are further separated based on m/z ratio (Moreno, Carvajal, López-Berenguer, & García-Viguera, 2006). The polyphenolic and flavonoid composition in the sample can be studied by liquid chromatography- UV diode-array detection (DAD) – electrospray ionisation mass spectrometry (ESI-MS) (Moreno, Carvajal, López-Berenguer, & García-Viguera, 2006). Currently, simultaneous detection of intact glucosinolates and polyphenols is possible by using UPLC-DAD-MS/MS technique with reduced time of analysis (Gratacós-Cubarsí, Ribas-Agustí, García-Regueiro, & Castellari, 2010)**.**

1.4 Biological Activity

1.4.1 Biological activity of glucosinolates

Glucosinolates and their degradation products are involved in the survival and defence system of crucifers against herbivores and pathogens (Radojčić Redovniković, Glivetić, Delonga, & Vorkapić-Furač, 2008). Consumption of glucosinolates is associated with various biological effects. It has been observed that excess consumption of glucosinolates is associated with toxic effects. For example, consumption of large quantities of cabbage by rabbits led to the development of goitre and growth retardation due to the negative effects of goitrogen (Stoewsand, 1995), but no harmful effects has been found on humans (Hanschen, Lamy, Schreiner, & Rohn, 2014). Whereas, glucosinolate hydrolysis products such as indole-3-carbinol and sulforaphane have positive health impact. *In vitro* and *in vivo* experimental studies indicate a positive correlation between the consumption of plants rich in glucosinolates and reduced indices of diseases such as cancer, mainly prostate, lung, bladder and colon (Latté, Appel, & Lampen, 2011), diabetes (Axelsson, et al., 2017), neurodegenerative and age related diseases (Lee, Kim, Seo, Choi, Han, Lee, et al., 2014) and coronary heart diseases (Ares, Nozal, & Bernal, 2013), due to their antimicrobial (Yanaka, Fahey, Fukumoto, Nakayama, Inoue, Zhang, et al., 2009), anti-inflammatory and anti-oxidant properties (Jeffery & Araya, 2009).

Over the last few years, cancer prevention and delaying the onset of cancer using natural products have received considerable attention. Glucosinolates and its degradation molecules such as ITC and sulphoraphane **(Figure 10)**, have anti-cancerous and anti-neoplastic effects by blocking carcinogens from initiating tumour in several organs (Moreno, Carvajal, López-Berenguer, & García-Viguera, 2006). Glucoraphanin, under the action of myrosinase forms the 4-methylsulfinylbutyl isothiocyanate, commonly known as sulforaphane, one of the most important natural compounds with known anti-cancer properties (Domínguez-Perles, Martínez-Ballesta, Carvajal, García-Viguera, & Moreno, 2010; Mithen, Faulkner, Magrath, Rose, Williamson, & Marquez, 2003; Moreno, Carvajal, López-Berenguer, & García-Viguera, 2006). Among the glucosinolates in the class of indoles present in broccoli, glucobrassicin is the main representative, which degrades into indole-3-carbinol, and is metabolized to bioactive compound such as 3,3 '-di-

indolemethane, which also demonstrated anti-carcinogenic effects (Moreno, Carvajal, López-Berenguer, & García-Viguera, 2006).

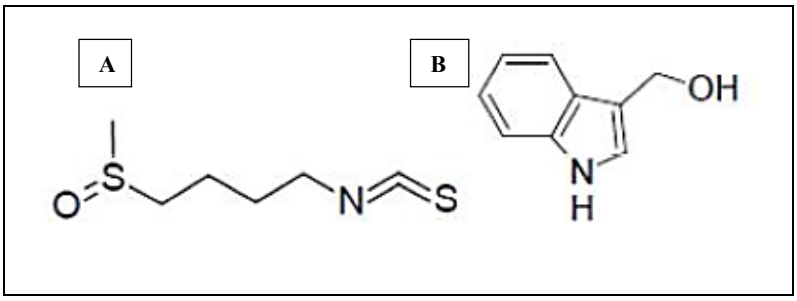


Figure 10: General structure of A: Sulforaphane and B: indole-3-carbinol, anti-cancer compounds derived respectively from the glucoraphanin and glucobrassicin (Moreno, Carvajal, López-Berenguer, & García-Viguera, 2006).

These isothiocyanates and indoles would act on enzyme metabolism, influencing several processes related to the metabolism of chemical carcinogenesis and carcinogen binding to DNA (Verhoeven, Verhagen, Goldbohm, van den Brandt, & van Poppel, 1997). Empirically, they potentially induce detoxification enzymes of phase II in mammalian cell cultures and rodents (Latté, Appel, & Lampen, 2011; Moreno, Carvajal, López-Berenguer, & García-Viguera, 2006; Verhoeven, Verhagen, Goldbohm, van den Brandt, & van Poppel, 1997). Sulforaphane has also shown an inductive effect of apoptosis, selectively to cancer cells (Mithen, Faulkner, Magrath, Rose, Williamson, & Marquez, 2003). By interfering with the rate of growth of cancer cells, the isothiocyanates are active chemoprotectants against cancer. The isothiocyanates have also shown significant antimicrobial effects against *Helicobacter pylori*, a bacterium that colonizes the stomach, which is a major risk factor for the development of gastric cancer (Ganin, Rayo, Amara, Levy, Krief, & Meijler, 2013).

1.4.2 Biological activity of phenolic compounds

Over the past few decades, great interest has been given to plant phenols and flavonoids by the scientific community. The main polyphenols present in broccoli are flavonoids and hydroxycinnamic acids. It has also been observed that plant polyphenols have health promoting properties such as anti-inflammatory, antimicrobial, anti-allergic, cytotoxic antitumor activity and most importantly antioxidant capacity (Chu, Chang, & Hsu, 2000; de Pascual-Teresa, Moreno, & García-Viguera, 2010; Fukumoto & Mazza, 2000; Plumb, Price, Modes, & Williamson, 1997; Podsędek, 2007). The antioxidant ability of plant

phenolics is associated with their chemical structure. The number and position of the hydroxyl groups on phenolic compounds confer the antioxidant ability and with an increase in the number of hydroxyl group leads to higher antioxidant activity (Cartea, Francisco, Soengas, & Velasco, 2010). It has been observed that various diseases like cancer, aging, atherosclerosis, inflammation and neurodegenerative diseases such as Parkinson's (PA) and Alzheimer's disease (AD) can be correlated to oxidative stress (Mullen, Marks, & Crozier, 2007; Shen, Callaghan, Juzwik, Xiong, Huang, & Zhang, 2010). Hence, antioxidant polyphenolic compounds provide protection against oxidative damage caused by Reactive Oxygen Species (ROS) thereby delaying the onset of various chronic diseases as well as prevent the oxidation of low-density lipoproteins (LDL), that plays an important role in atherosclerosis (Cartea, Francisco, Soengas, & Velasco, 2010).

Flavonoids, a plant phenolic compound can regulate a large variety of mammalian enzymes that are involved in the regulation of cell division, proliferation, platelet aggregation, detoxification, inflammatory and immune response. However, it has been noticed that, the health promoting effect of flavonoids can, not only be because of its antioxidant activity but also due to the modulation of various cellular pathway critical in the pathogenesis of various diseases (de Pascual-Teresa, Moreno, & García-Viguera, 2010; Skandrani, Limem, Neffati, Boubaker, Sghaier, Bhouri, et al., 2010).

1.5 Food Waste Recovery

World hunger and food security are the burning issues of today's society. According to United Nation's Food and Agricultural Organisation (FAO, Food Loss and Wastage Report 2011), 1/3 of the edible part of the food produced for human consumption is lost or wasted globally. There are serious economic and environmental impacts due to rampant food wastage and the disparity in the distribution of resources across developed and underdeveloped countries (Olsen, Aaby, & Borge, 2010). The journey of food from farm to fork are subjected to various losses during the agricultural production, post-harvest handling and storage, food processing, retail and consumer levels. In the developing countries, 40% of the food is lost during crop harvesting, postharvest handling and food processing. This is mainly due to the lack of infrastructure and storage facilities, improper crop handling skills and lack of agricultural and postharvest techniques. Whereas, in the

industrialized countries, food is wasted at the retail and consumer level due to stringent market regulations and consumer demands (Kummu, de Moel, Porkka, Siebert, Varis, & Ward, 2012). Around, 222 million tons of food are wasted by the industrialized countries, which is equivalent to the net food production in sub-Saharan Africa (230 million ton). Overall, 45% of fruits and vegetables produced for human consumption are lost or wasted globally (Lipinski, Hanson, Lomax, Kitinoja, Waite, & Searchinger, 2013).

In spite the nutritional loss across the food chain, rampant food loss and wastage leads to the generation of food residues that are disposed and discarded at great expense (Thomas, Badr, Desjardins, Gosselin, & Angers, 2017). Over the years, the most commonly used techniques for bioremediation are incineration, composting, use as landfills, animal feed and substrate for solid state fermentation. These modes of discard are not only expensive but also have adverse effects on the environment, for instance, release of greenhouse gases such as nitrous oxide and carbon dioxide (Domínguez-Perles, Martínez-Ballesta, Carvajal, García-Viguera, & Moreno, 2010; Laufenberg, Kunz, & Nystroem, 2003; Moreno, Carvajal, López-Berenguer, & García-Viguera, 2006). Recently, consumer awareness about the negative impact of food disposal and legislative pressure have led to its valorisation. The waste generated by the agro-food industry represents economic loss to the farmers. Hence, finding an alternative use to the lost crop can, not only be highly beneficial to the farmers and marketers, but also create a positive socio-economic impact, bridging us closer to sustainability (Otles, Despoudi, Bucatariu, & Kartal, 2015).

Despite the plethora of information available on fresh materials, scares information is available on the biorefining of by-products. The lost or wasted plant residues can be an excellent source of nutraceutical compounds, that may be recovered for the fabrication of food additives with positive health effects (Otles, Despoudi, Bucatariu, & Kartal, 2015). The global nutraceutical market is a 140 billion US $ industry, that is growing annually at an accelerated rate of 14.7% (Villarreal-García & Jacobo-Velázquez, 2016). Valorisation of industrial residues from the agro-food industry for the production of extracts have double sided benefits. One is the generation of good revenue out of the wasted materials and secondly to sustain the environment for its present and future needs (Charis Michel Galanakis, 2015).

Valorisation of food residues create new opportunities and benefits to everyone related to the food production system. Food residues for the fabrication of functional food ingredients reutilises the discards, enabling the fresh ingredients to be utilised as food rather than for the fabrication of fortified foods, thereby enhancing food availability. Waste recovery for the fabrication of functional foods also reduces the loss of energy, enhancing raw material usage, thus reducing the cost of production and enhancing environmental performance (Thomas, Badr, Desjardins, Gosselin, & Angers, 2017). Hence, food waste recovery brings us closer to food security and food sustainability, meeting the needs of the present generation without compromising the requirements of the future (Villarreal-García & Jacobo-Velázquez, 2016).

1.6 Dietary Supplements and Biotechnological Production

Health conscious consumers are becoming increasingly aware of the adverse effects of processed foods and the positive benefits of natural ingredients on health, to reduce the onset of diseases. Also, increasing research on identifying bioactive ingredients in natural diet have led to the concept of dietary supplements or functional food supplements. "Dietary supplements" or "functional food supplements" are the concentrated form of health beneficial bioactive ingredients (Bernhoft, 2010; Cartea & Velasco, 2008; Moreno, Carvajal, López-Berenguer, & García-Viguera, 2006). They are accompanied along with normal diet, in order to increase their consumption, which are claimed to have a desired biological effect (Radojčić Redovniković, Glivetić, Delonga, & Vorkapić-Furač, 2008).

1.6.1 Dietary supplement

1.6.1.1 Factors influencing the evolution of supplement fabrication (Charis Michel Galanakis, 2015)

1. Awareness among consumers regarding the positive health effects on the consumption of fresh fruits and vegetables
2. Increased awareness about the adverse effects of chemical preservatives and synthetic additives.
3. The necessity to replace synthetic additives with natural ingredients
4. Reduced availability of fresh fruits and vegetables throughout the year

5. Variations in the concentration of bioactive ingredients present in fruits and vegetables, thereby, making the standardization of intake dosage difficult
6. Unavailability of beneficial super food ingredients across the globe.
7. Special needs for immunocompromised patients, the elderly, sports personals and body builders to correct the nutritional deficiencies or to maintain an adequate intake of certain nutrients.
8. The need to acquire the right proportion of nutrients for the desired biological effects would necessitate the consumption of huge quantities of fruits and vegetables daily.

1.6.1.2 Constrains associated with the development of health supplements from industrial discards (Charis M. Galanakis, 2015)

1. Availability of reliable quality starting material throughout the year
2. Variability in the chemical composition of the bioactive compounds in the industrial discards.
3. Lower concentration of bioactive ingredients in the starting material when compared to the industrial or commercial plants, that are rich in the desired target molecules.
4. Augmented vulnerability, due to low biological stability of the starting material. Industrial discards are subjected to microbial contaminations, requiring immediate processing and treatment.
5. Increased processing cost, due to low recovery yields of the bioactive ingredients from the discards, thereby generating lower revenue.
6. Technical constrains in the development of an economically feasible and industrially viable extraction and purification process, using food grade solvents.

1.6.1.3 The limitations in the commercialisation of nutraceutical extracts from industrial discards

Over the years, increasing research on valorisation of industrial discards and technological development, have led to the advancement in the fields of food waste recovery and recycling. But, stringent consumer and marketing regulations make commercialisation of bioactive extracts from industrial discards difficult. The major limiting factors that retard

the advancement of this sector, is the development of an economically and environmentally feasible process for the fabrication of the extracts. The variability in the composition of the bioactive ingredients in the starting material brings in an inconsistency in the final product, making standardisation difficult. Despite, rampant food wastage; only limited number of marketable extracts have been fabricated from these industrial discards. Regardless of its advantage over synthetic compounds, these products are subjected to stringent consumer and legal regulations; to meet the quality standards of the food and nutraceutical industry (Charis M. Galanakis, 2015; Charis Michel Galanakis, 2015).

1.6.2 Extraction, purification and fabrication of extracts

1.6.2.1 Extraction of glucosinolates

Over the years, there has been considerable research on the extraction and isolation of glucosinolates from plants belonging to the brassica family, predominantly broccoli. Plethora of information is available on fresh materials; but, scarce information is available on valorization of industrial discards or residues. There is an increasing number of publication on the extraction of glucosinolates, using solvents as they are affordable and easy to perform (Otles, Despoudi, Bucatariu, & Kartal, 2015). Solid phase extraction (SPE), is another commonly used procedure for the extraction of target molecules from plant matrix, to obtain cleaner and purer extracts with the simultaneous concentration of the samples. Other sample extraction process includes supercritical fluid extraction (SFE), microwave-assisted extraction (MAE) and soxhelt extraction (Ares, Nozal, & Bernal, 2013).

Extraction of bioactive molecules from plant matrix is a complex process influenced by various process parameters such as nature of the starting material, structural characterization, solvent characteristics, localisation and stability of the target molecules.

1.6.2.1.1 Factors influencing the extraction process (Charis M. Galanakis, 2015; Gerschenson, Deng, & Cassano, 2015)

1. Nature of the starting material

   Nature of the starting material plays an important role in the extraction of the bioactive ingredients. Water content inside the sample, solid and oil ratio, activation

or deactivation of the enzymes, microbial load on the sample, porosity and permeability of the starting material plays a significant role in the ease of extraction of the plant bioactives. Plants with higher water content are either dried or lyophilized to maintain the stability and functionality of the target molecules, to maximize the yield of the bioactive molecule (Gerschenson, Deng, & Cassano, 2015).

2. Structural characterization

   Structural parameters such as plant matrix microstructure and its interaction with the target molecule, its molecular weight, intermolecular polarity, the presence of reactive functional groups and the isoelectric point of the bioactive molecule are the main factors influencing the extraction process (Charis M. Galanakis, 2015).

3. Solvent characteristics

   Selection of a desired solvent plays a crucial role in maximizing the extractability of glucosinolates or bioactive molecules from the plant matrix.

   Characteristics of an ideal solvent are (Charis M. Galanakis, 2015):

   1. Cheap and easily available
   2. Reusable and recyclable
   3. Capability to extract a wide range of molecules
   4. Ability to inhibit the enzyme activity maintaining the functionality and stability of the target molecule
   5. Location and stability of the target molecule

      Location and stability of the target molecule plays a pivotal role in the efficient extraction of the target molecule. Glucosinolates are anionic molecules sequestered inside the vacuole, that are separated from its degrading enzyme myrosinase, present in the cell cytosol. In nature glucosinolates exist as its potassium salt. Factors such as nature of the extracting solvent, temperature, pH, duration of extraction and composition of the vegetable matrix plays a significant role in the isolation of the target molecule. An efficient method for glucosinolates extraction should retain the stability of the glucosinolates along with the inactivation of the enzyme myrosinase, maximizing its yield (Clarke, 2010).

Alcohol or protic solvents, are the most popular solvent used for the extraction of the bioactive molecules from plant matrix. Methanol-water, ethanol-water, water, phosphate buffer, phosphoric acid, methanol-water- ammonia have been successfully employed for the extraction of glucosinolates from brassica species. Of all the solvents, methanol is the most widely used, as it facilitates cellular disruption, liberating the target molecules, easing the extraction process, but, the toxic nature of methanol limits its applicability in the food industry (Clarke, 2010). Aqueous ethanol is a feasible alternative, for the extraction of bioactive molecules, inhibiting the enzyme activity by lowering the pH to an acidic range of 2-3. Aqueous ethanol generates a powerful co-solvent, as water swells the extracting material, improving the extractability of the bioactive molecule. The major limitation of this technique is the co-extraction of water soluble compounds such as organic acids and sugars, requiring additional purification process (Prado, Vardanega, Debien, Meireles, Gerschenson, Sowbhagya, et al., 2015).

1.6.2.2 Purification of glucosinolates

Crude extracts of broccoli contain glucosinolates along with chlorophyll, plant pigments and oils that are highly labile; and therefore, subjected to various isolation and purification techniques (Schoefs, 2004). Solvent based and chromatographic separation of glucosinolates are the two commonly used techniques for the purification of glucosinolates.

***1.6.2.2.1 Solvent purification process*:** The solvent based purification process involves the use of highly salted and highly polar biphasic solvent system: 1-propanol/acetonitrile/saturated aqueous ammonium sulfate and water (1:0.5:1.2:1), for the purification of glucosinolates. This method of purification relies solely on the partition coefficient of the solute between the stationary phase and the mobile phase, separating gram quantities of structurally and chromatographically similar glucosinolates. The applicability of this technique in the fabrication of functional food ingredients are limited, due to the use of non-food grade solvents for the purification process (Fahey, Wade, Stephenson, & Chou, 2003; Toribio, Nuzillard, & Renault, 2007; Wang, Liang, & Yuan, 2012).

***1.6.2.2.2 Ion-exchange purification process*:** Chromatographic separation of glucosinolates using ion-exchange resins is based on the inherent anionic charge of the glucosinolates. The crude sample containing charged molecules, bind to the oppositely charged sites in the stationary phase. Anion exchanger resin is the most commonly used ion exchange resin for the purification of glucosinolates. Identification of an ideal operation process for the purification of glucosinolates using ion-exchange resins depend on various parameters (Bertin, Frascari, Domínguez, Falqué, Riera Rodriguez, & Blanco, 2015).

***1.6.2.2.2.1 Stationary phase selection*:** An ion exchange resin (stationary phase), have a positively or negatively charged functional group, covalently linked to a solid matrix. The most commonly used matrixes are cellulose, polystyrene, agarose and polyacrylamide. The two most common ion exchange resin are cation exchange and anion exchange (Gallaher, Gallaher, & Peterson, 2012; Toribio, Nuzillard, & Renault, 2007; Wang, Liang, & Yuan, 2012). In the anion exchange the stationary phase is positively charged and in the cation exchange it is negatively charged. The major factors that can influence the choice of the stationary phase are flow rate, pH, particle size, binding capacity, nature and stability of the target molecule. The most commonly used anionic ion exchange resins for the purification of glucosinolates is diethylaminoethyl- (DEAE) – Sephadex (Bertin, Frascari, Domínguez, Falqué, Riera Rodriguez, & Blanco, 2015; Mohn, Cutting, Ernst, & Hamburger, 2007).

***1.6.2.2.2.2 Displacer selection*:** The success of the ion exchange resin for the purification of glucosinolates depends on the selection of the displacer. The displacer ion selected, should have a greater affinity towards the ion-exchange resin than the target molecule. Sodium hydroxide, potassium hydroxide, potassium sulphate, ethyl acetate and ammonium hydroxide are the most commonly used displacers for ion-exchange resins. For the purification of glucosinolates ammonium hydroxide provides the best results (Toribio, Nuzillard, & Renault, 2007).

***1.6.2.2.2.3 Displacer solvent screening*:** An ideal solvent system for the displacer depends on the ability of the displacer in different solvent concentrations or solvent type to extract glucosinolates from the stationary phase. The use of polar solvents such as

methanol, water, ethanol, hexane, ethyl acetate, acetonitrile are usually preferred due to the hydrophilic nature of the glucosinolates (Toribio, Nuzillard, & Renault, 2007).

1.6.3 Fabrication of extract rich in glucosinolates

The liquid extract obtained after extraction and purification can be used for the extract preparation process. The purified extract rich in glucosinolates are further concentrated using a rota-vapour to remove the excess displacer solvent. Further, bulking agents such as maltodextrin, arab gum are added to enhance the solid content inside the sample, to improve the drying and extract formation process. The most commonly used extract formation techniques are vacuum drying, lyophilization and atomization (spray drying) (Desobry, Netto, & Labuza, 1997; Gerschenson, Deng, & Cassano, 2015; Souza & Oliveira, 2005).

1.6.3.1 Vacuum drying is the most commonly used technique partially because of its ease of operation and its affordability. Parameters such as temperature, solid content, co-extracted plant matrices and time of contact are the most critical parameters that influence the drying process. The major limitation of this process is that the target molecules are exposed to high heat for a longer duration, influencing its integrity and stability.

1.6.3.2 Atomization or spray drying requires the optimization of various parameters such as the solid content of the extract that can be modulated based on the carrier materials (maltodextrin and arab gum), the flow rate of the extract, the inlet and outlet temperature of the drying air, particle size and the moisture content and the concentration of the active molecule in the final powered extract. This is the most preferable technique for the fabrication of powdered extract due to minimal residence time inside the atomizer and reduced exposure time at high temperature, preventing the degradation of the target molecule.

1.6.3.3 Lyophilisation or freeze drying is the removal of ice or other frozen solvent from the extract through the process of sublimation and the removal of bound water molecule by the process of desorption. The major advantage of this technique is its short reconstitution time and the superior quality of the extracts. This technique is highly suitable

for heat sensitive materials such as proteins, microbes, pharmaceuticals, tissues and plasma. But, the major limitation of this technique is the cost of operation.

Hence, incorporation of bioactive ingredients from industrial discards into nutraceutical and functional food supplements provides an alternative route for food recovery, along with socio-economic sustainability.

Chapter 2

Problematic, Research Hypothesis, Principle Objective and Specific Objective

2.1 Problematic

Rampant food wastage and increasing environmental pollution are the burning issues of today's society. The increasing demand for fresh products have led to its increased production, generating of huge quantities of waste that are discarded at great expense. Also, the negative effects of artificial additives in ready to eat foods, have made consumers to shift to natural additives over synthetic ingredients. Plethora of information is available on the valorization of normally consumed plant parts using non-food grade solvents, whereas, scarce information is available on industrial discards using green solvents. Despite, the high extractability and ease of extraction using non-food grade solvents, the environmental and toxic effects of non-food grade solvents, limits its applicability in the food industry. The discards generated by the agrifood industry can be a potent source of bioactive molecules, that can be utilized for the fabrication of bioactive extracts. But, the high regulatory and consumer standards, along with the high cost of production limits its applicability, in the food industry. Therefore, developing a sustainable industrially viable green process for the fabrication of extracts from industrial discards can be challenging.

2.2 Research Hypothesis

It is hypothesized that downgraded vegetable residues of broccoli, rich in bioactive molecules can be extracted using green technology and further formulated as ingredients for the health food market. Developing such a technology can prove to be beneficial for health and also to the environment, with optimal utilization of the natural resources and minimizing wastage.

2.3 Principle Objective

The overall objective of this project is to develop an industrially feasible and sustainable glucosinolate extraction and purification process using 'green chemistry' from broccoli

industrial discards and develop a potential bioactive extract with specific bioactivity for its use in animal nutrition as health supplements.

2.4 Specific Objectives

1. To identify, characterize and quantify the glucosinolates and polyphenols content in broccoli co-products using UPLC MS/MS and spectrophotometric methods as a first step towards industrial biorefining.
2. To develop a robust, economic and environmental friendly glucoraphanin extraction process by optimizing the extraction parameters such as time and temperature, sample to solvent ratio, ethanol concentration and pH of the extracting solvent, using green technology.
3. To systematically investigate and optimize the most important factors that influence the purification of glucoraphanin from broccoli industrial discards, seeds and florets using ion exchange resins by response surface methodology.

Chapter 3

Characterization of Broccoli by-Products (*Brassica oleracea* var. italica) for Their Glucosinolates, Polyphenols and Flavonoids Content Using UPLC MS/MS and Spectrophotometric Methods

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Keywords: Broccoli by-products, glucosinolates, polyphenols, agrifood industry, valorization, functional food supplements, bio-refining, socio-economic sustainability

3.1 Foreword

Broccoli has been widely studied for its varied health effects due to its abundance in plant bioactives such as glucosinolates and polyphenols and most distinctively, glucoraphanin. In this chapter, we have mainly focused on the characterization and quantification of glucosinolates and polyphenols present in 10 rejected lots of broccoli seed cultivars and industrial residues of broccoli florets, stalk and mixture (the combined residual segments after processing). Further, the different cultivars were compared for their nutraceutical compounds, to identify a promising source for the commercial extraction of the bioactive molecules using green technology.

This chapter is presented as an article entitled: "Characterization of industrial broccoli discards (*Brassica oleracea* var. italica) for their glucosinolate, polyphenol and flavonoid contents using UPLC MS/MS and spectrophotometric methods" published in Food Chemistry of which the authors are Minty Thomas; Ashraf Badr; Yves Desjardins; André Gosselin; Paul Angers. (245, 1204-1211, doi.org/10.1016/j.foodchem.2017.11.021)

The authors are Minty Thomas (first author), who participated in the planning, interpretation, discussion and writing of the article. Dr. Paul Angers, director of the thesis, and Dr. André Gosselin, Dr. Yves Desjardins, co-directors of the thesis participated in the planning of the research, the discussion of the results at meetings and the revision of the article presented. Dr. Ashraf Badr, research professional, participated in the discussion of the results and in the revision of the article.

As the future perspective this work, the promising starting materials, as identified in this study, were utilized to optimize the extraction of the bioactive ingredients from broccoli industrial discards using green solvents.

3.2 Résumé

L'industrie agroalimentaire produit des tonnes de déchets et des produits de qualité inférieure qui sont jetés à grands frais. La valorisation des résidus industriels limite les problèmes liés à la sécurité alimentaire et aux problèmes environnementaux. Le brocoli (*Brassica oleracea* var italica) est associé à divers effets bénéfiques sur la santé, mais sa production produit plus de 25% de rejets. Le but de cette étude était de caractériser et de quantifier les sous-produits industriels du brocoli pour leur teneur en glucosinolates et en polyphénols comme première étape vers le bioraffinage industriel. Les segments de brocoli et les lots rejetés de 10 cultivars de graines ont été analysés en utilisant UPLC MS/MS. La variabilité du contenu des molécules bioactives a été observée entre les cultivars. Les sous-produits du brocoli étaient riches en glucosinolates (0,2-2% de poids sec), principalement en glucoraphanine (32-64% des glucosinolates totaux), alors que la teneur en polyphénols était inférieure à 0,02% en poids sec. La valorisation des résidus industriels facilite la production d'ingrédients alimentaires fonctionnels de grande valeur ainsi que la durabilité socio-économique.

3.3 Abstract

The agrifood industry produces tons of waste and substandard products that are discarded at great expense. Valorization of industrial residues curbs issues related to food security and environmental problems. Broccoli (*Brassica oleracea* var. italica) is associated with varied beneficial health effects, but its production yields greater than 25% rejects. We aimed to characterize and quantify industrial broccoli by-products for their glucosinolates and polyphenols content as a first step towards industrial bio-refining. Broccoli segments and rejected lots of 10 seed cultivars were analyzed using UPLC MS/MS. Variability in the contents of bioactive molecules was observed within and between the cultivars. Broccoli by-products were rich in glucosinolates (0.2-2% dry weight sample), predominantly glucoraphanin (32-64% of the total glucosinolates), whereas the polyphenolic content was less than 0.02% dry weight sample. Valorization of industrial residues facilitates the production of high value functional food ingredients along with socio-economic sustainability.

3.4. Introduction

Food security and environmental pollution are the burning issues in today's society. According to United Nation's Food and Agricultural Organization (FAO, Food Loss and Wastage Report 2011), "one third of the food that is produced for human consumption is lost or wasted globally", accounting for around 186 kilograms of food per person lost annually. By 2050, with increasing population, the demand for food will increase and food by-products are expected to reach 9 billion tons. Limitations in natural resources such as land, water and energy along with an increasing urbanization makes it difficult to meet the requirements of the ever growing population leading to food insecurity (Otles, Despoudi, Bucatariu, & Kartal, 2015).

The journey of food from farm to plate is subjected to various losses. First and foremost, food loss occurs at postharvest and processing levels mainly due to lack of technological advancement, improper agricultural practices and insufficient transportation and storage facilities. This kind of food loss mainly happens in developing countries. Whereas, in developed countries, squandering of food and resources appears mainly at consumer and retail levels due to stringent market and consumer requirements (Kummu, de Moel, Porkka, Siebert, Varis, & Ward, 2012). As reported by the FAO (2011 Global Food Loss and Wastage Report), the food wasted in developed countries (222 million tons) corresponds to the annual food production in sub-Saharan Africa (230 million tons). The huge quantities of waste generated not only raises food security concern but also environmental burden by increasing the emission of greenhouse gases such as carbon dioxide, nitrous oxide leading to climate change (Lipinski, Hanson, Lomax, Kitinoja, Waite, & Searchinger, 2013).

Over the years, extensive research has been performed on fresh fruits and vegetables to identify potent bioactive molecules that possess health promoting and disease preventive properties. There is sufficient evidence that plants belonging to the Brassicacea family, especially broccoli (*Brassica olerecea* var. italica), are rich in plant bioactives like glucosinolates and their degradation molecules, polyphenols (flavonoids and hydroxycinnamic acids), vitamins (ascorbic acid), minerals (manganese, potassium and selenium) and carbohydrates (dietary fibers) (Moreno, Carvajal, López-Berenguer, & García-Viguera, 2006; Vasanthi, Mukherjee, & Das, 2009).

In developed countries, the leading causes of death are cancer and cardiovascular diseases (Liu, 2003). Epidemiological and cohort studies indicate a positive correlation between the consumption of plants belonging to the brassica family and reduced indices of diseases such as cancer, mainly prostate, lung, bladder and colon (Latté, Appel, & Lampen, 2011), neurodegenerative and age related diseases (Lee, Kim, Seo, Choi, Han, Lee, et al., 2014), and coronary heart diseases (Ares, Nozal, & Bernal, 2013), due to their antimicrobial (Yanaka, Fahey, Fukumoto, Nakayama, Inoue, Zhang, et al., 2009), anti-inflammatory and anti-oxidant properties (E. H. Jeffery & Araya, 2009). The health promoting and disease preventive properties of the Brassicaceae family, especially broccoli, have resulted in intensified research on these vegetables.

Glucosinolates are sulphur containing plant secondary metabolites derived from amino acids. The core structure of a glucosinolate consists of a β-D-thio-glucose group linked to a sulfonated aldoxime moiety and a variable side chain derived from amino acids. Based on the amino acid precursor, glucosinolates are classified as 1. Aliphatic glucosinolates derived from leucine, isoleucine, methionine or valine; 2. Aromatic glucosinolates derived from tyrosine and phenylalanine and 3. Indole glucosinolates derived from tryptophan (Lelario, Bianco, Bufo, & Cataldi, 2012). The biosynthesis of glucosinolates proceeds via three independent steps: a. chain elongation of the precursor amino acids mainly methionine and phenylalanine, b. core glucosinolate structure formation and c. secondary modification of the amino acid side chain by oxygenation, hydroxylation, alkenylations and benzoylations (Sønderby, Geu-Flores, & Halkier, 2010). Glucosinolates are hydrophilic anionic molecules that are sequestered in the vacuoles as potassium salts (Moreno, Carvajal, López-Berenguer, & García-Viguera, 2006). These bioactive molecules, upon cellular disruption, come in contact with the endogenous enzyme myrosinase (EC 3.2.3.1 thioglucosidase), initiating a hydrolysis reaction. Upon hydrolysis, the D-glucose moiety from the glucosinolates is released, leading to the formation of an unstable intermediate thiohydroximate-O-sulfonate, which upon spontaneous rearrangement leads to varied degradation products such as isothiocyanates, thiocyanates, nitriles and epithionitriles. The nature of the hydrolysis products is governed by various intrinsic and extrinsic factors such as structure of the parent glucosinolates, pH, temperature, presence of cofactors like Fe^{2+} ions and epithiospecifier proteins (EPS) (Radojcic Redovnikovic *et al*., 2008). *In vitro* and

in vivo studies indicate that molecules from glucosinolate degradation, predominantly sulphoraphane from glucoraphanin, and attenuate cancer and cardiovascular diseases (Ares, Nozal, & Bernal, 2013).

Polyphenols are a large and diverse class of plant secondary metabolites, abundantly found in plants and characterized by their phenolic groups. They are biosynthesized via the shikimic acid pathway from aromatic amino acid phenylalanine, in response to plant stress, to protect the plants from both biotic and abiotic attacks through their anti-bacterial and anti-oxidant activity (Jaiswal, Abu-Ghannam, & Gupta, 2012). Based on the number of carbon atoms and molecular arrangements, polyphenols are classified as flavonoids and non-flavonoids. In *Brassica* species, the most common flavonoids are flavonols, mainly quercetin, kaempferol and isorhamnetin. The most predominant non-flavonoids are hydroxycinnamic acids, mainly *p*-coumaric, sinapic and ferulic acids, and they are mostly found conjugated with sugars and organic acids (Cartea, Francisco, Soengas, & Velasco, 2010).

The process of discarding food residues in a sustainable way with minimal environmental impact has led to a new area of research of food recovery. The agro-food industrial co-products may also be rich in plant bioactives that can be utilized to develop commercial extracts beneficial for human health, but scarce information is available with regards to valorization of by-products (Laufenberg, Kunz, & Nystroem, 2003). The main goal of the present study was to characterize and quantify the prominent bioactive molecules present in broccoli by-products, compare different varieties for their nutraceutical compounds and identify promising sources for a commercial extraction of their bioactive molecules using green technology.

3.5 Materials and Methods

3.5.1 Plant Material and Sampling

Rejected lots of ten broccoli seed cultivars were analyzed for their glucosinolate and polyphenol contents. They were: hybrid baro star (HBS), hybrid power dome (HPD), hybrid blue wind (HBW), broccoli sprouting (BS), marathon F1 hybrid (MF1), CND, SBA, VA1, VA2 and VA3. All the seeds varieties were kindly provided by Norseco Inc. (Laval,

QC, Canada). These seed cultivars were non-marketable for different reasons that comprise age and/ or quality of the seed lots, yield parameters that were below export and commercialization standards, poor germination rate, grain quality and size and the presence of yellow grains. Seed samples were flash frozen in liquid nitrogen, finely ground by means of mortar and pestle, and stored at -20°C until further use. The industrial residues of broccoli florets, stalk and mixture (the combined residual segments after processing) were graciously provided by Productions Maraîchères Mailhot Inc. (Saint-Alexis, QC, Canada). These broccoli residues were destined to discard after packing and processing of broccoli for commercial use, mainly because they were overripe or had some yellowish spots. They were lyophilized and ground using a Thermomix (Canada), vacuum sealed and stored at -20°C until further use.

3.5.2 Chemicals

Sinigrin monohydrade (99%), quercetin, rutin, caffeic, sinapic and gallic acids, aluminum chloride, potassium acetate and Folin & Ciocalteu's phenols reagent were purchased from Sigma-Aldrich (Mississauga, ON, Canada). Glucoraphanin potassium salt (>99%) was obtained from Chromadex (Irvine, CA). All the solvents used were LC-MS grade, unless mentioned otherwise. Formic acid (99.5%) LC-MS grade was from Fisher Scientific (Ottawa, ON, Canada). Ultrapure water was used throughout the experiment and was produced using milli-Q RG system from Millipore (Billerica, MA). Stock solutions of the standards were prepared by dissolving them in ultrapure water at a concentration of 1 mg/mL.

3.5.3 Total Phenolic Content

The polyphenols in broccoli by-products were extracted according to Singleton & Rossi (1965), from a dry sample (1 g) with aqueous methanol (20 mL; 80% v/v) in a sonicator at 37°C for 20 min. The mixture was centrifuged for 5 min at 4000 rpm and the resulting supernatant was collected. The precipitate was re-extracted using aqueous methanol (20 mL; 80% v/v), and the supernatants from both extractions were combined and utilized for the determination of total polyphenols using Folin-Ciocalteu's method (Singleton & Rossi, 1965). An aliquot (20 μL) of each sample, standard (Gallic acid) and blank (milliQ water)

was mixed with Folin-Ciocalteu's phenol reagent (100 µL; 10% v/v) in a 96 well microplate. After 8 min, an aqueous solution of Na_2CO_3 (80 µL; 7.5% w/v) was added to the mixture and left to incubate for 1 hour at room temperature before absorbance was read against a blank, at 765 nm with a FLOURstar omega microplate spectrophotometer (BMG labtech, Ortenberg, Germany). The total content in polyphenolics was expressed as milligram Gallic acid equivalent per gram of sample dry matter (mg GAE/g DW).

3.5.4 Total Flavonoid Content

The total flavonoids were extracted by a two-step extraction process from a dry sample (1 g) using aqueous ethanol (10 mL; 80% v/v) in a sonicator at room temperature for 10 min. The supernatant from the two extractions were combined and utilized for the estimation of total flavonoids using the aluminum chloride colorimetric assay, adapted from Chang *et al*., (2002). An aliquot (100 µL) of each sample, standard (Quercetin) and blank (milliQ water) were thoroughly mixed with ethanol (300 µL; 95% v/v), aqueous aluminum chloride (20 µL; 10% w/v), and potassium acetate (20 µL; 1M w/v) solutions, and milliQ water (600 µL). An aliquot (300 µL) of the sample was transferred into a 96 well microplate and allowed to incubate for 40 min at room temperature, before absorbance was read against blank at 415 nm with a FLOUstar omega microplate reader. The total flavonoid content was expressed as milligram quercetin equivalent (QE) per gram of sample dry matter (mg QE/g DW).

3.5.5 Simultaneous Identification and Characterization of Glucosinolates and Polyphenols

The glucosinolates and polyphenols content in broccoli by-products were simultaneously analyzed according to Gratacos-Cubarsi *et al*., (2010). Glucosinolates and polyphenols were extracted from a dry sample (1 g) with ice cold methanol (7.5 mL; 100% v/v) in a sonicator set at 70°C. After 15 min, the samples were placed in ice for an additional 15 min with intermittent vortexing every 5 min. The mixture was centrifuged at 5000 rpm for 10 min at 4°C and the resulting supernatant was collected. Samples after centrifugation (1 mL) were evaporated to dryness using nitrogen and reconstituted with an aqueous solution of formic acid (0.1% v/v) in aqueous acetonitrile (5% v/v; 1 mL, mobile phase A). Aliquots (300 µL) of each standard solution of sinigrin, glucoraphanin, rutin, quercetin, caffeic and

sinapic acids (0 to 200 ppm), blank (mobile phase A) and samples were filtered (0.2 µm, nylon filter) and analyzed by UPLC MS/MS.

3.5.6 UPLC MS/MS

The chromatographic analysis was carried out on an AQUITY UPLC HSS T3 1.8 µm (18 µm particle 2.1 id X 150 mm) column (Waters, Millford, USA). The system was equipped with a Triple Quadrupole Mass Spectrometer (TQD); operated in negative electron-spray ionization mode (ESI^-) and Multiple Reaction Monitoring (MRM). The compounds were separated by varying the composition of the mobile phase from 100% A (formic acid 0.1% v/v in 5% v/v acetonitrile) and 0% B (formic acid 0.1% v/v in acetonitrile 60% v/v) to 50% A and 50% B at 15 min and finally returning to the initial condition at 21 min. The flow rate was set at 0.4 mL/min, with a sample injection volume of 2.5 µL, on a column maintained at 40°C. The ionization was carried out by adjusting the respective source and desolvation temperatures at 140°C and 350°C, with flow rates of the desolvation gas, cone gas and collision gas set at 800 L/h (nitrogen), 50 L/h (nitrogen) and 0.10 mL/min (Argon) respectively. The capillary, extractor and RF lens voltages were set at 2.5 kV, 3 V and 0.2 V, respectively. The operation conditions of the instrument were optimized using an Autotune Wizard Software (Waters corporations, USA) and manually by infusing the concentrated solution of each compounds separately. Identification and quantification of the compounds were performed by comparing the retention time, MS, MS/MS fragmentation pattern of pure standards solutions and existing literature data (Gratacós-Cubarsí, Ribas-Agustí, García-Regueiro, & Castellari, 2010). Intact glucosinolates were expressed as sinigrin equivalent (SE), glucoraphanin as both sinigrin (SE) and glucoraphanin (GE) equivalents, flavonoids as rutin equivalent (RE), sinapic acid as sinapic acid equivalent and chlorogenic acid as caffeic acid equivalent. The total analyte content of glucosinolates and polyphenols in seeds and vegetative segments of broccoli were expressed as milligrams per kilogram dry matter (mg/kg DW).

3.5.7 Statistical Analysis

All the assays were conducted in triplicate and the results were expressed as mean±SD. The data was processed using SAS 9.4 (SAS Institute Inc., USA). The analysis of variances

(ANOVA) and multiple comparisons (MANOVA) were performed using Wilk's Lambda analysis and Dunnete's multiple comparison method to determine significant differences at $p< 0.05$. The data set were hierarchically clustered and visually analyzed through a heatmap generated using MeV 4.9.0 – Multiple Experiment Viewer (J.Craig Venter^{TM} Institute, CA, USA).

3.7 Results and Discussion

3.7.1 Total Content in Phenols and Flavonoids

The total polyphenol and flavonoid content **(Table 2)** between different plant segments of broccoli by-products varied from 3.8±0.2 to 6.1±0.3 mg GAE/g DW for total phenols and 2.4±0.1 to 5.4±0.6 mg QE/g DW for total flavonoids. Florets had the highest total phenol and total flavonoid content compared to stalk and mix by 63% and 13%, respectively. The results found in the present study was within the range as observed by Jaiswal *et al*., (2012) and Sosnowska *et al*., (2006), where the polyphenol content in various vegetables varied from 1.5-33.7 mg GAE/g DW (estimated from fresh weight (FW), considering 90% of broccoli is water (Latté, Appel, & Lampen, 2011). In broccoli florets, the total polyphenol content varied from 23.6 mg GAE/g DW to 1.5-12.1 mg GAE/g DW (estimated from FW) as reported by Jaiswal, Abu-Ghannam & Gupta (2012) and Koh, Wimalasiri, Chassy & Mitchell (2009). Commercial cultivars have 2-4 times higher total polyphenolic content in florets than broccoli floret by-products, indicating possible variability between cultivars and loss due to degradation of glucosinolates by myrosinase (Koh, Wimalasiri, Chassy, & Mitchell, 2009). The total flavonoid content in the present study was within the range reported by Lin & Tang (2007) (0.4-13.3 mg QE/g DW, estimated from fresh weight). Our results on by-products were lower than that reported by Jaiswal, Abu-Ghannam & Gupta (2012) (17.5 QE/g DW) but, higher than values reported by Koh, Wimalasiri, Chassy & Mitchell (2009) (0.003-1.1mg QE/g DW, estimated from FW) for commercial broccoli florets.

Analysis of the rejected lots of ten broccoli seed cultivars indicated that the content of total phenols varied from 6.3±0.04 to 11.8±0.1 mg GAE/g DW, in VA2 and MF1, respectively, and that of total flavonoids ranged from 0.17±0.01 to 0.67±0.01 mg QE/g DW, in HPD and

HBS, respectively **(Table 2)**. These values are in the same range that those reported by Pajak, Socha, Galkowska, Roznowski & Fortuna (2014) at 5 mg GAE/g DW and lower than those reported by Natella, Maldini, Nardini, Azzini, *et al*., (2016) at 35 mg GAE/g DW (estimated from FW). The total flavonoid content in the seed cultivars was lower than 16 mg QE/g DW as reported by Pajak, Socha, Galkowska, Roznowski & Fortuna (2014) (16 mg QE/g DW) and at par with the values reported by Natella, Maldini, Nardini, Azzini, *et al*., (2016) in broccoli sprouts at 15 mg catechin/g DW (estimated from FW). Studies by Natella, Maldini, Nardini, Azzini, *et al*., (2016) showed that broccoli sprouts have a 50% higher flavonoid content than seeds, indicating that the similarity in the flavonoid content in broccoli seed cultivars and sprouts is purely genetic variability. The variations in the content of total phenols and flavonoids might be due to genetic variability between samples, differences in the developmental stage of the plant, response towards stress (biotic and abiotic) and environmental variability as reported by E. Jeffery, Brown, Kurilich, Keck, *et al*., (2003).

Table 2: Total phenolic and flavonoid contents in broccoli by-products (Brassica oleracea var. italica)

Plant part	Total phenols (mg GAE/g DW)	Total flavonoids (mg QE/g DW)
Broccoli segments		
Stalk	3.8±0.2	2.4±0.1
Mixture	5.4±0.3	4.3±0.3
Florets	6.1±0.3	5.4±0.6
Seed cultivar		
MF1	6.32±0.04	0.23±0.01
HBS	7.2±0.2	0.67±0.01
SBA	8.32±0.04	0.26±0.03
HPD	8.8±0.1	0.17±0.01
VA3	8.0±0.1	0.30±0.01
VA1	9.2±0.1	0.30±0.01
BS	9.37±0.02	0.40±0.01
HBW	9.4±0.1	0.61±0.01
CND	9.7±0.2	0.33±0.03
VA2	11.8±0.1	0.55±0.03

Values represent the mean of three replicates ± standard deviation.

3.7.2 Simultaneous identification and quantification of glucosinolates and polyphenols

UPLC-MS/MS technique enabled a precise and concurrent identification of glucosinolates and polyphenols in broccoli by-products which previously needed separate sample treatment and chromatographic conditions **(Figure 11)**. Characterization of these compounds was based on identifying the parental molecular ions $[M-H]^-$, the fragmentation patterns of the daughter ions (m/z) and the retention time of these molecules and their standards (Gratacós-Cubarsí, Ribas-Agustí, García-Regueiro, & Castellari, 2010). Identification of glucosinolates was facilitated by the presence of certain dominant MS product ions $[M-H]^-$ and *m/z* 97 fragment ion that corresponds to the sulphate group in glucosinolates that is formed in abundance in ESI negative mode. Indolic glucosinolates neoglucosbrassicin and 4-methoxyglucobrassicin have identical molecular weight (478) and similar fragmentation patterns, and were identified based on their elution order in reverse phase chromatographic technique. From previous studies on *Brassica* species, a deprotonated molecular ion $[M-H]^-$ (787) and it's a fragment $[M-H-162]^-$ indicated the loss of glycosidic residue (mainly a hexoside) at position 7 and the presence of di-/tri-hexoside at position 3, therefore tentatively identifying the peak at 6.4 min as quercetin-3-diglucoside-7-glucoside. Fragmentation ion $[M-H]^-$ (609), $[M-H-180]^-$ (429) from the loss of glucose moiety and an aglycone fragment $[Agl-H]^-$ (285), indicate the presence of a flavonol-O-diglycosidics, tentatively identifying the peak at 6.27 min as kaempferol-3-O-sophoroside. Hydroxycinnamic acids such as 3-caffeoylquinic acid and 5-caffeoylquinic acid have similar molecular mass and fragmentation pattern $[M-H]^-$ (353) and *m/z* (191). Based on their elution sequence, the peaks at 5.2 min and 6.8 min were identified as 3-caffeoylquinic acid and 5-caffeoylquinic acid, respectively. Sinapic acid was identified by comparing the retention time and fragmentation patterns obtained from its standard solution.

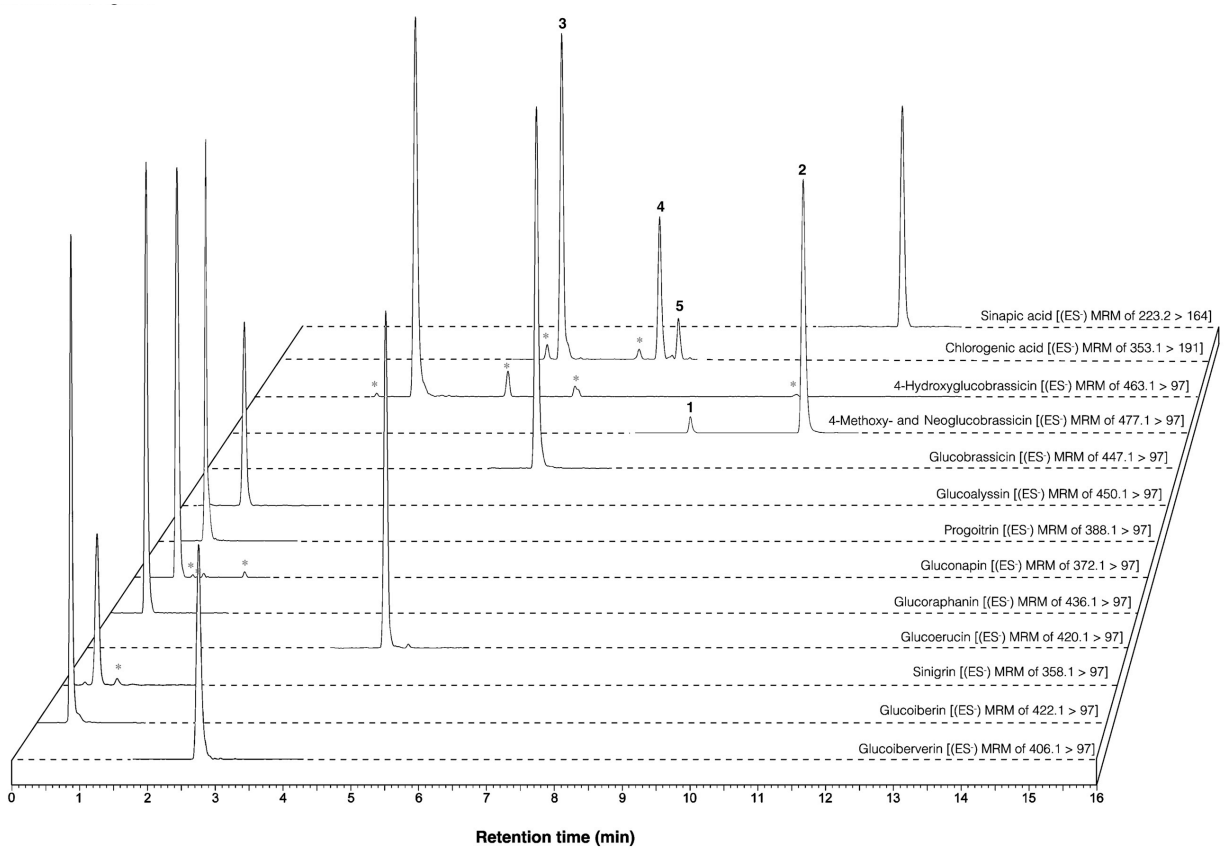

Figure 11*:* MRM profiles of intact glucosinolates and polyphenols corresponding to seed cultivar MF1 except progoitrin from seed cultivar SBA.

The chromatograms correspond to varied sample dilutions and the peaks marked (*) were unidentified. For multiple-peak chromatograms, identification is 1) 4-methoxyglucobrassicin; 2) neoglucobrassicin; 3) 3-caffeoyl-caffeic acid; 4) 5-caffeoyl-caffeic acid; and 5) 4-caffeoyl-caffeic acid.

Characterization of different plant segments of broccoli by-products and broccoli seed cultivars allowed the identification of 12 glucosinolates, of which three aliphatic glucosinolates of the propyl group (C-3), glucoiberverin (GIV), glucoiberin (GIB), and sinigrin (SIN), all four aliphatic glucosinolates of the butyl group (C-4), glucoerucin (GER), glucoraphanin (GRA), gluconapin (GNA) and progoitrin (PRO), and only one aliphatic glucosinolate of the pentyl group (C-5), glucoalyssin (GAL). In the identification process, we closely looked for the missing glucosinolates from the biosynthetic pathway of both the propyl and pentyl groups, such as the analogues of progroitrin, 1-hydroxy-2-propenyl and 3-hydroxy-4-pentenyl, respectively, and other glucosinolates of the pentyl group, glucoberteroin and glucobrassicanapin, but their level was below the detection limit of the analytical instrument. Among the indolic glucosinolates, glucobrassicin (GBS), neoglucobrassicin (NGBS), 4-hydroxyglucobrassicin (OHGBS) and 4-

methoxyglucobrassicin (MGBS) were identified. Five different polyphenols were also found. They are quercetin-3-diglucoside-7-glucoside (QtriG), kaempherol-3-O-sophoroside (KdiG), sinapic acid (SA), 3-caffeoylquinnic acid (3CQA) and 5-caffeoylquinnic acid (5CQA) **(Table 3)**.

Table 3: Glucosinolates and polyphenols identified with their corresponding retention time, MS data and conditions of detection in extracts of broccoli by-products (Brassica oleracea var. italica).

	Compounds	R_t (min)	MS^1 $[M-H]^-$ (m/z)	MS^2 $[M-H]^-$ (m/z)	Time window (min)	Dwell time (min)	Cone voltage (V)	Collision Energy (eV)
Aliphatic Glucosinolates								
C-3	Glucoiberverin	3.12	406	97	2.2-4.2	0.047	33	23
	Glucoiberin	1.02	422	97	0-2	0.05	42	22
	Sinigrin	1.33	358	97	0.5-2.5	0.05	37	20
C-4	Glucoerucin	5.03	420	97	4-6	0.047	46	21
	Glucoraphanin	1.15	436	97	0.2-2.2	0.05	32	30
	Gluconapin	1.17	372	97	0.3-2.3	0.05	38	18
	Progoitrin	1.22	388	97	0.3-2.3	0.05	36	23
C-5	Glucoalyssin	1.52	450	97	0.6-2.6	0.05	33	23
Indolic Glucosinolates								
	Glucobrassicin	5.95	446	97	4.8-6.8	0.047	33	23
	Neoglucobrassin	9.68	477	97	6.8-10.5	0.009	33	23
	4-Hydroxyglucobrassicin	4.79	463	97	2-12	0.047	33	25
	4-Methoxyglucobrassicin	7.89	477	97	6.8-10.5	0.009	33	23
Polyphenols								
Flavonoids								
	Kaempherol-3-o-sophoroside[1]	6.27	609	285, 429	4-7.2	0.047	42	29
	Quercetin-3-diglucoside-7-glucoside[1]	6.40	787	625	4-7.2	0.047	33	28
Hydroxycinnamic acids								
	3-Caffeoyl quinic acid	5.19	353	191	4-7.2	0.047	24	15
	5-Caffeoyl quinic acid	6.86	353	191	4-7.2	0.047	24	15
	Sinapic acid	10.84	223	164, 208	9-11.5	0.172	47	38

[1] Tentative identification.

Quantification results show significant variations in the profile of glucosinolates and polyphenols within different segments of industrial broccoli discards **(Table 4)** and rejected lot of broccoli seed cultivars **(Table 5).** Glucosinolates are the most predominant bioactive compound present in broccoli by-products and unused seed cultivars (0.6-2% DW). While the polyphenolic content varied from 0.015-0.025% DW in seeds and different segments of broccoli, glucosinolates content was on average 3.3 times more in seeds than various segments of broccoli by products. In broccoli plant segments, florets had the highest glucosinolate (2-3 times more) and polyphenol (1.5–3.4 times more) content compared to the mix and the stalk. Seed cultivars HBS and SBA surpassed the other 9 cultivars for their total content in glucosinolates, at 5 to 100% more, and total polyphenols content, at 54 to 370% more.

Characterization and quantification of broccoli by-products, identified glucoraphanin as the most predominant glucosinolate. In the present study, quantification of glucoraphanin was performed both in terms of sinigrin equivalent (SE) and glucoraphanin equivalent (GE) **(Table 5)**. The results demonstrate that when glucoraphanin is quantified as sinigrin equivalent, the value obtained highly underestimate its actual content in the sample. In the present work, the mean ratio of glucoraphanin quantified as SE over GE was approximately 0.45. For example, value for glucoraphanin in seed variety HBW, quantified as SE, was 9443 mg/kg DW, whereas a value of 19957 mg/kg DW was obtained for a quantification as GE.

Characterizing the plant segments of the industrial broccoli discards for their content in glucosinolates resulted in the quantification of 11 glucosinolates (**Table 4**). The results show that the glucoraphanin content accounted for 32 to 61% of the total glucosinolates followed by indole glucosinolates (mainly glucobrassicin and neoglucobrassicin) whose concentration ranged from 31 to 49% of the total glucosinolates. Of the three broccoli plant by-products, the florets have 1.5-4.2 times higher glucoraphanin and 3 times higher total indole glucosinolates content than the mix and the stalk. Despite the lower concentration of glucosinolates in broccoli stalks, glucoerucin and glucoiberverin content was significantly higher than in the florets and the mix. In the present study, we did not detect the presence of

progoitrin and 4-hydroxyglucobrassin in stalks although they are present in the other segments of broccoli by-products.

Table 4: Total and individual glucosinolates and polyphenols content in the various segments of broccoli by-products (Brassica oleracea var. italica).

Molecular Compounds (mg/kg DW)		Broccoli part		
		Stalk	Mix	Florets
Aliphatic Glucosinolates				
C-3	Glucoiberverin[1]	4.5a	1.1a	1.0a
	Glucoiberin[1]	86.1a	77.8ab	242.9b
	Sinigrin[1]	N.D	N.D	N.D
	Total C-3 glucosinolates	90.6	78.9	243.9
C-4	Glucoerucin[1]	245.2a	82.0ab	45.0b
	Glucoraphanin[1]	591.8	1646.6a	2536.8a
	Glucoraphanin[2]	1586.6	3374.3a	5076.3a
	Gluconapin[1]	34.3	30.8a	119.3a
	Progoitrin[1]	N.D	0.84	2.0
	Total C-4 glucosinolates	*2468*	*5135*	*7779*
C-5	Glucoalyssin[1]	7.7a	5.5ab	22.6b
Indolic Glucosinolates				
	Glucobrassicin[1]	100	184.9	868.1
	Neoglucobrassin[1]	472.4a	491.3ab	1488.3b
	4-Hydroxyglucobrassicin[1]	N.D	2.8a	18.5a
	4-Methoxyglucobrassicin[1]	294.8a	159.4a	431.0a
	Total indolic glucosinolates	*867*	*838*	*2806*
Total Glucosinolates[1]		**1836.6**	**2683**	**5775.6**
Polyphenols				
Flavonoids[3]				
Kaempherol-3-o-sophoroside		N.D	3.7	39.4
Quercetin-3-diglucoside-7-glucoside		N.D	0.6	17.2
Hydroxycinnamic acids				
3-Caffeoyl quinic acid[4]		35.0ab	25.0b	115.1a
5-Caffeoyl quinic acid[4]		18.3a	17.0a	24.4a
Sinapic acid[5]		21.3	30.7a	54.3a
Total Polyphenols		**74.5**	**77.0**	**250.4**

[1] mg sinigrin equivalent/kg; [2] mg glucoraphanin/kg; [3] mg rutin equivalent/kg; [4] mg caffeic acid equivalent/kg; [5] mg sinapic acid equivalent/kg.

Values represent the mean of three replicates per cultivar. Means followed by same letter within a row are not significantly different according to MANOVA test ($p<0.05$). N.D. not detected

Polyphenolic profiling of broccoli plant by-products showed higher levels of hydroxycinnamic acids (HCA) **(Table 4)**, of which chlorogenic acids, representing over 92% of the total polyphenolic content. Florets predominated in its total polyphenolic content (250 mg/kg DW) followed by stalk (75 mg/kg DW) and mix (77 mg/kg DW). Also, flavonoid derivatives like quercetin-3-diglucoside-7-glucoside and kaempferol-3-o-sophoroside present in the florets and mix segments were absent in the broccoli stalks by-products.

The present study supports the data of Wang, Gu, Yu, Zhao, Sheng & Zhang (2012) and Baenas, Moreno & Garcia-Viguera (2012), that the content of glucosinolates and polyphenols vary both within and between broccoli cultivars. The total glucosinolate content in the plant segments of broccoli by-products varied form 1836.6-5775.6 mg/kg DW, which were in accordance to the results obtained by Dominguez-Perles, Martinez-Ballesta, Carvajal, Grarcia-Viguera & Monreno (2010b) and Wang, Gu, Yu, Zhao, Sheng & Zhang (2012). Whereas, glucosinolates content in broccoli derived by-products (stalk and leaves) varied from 2026.7-5404.6 mg/kg DW (Dominguez-Perles *et al*., 2010b) and was 5305.3 mg/kg DW in commercial florets (Wang, Gu, Yu, Zhao, Sheng, & Zhang, 2012). Vallejo *et al*. (2002) reported a two-fold higher total glucosinolates content (1192-11127 mg/kg DW) in experimental cultivars of broccoli than that reported in this study. The progoitrin (anti-nutritive agent) content observed in the present study (N.D. to 2 mg/kg DW) was lower than that reported by Wang, Gu, Yu, Zhao, Sheng & Zhang (2012) for commercial cultivars 703.3-2412.2 mg/kg DW. The lower glucosinolates content in broccoli by-products might be due to its degradation by the enzyme myrosinase that is activated in response to stress (biotic and abiotic) or during processing. Variations in the glucosinolates content within different segments of broccoli as observed in this study was also reported by other authors (Clarke, 2010; Vallejo, Tomás-Barberán, & García-Viguera, 2002). Increased localization of glucosinolates inside broccoli florets, as observed in our study, might be to protect them from animals, pests, and environmental factors such as irradiation (Wang, Gu, Yu, Zhao, Sheng, & Zhang, 2012). Glucoraphanin and indolic glucosinolates were the most predominant glucosinolates in broccoli plant segments (Dominguez-Perles *et al*., 2010b), as found in the present study, indicating their role in plant defence mechanism.

Table 5: Total and individual glucosinolates (GLS) and polyphenols content in 10 broccoli seed cultivars (Brassica oleracea var. italica).

Molecular Compounds (mg/kg DW)		Broccoli Seed Cultivar									
		HPD	HBS	HBW	BS	VA1	VA2	VA3	CND	SBA	MF1
Aliphatic Glucosinolates											
C-3	Glucoiberverin[1]	17a	20a	30a	455a	377a	545a	307b	651a	386a	1279ab
	Glucoiberin[1]	68	131	92	2466ab	2162a	3423b	1850	4248b	3454ab	5089
	Sinigrin[1]	3.6	N.D	N.D	354b	346	462a	327b	705a	874a	N.D
	Total C-3 glucosinolates	*88.6*	*151*	*122*	*3275*	*2885*	*4430*	*2484*	*5604*	*4714*	*6368*
C-4	Glucoerucin[1]	9766	6299a	6593a	3784c	2106	2761b	2903b	4471c	3050b	5388a
	Glucoraphanin[1]	9661a	13376	9443a	6811bc	4614	6110bc	5946b	8514bc	7060b	9998a
	Glucoraphanin[2]	23226a	31495	19957a	16574bc	10754	14776bc	13885b	16986bc	13980b	21513a
	Gluconapin[1]	233a	738	223a	185	156b	174b	180b	172b	146b	238a
	Progoitrin[1]	1.9	N.D	N.D	94.5b	148.2b	235.3b	86b	288a	334a	N.D
	Total C-4 glucosinolates	*19662*	*23413*	*16259*	*10874*	*7024*	*9280*	*9115*	*13445*	*10590*	*15624*
C-5	Glucoalyssin[1]	72a	91.5	67.5a	50ab	35.4c	39.4bc	46.6b	56.8b	48.5b	66.1ab
Indolic Glucosinolates											
	Glucobrassicin[1]	16.3	92c	60.4b	47.7b	61.3b	62.6b	56.3b	130.3c	41ab	31.8a
	Neoglucobrassin[1]	11.3	114b	36.9c	118b	59.9ac	62.6a	108ab	117b	49.5ac	66.4ac
	4-Hydroxyglucobrassicin[1]	0.9 a	1.3 a	0.8 a	2.3 a	1.7 a	1.5 a	1.8 a	2.5 a	0.8 a	1.3 a
	4-Methoxyglucobrassicin[1]	1.8b	2.3b	9.8a	5a	8.6a	6.2a	6.7a	11.8a	4.8a	9a
	Total indolic glucosinolates	*30.3*	*209.6*	*107.9*	*17.0*	*131.5*	*132.9*	*172.8*	*261.6*	*96.1*	*108.5*
Total Glucosinolates[1]		**19853**	**20865**	**16557**	**14374**	**10076**	**13883**	**11818**	**19368**	**15447**	**22165**
Polyphenols											
Flavonoids[3]											
Kaempherol-3-o-sophoroside[4]		22.7a	13.4	9.6	42.0b	37.7b	36.5b	28.5a	23a	21.8a	14.03
Quercetin-3-diglucoside-7-glucoside[4]		1.13a	2.08a	0.50a	0.83a	0.55a	0.70a	0.7a	0.33a	0.71a	0.76a
Hydroxycinnamic acids											
3-Caffeoyl quinic acid[5]		0.28a	0.27a	0.33ab	0.39ab	0.26a	0.31a	0.7b	0.20a	2.7	0.31ab

5-Caffeoyl quinic acid[5]	1.09ab	1.48a	0.45c	1.45ab	1.02ab	0.69bc	1.43a	0.68b	0.36c	1.27ab
Sinapic acid[6]	24.9	15.7	38.6a	90.3c	57.5bd	48.6bd	114.7c	60.3d	126.8bc	33.6a
Total Polyphenols	**50.1**	**32.9**	**49.5**	**135.0**	**97.0**	**86.8**	**146.0**	**84.5**	**152.4**	**50.0**

[1] mg sinigrin equivalent/kg; [2] mg glucoraphanin/kg; [3] mg rutin equivalent/kg; [4] tentative identification; [5] mg caffeic acid equivalent/kg; [6] mg sinapic acid equivalent/kg.

Values represent the mean of three replicates per cultivar. Means followed by same letter within a row are not significantly different according to MANOVA test ($p<0.05$)

The polyphenolic concentration in the different plant segments of broccoli by-products ranged from 75 to 250 mg/kg DW, which is higher than the values reported by Vallejo, Tomas-Barberan & Garcia-Viguera (2002) of 1.5-17.6 mg/kg DW (estimated from FW)**.** In the present study, florets had the highest content of polyphenols of which HCA were the most predominant and segments like stalk lacked the presence of flavonoid derivatives, which was also reported by Dominguez-Perles, Martinez-Ballesta, Carvajal, Garcia-Viguera & Moreno (2010b) and Lopez-Berenguer, Martinez-Ballesta, Moreno, Carvajal & Garcia-Viguera (2009).

Determination of the glucosinolate content in different broccoli seed cultivars **(Table 5)** indicated high levels of aliphatic glucosinolates, mainly glucoraphanin, accounting for 44 to 64% of total glucosinolates, glucoerucin, at 20-49% of total glucosinolates, and glucoiberin, at 0.3% to 22% of total glucosinolates. Indolic glucosinolates corresponded to less than 1.46% of the total glucosinolates. There is a clear difference in the glucosinolate content between the hybrid cultivars (HPD, HBS, HBW and MF1) and the non-hybrid cultivars. In particular, the levels of C-3 glucosinolates are lowest in HPD, HBS and HBW and highest in MF1. Values for glucoiberverin were 17 to 30 mg/kg DW in the hybrid cultivars HPD, HBS and HBW, compared to 307-651 mg/kg DW in the non-hybrid cultivars. Similarly, values for glucoiberin ranged from 68 to 131 mg/kg DW in the seeds of HPD, HBS and HBW, whereas values in the non-hybrid varieties were higher and ranged from 1850 to 4248 mg/kg DW in VA3 and CND, respectively. The seed of MF1 had the highest content of the aliphatic C-3 glucosinolates glucoiberverin and glucoiberin, at 1279 and 5089 mg/kg DW respectively. For the C-4 glucosinolates, the seeds of MF1 cultivar showed 0.7-2 times higher glucoraphanin content than the 9 other seed cultivars analyzed. The absence or low concentration of glucosinolates such as sinigrin and progoitrin was observed in the four hybrid cultivars HPD, HBW. HPW and MF1. The seed cultivar HPD was quite different from the other hybrid cultivars, with its higher levels of glucoerucin at 9766 mg/kg and lower levels of indolic glucosinolates such as glucobrassicin (16.3 mg/kg), neoglucobrassicin (11.3 mg/kg) and 4-methoxyglucobrassicin (1.8 mg/kg). The hybrid seed cultivars HBS and HBW showed higher glucoraphanin to total glucosinolates content of 64% and 57% respectively.

Polyphenolic profiling in the seed cultivars showed the abundance of HCA, most predominantly sinapic acid, that accounted for over 75% of the total polyphenolic content. Seeds of the cultivar SBA showed 1-5.8 times higher polyphenolic content than the other 9 cultivars of broccoli seeds, at 152.4 mg/kg DW **(Table 5)**. Relatively lower levels of flavonoid derivatives were observed in the seed cultivars, the predominant one being Kaempferol-3-o-sophoroside, at more than 86% of the total flavonoid content.

The heat map and hierarchical cluster analysis of glucosinolates and polyphenols in broccoli products indicated that vegetative segments of broccoli by-products showed similar glucosinolates and polyphenols profile as hybrid seed cultivars MF1, HBS and HBW **(Figure 12)**.

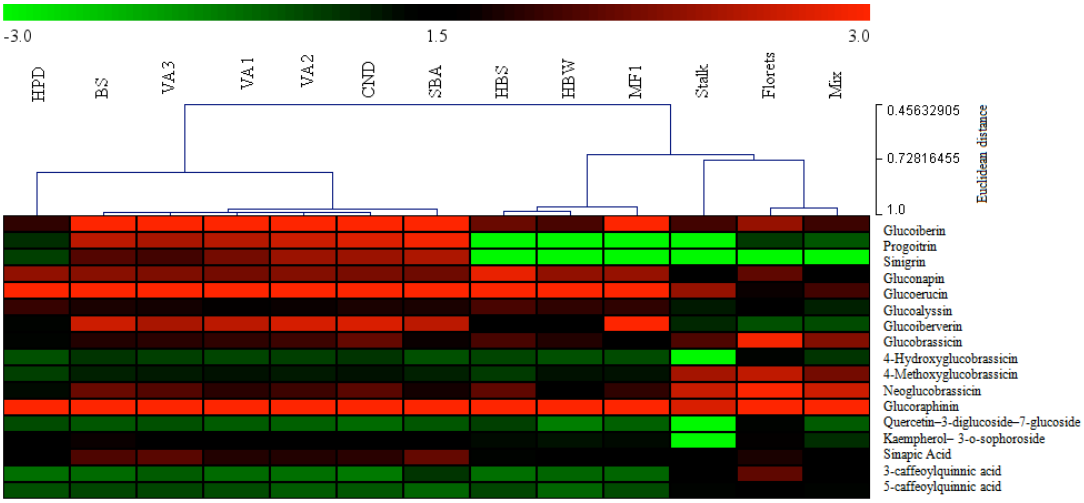

Figure 12: Heatmap and hierarchical cluster analysis of glucosinolates and polyphenols in broccoli by-products.
Mean data were normalized by logarithmic transformations and displayed in color scale where, red indicates high, black intermediate and green low compounds concentration values.

Glucosinolates are present all across the plant body at varying proportion with maximal accumulation inside seeds (Clarke, 2010; Prestera, Fahey, Holtzclaw, Abeygunawardana, Kachinski, & Talalay, 1996)**.** In the seed cultivars, the total glucosinolate content varied from 10076-20865 mg/kg, which was within the range as reported by Bellostas, Kachlicki, Sorensen & Sorensen (2007). The glucosinolate profile observed in the present study, was similar to that observed by Bellostas, Kachlicki, Sorensen & Sorensen (2007), who reported the abundance of glucoerucin, glucoiberverin and glucoraphanin and low levels of indolic

glucosinolates in broccoli seed cultivar Jade Crosse. The low levels of glucobrassicn in broccoli seeds and the enhanced content in the glucosinolates content in the hybrid cultivars compared to the wild cultivars were also observed by Kushad *et al*., (1999), who reported the absence of glucobrassicin in broccoli seedlings and a 10 fold increase in the glucoraphanin levels in the hybrid lines of broccoli. The variability in the glucosinolate content may be due to genetic variations between the cultivars. In the present study, we found broccoli seeds to be rich in aliphatic glucosinolates and with plant development its content decreases with an increase in indole glucosinolates in the vegetative tissues as reported by E. Jeffery (2003). The reduction in the proportion of glucosinolate content in the various segments of broccoli compared to the seeds was likely due to the dilution of glucosinolates and active catabolism of glucosinolates by myrosinase during tissue expansion as reported by Bellostas, Kachlicki, Sorensen & Sorensen (2007).

In this study, the polyphenolic content of the seed cultivars, varied form 33-152 mg/kg which was lower than that described by Pérez-Balibrea, Moreno & Garcia-Viguera (2011) and Sousa, Lopes, Seabra *et al*., (2007) for broccoli seed cultivars. High levels of HCA, predominantly sinapic acid as observed in this study, coincided with the results of Sousa, Lopes, Seabra *et al*., (2007) and Pajak, Socha, Fortuna *et al*., (2014), who reported that *Brassica oleracea* seed cultivars were rich in phenolic acid derivatives. Whereas, studies of Perez-Balibrea, Moreno & Garcia-Viguera (2011), reported values lower than 15% of total polyphenols as hydroxycinnamic acids, contrary to what was observed in our study. Genetic variability is the major factor that influences the variations in the concentration of polyphenolic compounds within and between the broccoli cultivars. There are various other factors that influence the polyphenolic content in plants such as environmental pressure, cultivation techniques, age and maturity of the plants and postharvest treatments. In this study, since we are dealing with industrial by-products from food processing; microbial contamination, handling and storage conditions influences the variability in bioactive molecules (Koh, Wimalasiri, Chassy, & Mitchell, 2009)**.** Overall, broccoli by-products are also a potent source of health beneficial bioactive ingredients, which are produced by the plant defense system to combat stress and the variations depend on the developmental stage

of the plant, tissue, genetic, environmental and socio-economic factors (Williams, Critchley, Pun, Nottingham, & O'Hare, 2008).

3.8 Conclusion

The present study provides a thorough insight on the variations in glucosinolate and polyphenol profile in broccoli derived by-products i.e. florets, mix, stalk and seeds. The concentrations of these bioactive ingredients are at par with commercial cultivars, especially for their content in glucosinolates (0.2-2% dry weigh sample), whereas their content in polyphenols was very low (less than 0.02% dry weigh sample). The major glucosinolate identified was glucoraphanin, which upon hydrolysis lead to the formation of sulforaphane with known positive health effects. Broccoli seeds and florets had the highest proportion of bioactive compounds compared to other industrial by-products. Hence, this study sheds light on the possible mode of reutilization of the usually discarded and underutilized industrial residues, bringing us closer to food security and sustainability. Further studies have to be undertaken to check the applicability of these residues on the fabrication of health beneficial extracts using food grade solvents.

3.9 Acknowledgements

The authors acknowledge the financial support of the Natural Science and Engineering Research Council of Canada (NSERC), Diana Food Canada Inc. and Productions Maraîchères Mailhot Inc. We would also like to express our gratitude to Norseco Inc., Québec, Canada for providing the seed samples and to Pascal Dubé for his support with the chemical analysis.

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Chapter 4

Optimization of glucosinolates extraction process from broccoli (*Brassica oleracea* var. italica) industrial discards using green technology

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Keywords: Agrifood waste, broccoli by-products, glucoraphanin, anti-cancerous, eco-friendly, sustainable method

4.1 Foreword

Broccoli by-products are a rich source for glucosinolates most distinctively glucoraphanin. The most commonly used technique for the extraction of glucosinolates involves the use of non-food grade solvents. In this chapter, we have optimized a green process for the extraction of glucoraphanin from industrial discards. Further, the glucoraphanin extractability was compared between the optimized extraction process and the conventional extraction technique using methanol as its solvent.

This chapter is presented as an article entitled: "Optimization of glucosinolates extraction process from broccoli (*Brassica oleracea* var. italica) industrial discards using green technology" has been submitted to the journal of Food Science and Technology.

The authors are Minty Thomas (first author), who participated in the planning, interpretation, discussion and writing of the article. Dr. Paul Angers, director of the thesis, and Dr. André Gosselin, Dr. Yves Desjardins, co-directors of the thesis participated in the planning of the research, the discussion of the results at meetings and the revision of the article presented. Dr. Ashraf Badr, research professional, participated in the discussion of the results and in the revision of the article and Pascal Dubé, research professional, participated by establishing the UPLC MS/MS protocol.

The extract obtained after the extraction process using green technology, was rich in glucoraphanin, but not significantly high enough to generate profit on the commercial scale. Hence, the glucoraphanin rich extracts were further purified using the ion-exchange resins to isolate and fabricate an extract rich in bioactive molecules.

4.2 Résumé

L'augmentation de la demande de nourriture ainsi que la production alimentaire génèrent des déchets agroalimentaires aux effets socio-économiques défavorables, qui sont gérés à grands frais. Les sous-produits du brocoli (*Brassica oleracea* var italica) sont riches en ingrédients bioactifs bénéfiques pour la santé; principalement la glucoraphanine, connue pour son activité anticancéreuse. Dans ce travail, notre objectif était d'étudier l'effet de temps-température, échantillon:solvant, concentration d'éthanol et le pH du solvant d'extraction pour maximiser l'extractibilité de glucoraphanine à partir des graines de brocoli et sous-produits de fleurons. Les conditions d'extraction optimales en utilisant un lot unique, évaluées à l'aide d'un extracteur à agitation magnétique, se sont révélées être; Solvant aqueux à 50 et 70% d'éthanol à 60 et 23°C, pour un rapport de matière (MS): solvant de 1:20 pendant 60 et 30 min à partir de graines et de sous-produits floraux, respectivement. Les conditions optimales d'extraction écologiques et durables ont permis d'obtenir un rendement en glucoraphanine de 55,5 g/kg de graines et de 4,3 g/kg de sous-produits de fleurons (MS). Ces valeurs étaient 2,6 et 1,4 fois plus élevées que les valeurs obtenues par la technique conventionnelle d'extraction au méthanol, indiquant une sous-estimation significative de la teneur en glucosinolates avec cette dernière méthode.

4.3 Abstract

The escalating demand for food and increased food production generates agrifood waste and substandard products with adverse socio-economic effects that are managed at great expense. Broccoli by-products (*Brassica oleracea* var. italica) are rich in health beneficial bioactive ingredients, predominantly glucoraphanin, known for its anti-cancerous activity. We aimed to study the effect of time-temperature, feed:solvent, ethanol concentration and pH of the extracting solvent to maximize the glucoraphanin extractability from broccoli seeds and florets by-products. The optimal extraction conditions, evaluated using a single batch magnetically stirred extractor, for seeds and florets by-products were 50 and 70% aqueous ethanol solvent at 60 and 23°C, for a feed (DW) to solvent ratio of 1:20 extracted for 60 and 30 min, respectively. The optimized green process provided a glucoraphanin yield of 55.5 g/Kg from seeds and 4.3 g/kg (DW) from florets by-products. These values were 2.6 and 1.4 times higher than the values obtained by the conventional boiling methanol extraction technique, indicating a significant underestimation of the content in glucosinolates, in general, and glucoraphanin, in particular, with the latter widely used method.

4.4 Introduction

Over the years, epidemiological studies indicated that plants belonging to the *Brassicaceae* family, predominantly broccoli (*Brassica oleracea* var. italica), have health promoting and disease preventive properties. The health promoting attributes of broccoli are due to its abundance in glucosinolates, most distinctively glucoraphanin and its conversion product sulphoraphane, polyphenols (flavonoids and hydroxycinnamic acids), and various nutrients such as vitamins (ascorbic acid), minerals (manganese, potassium and selenium) and carbohydrates (dietary fibers) (Ares, Nozal, Bernal, & Bernal, 2014). These bioactive molecules protect humans against various diseases such as cancer (Latté, Appel, & Lampen, 2011), neurodegenerative and age related diseases (Lee, Kim, Seo, Choi, Han, Lee, et al., 2014), and coronary heart diseases (Ares, Nozal, & Bernal, 2013).

Extensive research and increasing awareness about the positive health effects of broccoli (superfood), have led to its increased demand and consumption. It is interesting to note that the marketable broccoli florets represent less than 25% of its yield, generating huge quantities of substandard products (Domínguez-Perles, Martínez-Ballesta, Carvajal, García-Viguera, & Moreno, 2010). Broccoli derived by-products have similar composition as the edible plant parts, hence, residues derived from the agrifood industry can act as potent functional ingredients in the food and nutraceutical industries. Valorization of vegetable residues not only reduces the negative environmental impact but also brings us closer to sustainability with an increase in the economic value (Thomas, Badr, Desjardins, Gosselin, & Angers, 2017).

There is a deficit of published literatures on valorization of broccoli by-products using green solvents as compared to the normally consumed plant parts. The most commonly reported glucosinolates extraction conditions from plant materials involves the use of heated methanol (Gratacós-Cubarsí, Ribas-Agustí, García-Regueiro, & Castellari, 2010), methanol and water (Gallaher, Gallaher, & Peterson, 2012), ethanol and water (Campos, Chirinos, Barreto, Noratto, & Pedreschi, 2013; Wang, Liang, & Yuan, 2011). The main aim of the present study was to develop a robust, economic and environmentally friendly glucoraphanin extraction process by optimizing the extraction parameters such as time and temperature, sample to solvent ratio, ethanol concentration and pH of the extracting

solvent, using green technology; to utilize the optimized extraction process to characterize and quantify the glucosinolates and polyphenols in broccoli seeds and florets by-products by UPLC MS/MS, and to compare the optimized green process conditions to the conventional extraction conditions with boiling methanol and validate the higher scale feasibility of glucosinolate extraction technique from broccoli industrial discards using green chemistry.

4.5. Materials and Methods

4.5.1 Plant Material and Sampling

A rejected lot of broccoli seed cultivar Marathon F1 hybrid (MF1) was kindly provided by Norseco Inc. (Laval, QC, Canada) and the seed lot was non-marketable because it was below export and commercialization standards. The seed samples were finely ground by means of mortar and pestle, and stored at -20°C until further use. Whereas, the industrial discards of broccoli residues, provided by Productions Maraîcheres Mailhot Inc. (Saint-Alexis, QC, Canada), were destined to discard after packing and processing of broccoli for commercial use, mainly because they were overripe or had some yellowish spots. The floret industrial discards were lyophilized and ground using a Thermomix (Canada), vacuum sealed and stored at -20°C until further use.

4.5.2 Reagents and Materials

Sinigrin monohydrade (99%), rutin, caffeic and sinapic acids were purchased from Sigma-Aldrich (Mississauga, ON, Canada). Glucoraphanin potassium salt (>99%) was obtained from Chromadex (Irvine, CA). LC grade formic acid (99.5%) and methanol were acquired from Fisher Scientific (Ottawa, ON, Canada). Ethanol (95%) was purchased from Les Alcools du Commerce (Boucherville, QC, Canada). Ultrapure water was used throughout the experiments and was produced using the Milli-Q RG system from Millipore (Billerica, MA). Syringe filters (Nylon 0.45µm and Nylon 0.22µm) were purchased from Chromatographic Specialties Ltd. (Brockville, ON, Canada), qualitative filter paper (Whatman Grade 1) was obtained from VWR International (Montréal, QC, Canada). All the solvents used were on the GRAS list, unless mentioned otherwise.

4.5.3 Moisture Content in the Starting Materials

To determine the moisture content, broccoli seeds and fresh florets (5g each) were ground and dried at 105°C for at least 16 hours, until a constant weight was obtained (Miglio, Chiavaro, Visconti, Fogliano, & Pellegrini, 2008). The moisture content was expressed in percentage (%).

4.5.4 Glucoraphanin Stability in Ground Broccoli Seeds During Storage

The glucoraphanin stability in ground broccoli seeds were analyzed by estimating its concentration over a period of 7 days at room temperature, according to Thomas *et al.,* (2017). Seed sample (1g) was extracted with ice cold methanol (7.5 mL; 100% v/v) in a sonicator set at 70°C for 15 min. The resulting mix was cooled in ice for 15 min with intermittent vortexing every 5 min, and further centrifuged at 5000 rpm for 10 min at 4°C. A volume of 1 mL of the supernatant was retrieved, evaporated to dryness using a flow of dry nitrogen, and reconstituted with an aqueous solution of formic acid (0.1% v/v) in aqueous acetonitrile (5% v/v; 1 mL, mobile phase A). Aliquots (300 µL) of standard solutions of glucoraphanin (0 to 200 ppm), blank (mobile phase A) and samples were filtered (0.2 µm, nylon filter) and analyzed by UPLC MS/MS.

4.5.5 Optimization of Glucoraphanin Extraction Parameters

Glucoraphanin extraction parameters of contact time-temperature, ethanol concentration, sample to solvent ratio and pH of the extracting solvent, were optimized by multivariate analysis. The predominate process variable for the optimization study was identified based on experimental data and literature results. Subsequently, the optimal levels of each variable were updated to obtain the desired experimental parameters that maximize the extractability of glucoraphanin from the samples (Powell, Hill, Juurlink, & Carrier, 2005).

4.5.5.1 Variations in Time-Temperature. The influence of the variable reaction kinetics on glucoraphanin extractability was analyzed by extracting dry broccoli by-product samples of seeds and florets (5g) with aqueous ethanol (100 mL; 70% v/v), at 23, 40, 60 and 80°C, on a magnetic stirrer set at 400 rpm. Aliquots (3 mL) from each reaction mixture for time periods of 0, 15, 30, 60, 120, 240, 360 and 1440 min were removed and stored at -

20°C until sample preparation and quantification by UPLC MS/MS. The isolated sample solutions (3 mL) were filtered using Whatman No: 1 and further utilized to determine the glucoraphanin and dry matter content in the extracts. For the dry matter content in the extraction solutions, aliquots (2 mL) of each sample were filtered and oven dried at 105°C for at least 3 hours, until a constant weight was obtained. The dry matter content was expressed in gram per liter (g/L).

4.5.5.2 Variations in Ethanol Concentration. Dry samples of broccoli by-products (2g) were extracted with aqueous ethanol solvent (40 mL) at concentrations of 0, 50, 70 and 95% (v/v), on a magnetic stirrer set at 400 rpm using the optimized time and temperature. The dry matter content in the extracts was determined as described in section 2.5.1 and the glucoraphanin content using UPLC MS/MS as reported in section 2.6.

4.5.5.3 Variations in Sample to Solvent Ratio. Dry samples of broccoli by-products were extracted using the optimized ethanol concentration and time-temperature combination obtained for non-commercial seed cultivars and florets by-products. The sample to solvent ratio analyzed for the rejected lot broccoli seed cultivar MF1 were 1:2.5, 1:5, 1:10, 1:20, 1:30, 1:40 and for florets by-products 1:10, 1:20, 1:30, 1:40. Extraction parameters and determination of the dry matter in the extracts were performed as described in section 2.5.1. Quantification of was performed by UPLC-MS/MS as reported in section 2.6.

4.5.5.4 Variation in pH of Extraction Solutions. Samples of broccoli by-products were extracted with acidified and alkalized aqueous ethanol using the optimized ethanol concentration, time, temperature and sample to solvent ratio. The pH of the extracting solvent was 2,7,10 and 12 obtained using HCl (0.1N, v/v) in deionized water or NaOH (0.1N, v/v) in deionized water. Extraction parameters and the dry matter content in the extracts were determined as described in section 2.5.1. Quantification was performed by UPLC-MS/MS as reported in section 2.6.

4.5.5.5 Multiple Extractions and Process Optimization. Samples of broccoli by-products (seeds and lyophilized floret discards) were extracted thrice using the optimized extraction conditions to determine the levels of glucoraphanin extractability, with the

addition of fresh solvents at each step. The optimized extraction process as described in section 2.5 for lyophilized broccoli florets were extrapolated for fresh broccoli florets by-products to determine the industrial applicability of the study. Frozen broccoli florets were extracted with 70% aqueous ethanol with a sample to solvent ratio of 1:2 at 23°C and 60°C and extraction durations of 0, 15, 30, 60, 120, 240, 360 and 1440 min. The dry matter and the glucoraphanin content in the extracts were determined as described in section 2.5.1 and 2.6 respectively.

4.5.5.6 Conventional Extraction Method

The conventional extraction method was adapted from Gratacos-Cubarsi *et al*., (2010). Glucosinolates and polyphenols were extracted from a dry sample (1 g) with ice cold methanol (7.5 mL; 100% v/v) in a sonicator at 70°C. After 15 min, the samples were placed in ice for an additional 15 min with intermittent vortexing every 5 min. The mixture was centrifuged at 5000 rpm for 10 min at 4°C and the resulting supernatant was analyzed by UPLC MS/MS, for its glucosinolate and polyphenol content.

4.5.6 Simultaneous Characterization and Quantification of Glucosinolates and Polyphenols Using UPLC MS/MS

4.5.6.1 Sample preparation. Samples (500 µL) after centrifugation (5000 rpm for 10 min at 4°C) were evaporated to dryness using nitrogen and reconstituted with an aqueous solution of formic acid (0.1% v/v) in aqueous acetonitrile (5% v/v; 500 µL, mobile phase A). An aliquot (300 µL) of each standard solution of sinigrin, glucoraphanin, rutin, quercetin, caffeic and sinapic acids (0 to 200 ppm each), blank (mobile phase A) and samples from each extraction processes were filtered (0.2 µm nylon filter) and passed into UPLC MS/MS.

4.5.6.2 Analysis. The chromatographic analysis was carried out on AQUITY UPLC HSS T3 1.8 µm (18 µm particle 2.1 id X 150mm) column (Waters, Millford, USA). The system was equipped with a Triple Quadrupole Mass Spectrometer (TQD), operated in negative electron-spray ionization mode (ESI^-) and Multiple Reaction Monitoring (MRM). The compounds were separated by varying the composition of the mobile phase from 100% A (formic acid 0.1% v/v in 5% v/v acetonitrile) and 0% B (formic acid 0.1% v/v in

acetonitrile 60% v/v) to 50% A and 50% B at 15 min and finally returning to the initial condition at 21 min. The flow rate was set at 0.4 mL/min, with a sample injection volume of 2.5 µL. Intact glucosinolates were expressed as sinigrin equivalent (SE), glucoraphanin as both sinigrin (SE) and glucoraphanin equivalent (GE), flavonoids as rutin equivalent (RE), sinapic acid as sinapic acid equivalent and chlorogenic acid as caffeic acid equivalent. Values are expressed as milligrams per kilogram dry matter (mg/kg DW).

4.5.7 Statistical Analysis

All the assays were conducted in triplicate and the results were expressed as mean±SD. Statistical analysis on the experimental data was processed using SAS 9.4 (SAS Institute, Cary, NC). The analysis of variances (ANOVA) and multiple comparisons (MANOVA) were performed using Wilk's Lambda analysis and Dunnete's multiple comparison method to determine significant differences at $p< 0.05$.

4.7 Results and Discussion

4.7.1 Moisture Content in the Starting Material

The moisture content in broccoli seed cultivar MF1 and fresh florets by-products corresponded to 4.8±0.1% and 88.7±0.5% of the total biomass, respectively. The high moisture content in the fresh broccoli florets by-products necessitated an additional sample processing step such as lyophilization, to retain the stability of the bioactive compounds.

4.7.2 Glucoraphanin Stability in Ground Broccoli Seeds During Storage

Ground broccoli seeds were stored at room temperature for up to one week to monitor the stability of glucoraphanin during short term storage. The results **(Table 6)** show that over a seven day period, the glucoraphanin content in ground raw broccoli seeds decreased by 13%, from 19.6 to 17.1 g/Kg, indicating its relative stability. The low moisture content in the seeds (5%) is likely a significant factor, that contributed to the stability of glucosinolates over time, as water is an important co-factor for the activation of glucosinolate hydrolyzing enzyme, myrosinase (D. B. Clarke, 2010).

Table 6: Glucoraphanin stability in ground broccoli (Brassica oleracea var. italica) seed cultivar (MF1) for a period 7 days

Time (hours)	Glucoraphanin content (g/Kg)
0	19.61 ± 0.09 a
0.75	18.59 ± 0.06 b
1.25	19.3 ±0.5 b
3	18.2 ±1.1 b c
6	18.68 ±0.10 b
12	19.56 ±0.10 a
24	19.4 ±0.1 a
96	18.8 ±0.8 a
120	17.9 ±1.5 a d
144	17.5 ±0.3 c
168	17.1 ±0.3 c d

Values represent mean±SD. Values followed by the same letter within a column are not significantly different according to Wilk's Lambda analysis ($p < 0.05$).

4.7.3 Optimization of Glucoraphanin Extraction Parameters

4.7.3.1 Effect of Time-Temperature on Glucoraphanin Extraction

Standardizing the extraction parameters to maximize the yield of glucoraphanin from broccoli seeds and florets by-products is crucial. Results for the extraction of glucoraphanin from the rejected broccoli seed lot indicated an increased glucoraphanin recovery with temperature **(Table 7)**. Temperatures higher than 60°C provided better extraction of glucoraphanin than lower temperatures, with values ranging from 43.3 to 46.2 g/Kg. But the difference in the extractability of glucoraphanin became non-significant after 60 minutes of extraction at 60°C and 80°C. However, the high volatility of ethanol at 80°C made it unpractical to operate at 80°C, despite the high yield at short extraction duration. The stability of glucosinolates at the higher temperatures are in agreement with the results of Mohn *et al.*, (2007) who reported that aliphatic glucosinolates are relatively stable to thermal degradation. The higher extractability of glucosinolates from plant materials at elevated extraction temperatures is due to increased rate of mass transfer enabling

equilibrium to be reached more quickly (H. C. Fauduet, J. P Lessire, M., Quinsac, Ribaillier, & Rollin, 1995; Powell, Hill, Juurlink, & Carrier, 2005). Similar temperature responses were reported by Powell *et al*., (2005), Fauduet *et al*., (1995) and Wang *et al*., (2011) for the extraction of glucoraphanin from *Cardaria draba*, glucosinolates from rapeseed meal and sinigrin from mustard seeds, respectively. Studies on the extracted dry matter (DM) content from seeds extract indicated an increased extractability with time and temperature. The extracted DM increased with contact time at lower temperatures. Whereas, at higher temperatures an extraction time of 30 - 60 minutes was sufficient to maximize the quantity of extractable matter. The augmented extractability of DM, reduces the concentration of glucosinolates in the final extract. Similar results were reported by Fauduet *et al*., (1995) for rapeseed meal, where a maximal DM content was observed for a contact time of 15-20 minutes. Hence, an extraction time of 60 min at a temperature of 60°C was chosen as the optimal reaction conditions, as it maximized the recovery of glucoraphanin, making the process viable without significant dilution of glucoraphanin in the dry matter of the extract.

Table 7: Effect of reaction kinetics (time of contact (min), temperature (°C)) on glucoraphanin extraction and dry matter content (g/L) from broccoli seeds by-products.

	Seeds							
	Glucoraphanin Content (g/Kg)				**Dry Matter Content** (g/L)			
	(Temperature °C)				(Temperature °C)			
Time (min)	23	40	60	80	23	40	60	80
0	10.2±0.3a	10.3±0.1a	18.1±1.2b	19.7±0.8b	2.0±0.1	1.9±0.1	3.3±0.1	3.9±0.2
15	22.6±0.2	32.2±0.3	37.5±0.4	41.6±0.6	3.5±0.3	5.9±0.3	7.3±0.2a	7.5±0.2c'a
30	29.6±0.4	37.4±0.7	40.7±0.9e'	44.1±0.9h'i'	4.9±0.2	6.7±0.0	7.7±0.1	7.5±0.0c'
60	34.4±0.3	39.0±0.8b'd'	43.3±1.8e	43.9±0.2eh'	5.7±0.4	6.9±0.1	8.1±0.0b	8.0±0.1b
120	37.7±0.5	40.0±0.2b'd'	44.2±0.02fg'	44.5±0.2fi'	6.6±0.1	7.3±0.0	8.5±0.5b'c	8.5±0.1d'c
240	41.6±1.6ha'	40.68±0.02hb'c'	41.3±1.3he'	45.8±0.2j'	7.3±0.1	7.8±0.1a'	8.7±0.3b'd	8.8±0.3d'd
360	41.8±0.5ia'	42.8±1.5ic'	46.2±0.1jf'	45.5±1.9jj'	7.1±0.1	7.9±0.1a'	8.6±0.1e	8.6±0.4d'e
1440	42.7±0.1a'	39.4±0.5d'	45.1±0.9f'g'	NA	7.3±0.0f	8.2±0.1	7.3±0.3f	NA

Extraction at 70% ethanol concentration, feed: solvent 1:20, mixed on a magnetic stirrer at 400 rpm. Values represent mean±SD. Values followed by the same letter within a column and row are not significantly different according to Wilk's Lambda analysis ($p < 0.05$).

For the industrial discards of broccoli florets, the results **(Table 8)** showed no significant increase in glucoraphanin yield with temperature. Glucoraphanin extractability reaches an equilibrium after 15 – 30 minutes. Studies on the dry matter content in broccoli florets extracts, showed its direct correlation with contact time and extraction temperatures. At higher temperatures and shorter contact time, maximum quantity of extractable material was dissolved. Hence, in the present study, extraction for 30 minutes at room temperature (23°C) was the optimized time-temperature combination for glucoraphanin recovery from broccoli florets by-products. Ambient temperature for the extraction of glucosinolates was also reported by Mohn *et al*., (2007) from *Isatis tinctoria* leaves.

Table 8: Effect of reaction kinetics (time of contact (min), temperature (°C)) on glucoraphanin extraction and dry matter content (g/L) from broccoli florets by-products.

	Florets							
	Glucoraphanin Content (g/Kg)				**Dry Matter Content** (g/L)			
Time (min)	(Temperature °C)				(Temperature °C)			
	23	40	60	80	23	40	60	80
0	3.4±0.3aa'	3.6±0.6ae'f'g'	3.4±0.4ah'	3.43±0.06a	16.5±0.3	18.9±0.2	19.7±0.5a	20.1±0.1a
15	4.0±0.2b'	3.2±0.2ae'	3.6±0.2ch'i'	3.80±0.03c	20.7±0.1a'b	20.9±0.2d'b	23.2±0.1g'c	23.0±0.3i'c
30	4.0±0.3db'd'	3.7±0.3df'	4.9±0.8d	4.1±0.5dj'	20.9±0.5a'c'd	21.2±0.2d'f'd	23.8±0.1h'e	23.4±0.5i'j'e
60	4.1±0.4ec'd'	3.1±0.6e'	3.8±0.5eh'i'	4.0±0.5ej'	21.0±0.2a'	22.3±0.1d'	23.2±0.3g'	23.9±0.1j'
120	4.1±0.6fgc'd'	4.5±0.4fg'	3.9±0.4gh'	4.1±0.4fgj'	22.6±0.2b'f	23.1±0.3e'fg	23.4±0.2g'h'g	24.2±0.1k'
240	4.1±0.3hc'd'	3.4±0.2e'	4.0±0.4hh'	4.2±0.09hj'	21.7±0.8a'b'h	22.6±0.2d'e'h	23.6±0.1h'	24.0±0.2k'j'
360	3.8±0.8ija'b'c'd'	3.6±0.6ie'	4.1±0.4ijh'	4.3±0.2jj'	21.9±0.5b'c'i	21.7±0.3f'i	23.4±0.2g'h'	24.4±0.1k'
1440	4.3+0.7kd'	3.6±0.3e'	4.0±0.2kh'	NA	22.2±0.2b'j	22.1±0.1j	22.8±0.1	NA

Extraction at 70% ethanol concentration, feed: solvent 1:20, mixed on a magnetic stirrer at 400 rpm. Values represent mean±SD. Values followed by the same letter within a column and row are not significantly different according to Wilk's Lambda analysis ($p < 0.05$).

4.7.3.2 Effect of Ethanol Concentration on Glucoraphanin Extraction

In the present study, the glucosinolate degrading enzyme myrosinase was not inactivated prior to its extraction process and the eco-friendly nature of the study forbade the use of anhydrous ethanol. Broccoli seeds upon grinding and storing at room temperature were found to be relatively stable for their glucoraphanin content for a period of 7 days **(Table 6)**. The broccoli seed sample preparation process, did not involve the use of any energy or

cost intensive techniques. The results obtained in the present study was contrary to the studies of Toribio *et al*., (2007) and Wang *et al*., (2011), where broccoli seeds were stirred for 2 hours, with water at reflux temperature and ground mustard seed was defatted using petroleum ether respectively, to inactivate the enzyme myrosinase.

Studies on solvent concentration indicated that glucoraphanin extractability was significantly influenced by ethanol concentration. The extractability of glucoraphanin in broccoli seeds and florets by-products increases with solvent concentration, reaches a maximum and then decreases **(Table 9)**. Analysis of the rejected lot of broccoli seed cultivars MF1, indicated that glucoraphanin yield increases with ethanol concentration, reaching a maximum of 45.8 g/kg at 50% ethanol concentration and further decreasing. It is interesting to observe that glucoraphanin extractability at 0% and 95% ethanol concentration was very low. The zero yield at 0% ethanol concentration might be due to the degradation of glucosinolates by the degrading enzyme myrosinase, as it is only partially inactivated with the optimized extraction temperature of 60°C. Studies by Dal Pra *et al*., (2013) reported that myrosinase in mustard and rapeseed extract and flaked seeds were active at a temperature of 60-90°C. in this study, at high ethanol concentrations, the glucoraphanin extractability was very low, this may be due to coagulation of proteins or precipitation of polysaccharides, carbohydrates, fats and water insoluble cell wall components, as reported by Finnigan & Lewis (1988) in rapeseed meal and Campos *et al*., (2013) from maca. The trapped glucosinolates in the coagulate and precipitate inhibits mass transfer, thereby, reducing its yield (Campos, Chirinos, Barreto, Noratto, & Pedreschi, 2013).

Studies on the dry matter content in broccoli seeds extracts indicated an inverse correlation. Where, the quantity of dry matter present in the seeds extract decreases with ethanol concentration. Similar results were observed by Fauduet *et al*., (1995), who reported an increases extractability of dry matter content, with water concentration. Ethanol concentration of 50% provided a dry matter content of 7.9 g/L in the broccoli seed extract, at par with an ethanol concentration of 70%. Hence, in this study the enhanced glucoraphanin extractability, reduced dry matter content in the extract, requisite the use of 50% as the optimized ethanol concentration range. Similar results were observed for

broccoli florets industrial discards with respect to glucoraphanin extractability and dry matter content in the extract. In the present study, maximal glucoraphanin extractability of 2.0 g/kg and the dry matter content of 22.2g/L were observed at 70% ethanol concentration. In this study, high concentration of ethanol inactivated the glucosinolates degrading enzyme myrosinase when operated at ambient temperatures for 30 min. Similar results were observed by Fauduet *et al*., (1995) and Mohn *et al*., (2007), who reported that high solvent concentration was sufficient to inactivate myrosinase, instead of the use of high extraction temperatures, thereby, enhancing glucosinolate stability. Majority of the studies on glucosinolate extraction involve the use of non-food grade solvents, most predominately methanol (D. B. Clarke, 2010; Cools & Terry, 2012; Devi & Thangam, 2010). The industrial applicability and green nature of this study, made the use of solvents in the GRAS list with minimal number of sample processing steps necessary. Hence, in the present study, an ethanol concentration of 70%, extracted at ambient temperature for 30 min was identified as the optimized process parameter to maximize the yield form broccoli florets by-products.

The use of high temperature for the extraction of glucosinolates from broccoli industrial discards can denature the glucosinolate degrading enzyme myrosinase and degrade the heat sensitive indolic glucosinolates. Inactivation of myrosinase can enhance glucosinolate extractability and stability, but drastically reduces glucoraphanin bioavailability when orally consumed. In the present study, the concentration of ethanol in the extracting media inactivates the glucoraphanin hydrolysis enzyme myrosinase, that not only enhances the stability and yield of glucoraphanin but also, maintains the integrity of heat labile indolic glucosinolates (Mohn, Cutting, Ernst, & Hamburger, 2007). Functional food supplements rich in glucoraphanin, usually obtained using high extraction temperatures degrades the myrosinase present in the plant material, reducing its bioavailability when orally consumed (Villarreal-García & Jacobo-Velázquez, 2016). In this present study, the inactivation of myrosinase by ethanol, enables us fabricate an extract rich in glucoraphanin, when consumed orally, activates the myrosinase. The gut microbiota along with the activated myrosinase acts synchronously, to degrade glucoraphanin, thereby, enhancing its bioavailability (J. D. Clarke, Hsu, Riedl, Bella, Schwartz, Stevens, et al., 2011; J. D. Clarke, Riedl, Bella, Schwartz, Stevens, & Ho, 2011).

Table 9: Effect of solvent concentration, feed to solvent ratio and pH on glucoraphanin extractability and dry matter content from broccoli seeds and florets by-products.

Extraction Parameter		Seeds		Florets	
		Glucoraphanin content (g/Kg)	**Dry Matter Content** (g/L)	**Glucoraphanin content** (g/Kg)	**Dry Matter Content** (g/L)
Ethanol concentration (% v/v) [1]	0	ND	10.7±0.0	0.02 ±0.01	27.8±0.3
	50	45.8±0.0	7.9±0.1a	2.0±0.1	23.5±0.3
	70	40.7±0.5	8.2±0.2a	3.9±0.6	22.2±0.1
	95	12.1±0.1	5.8±0.0	0.40±0.05	7.8±0.0
Sample to solvent ratio [2]	1:2.5	38.0±0.7a	59.7±0.3	NA	NA
	1:5	37.5±0.2a	30.5±0.2	NA	NA
	1:10	41.7±0.4	15.2±0.2	3.1±0.4a	41.2±0.1
	1:20	43.5 ±0.1b	7.7±0.0	3.6 ±0.3ab	22.1±0.1
	1:30	44.5 ±1.8bc	5.1±0.1	3.8 ±0.1b	16.6±0.4
	1:40	45.5±1.0c	3.9±0.1	3.8 ±0.7b	12.7±0.1
pH [3]	2	46.5 ±1.0a	11.7±0.2	3.2±0.4a	24.7±0.6
	7	44.4 ±1.3b	7.8±0.0a	3.5 ±0.2a	22.8±0.1a
	10	45.1 ±0.1ab	7.5±0.0	3.97 ±0.08	23.3±0.5ab
	12	44.74 ±0.03	7.8±0.0a	4.5 ±0.4a	23.8±0.2b

Values represent mean±SD. Values followed by the same letter within a column are not significantly different according to Wilk's Lambda analysis ($p < 0.05$).

[1] Seeds: Extraction at 60^0C for 60 min and florets: extraction at room temperature for 30 min; feed:solvent 1:20, mixed on a magnetic stirrer at 400 rpm.

[2] Seeds: extraction at 60^0C for 60 min, solvent concentration of 50% and Florets: extraction at room temperature for 30 min, solvent concentration of 70%; feed:solvent 1:20, mixed on a magnetic stirrer at 400 rpm.

[3] Seeds: extraction at 60^0C for 60 min, solvent concentration of 50% and Florets: extraction at room temperature for 30 min, solvent concentration of 70%; sample to solvent ratio 1:20, mixed on a magnetic stirrer at 400 rpm.

4.7.3.3 Effect of Sample to Solvent Ratio on Glucoraphanin Extraction

The influence of sample to solvent ratio on glucoraphanin extractability and dry matter content from broccoli seeds and florets by-products is shown in **Table 9**. The lowest sample to solvent ratio of 1:2.5 and 1:5, were less efficient in extracting glucoraphanin from the discarded lot of broccoli seeds, at 38.0±0.7 and 37.5±0.2 g/Kg (DW), respectively. Further, an increase in the sample to solvent ratio from 1:10 to 1:40, resulted in an increase in the glucoraphanin extraction yield, from 41.7±0.4 to 45.5±1.0 g/Kg (DW), that plateaued at 1:20. Whereas, for broccoli florets by-products, glucoraphanin extractability increased

with an increase in sample to solvent ratio from 1:10 to 1:40, with values ranging from 3.1±0.4 to 3.8±0.7 g/Kg (DW), for the lowest and highest sample to solvent ratios, respectively. In this study, no statistical difference in glucoraphanin extraction yield with sample to solvent ratio was observed. A sample to solvent ratio of 1:10 on dry weight basis for broccoli florets by-products corresponded to a sample to solvent ratio of 1:1 fresh weigh basis, considering 90% of fresh broccoli is water. In the present study, a lower sample to solvent ratio was industrially unpractical, due to the difficulty in attaining the optimized ethanol concentration of 70%, estimated from fresh weight. Hence, the industrial applicability of the present study necessitated the use of a sample to solvent ratio of 1:20 (DW) or 1:2 (FW) for broccoli florets by-products.

Studies on the influence of sample to solvent ratio on the dry matter content of the extracts from broccoli seeds and florets by-products indicated that with an increase in the sample to solvent ratio the dry matter content in the extract decreased. The dry matter content in the extract is proportional to the solid loading, i.e. as the weight of the feed sample increases, more extractable plant material was dissolved into the solution. Therefore, the optimized sample to solvent ratio of 1:20, provided a high glucoraphanin yield and low dry matter content of 43.5 ±0.1 g/Kg and 7.7±0.0 g/Kg; and 3.6 ±0.3 g/Kg and 22.1±0.1 g/Kg respectively; from broccoli seed and florets industrial discards respectively.

The sample to solvent ratio optimized in this study, was at par with the values reported by Finnigan & Lewis (1988) for rapeseed meal and by Powell *et al*., (2005), for *Cardaria draba*. In this study, a low sample to solvent ratio of 1:5 or less resulted in reduced glucoraphanin extraction yield, which may be due to the formation of thick slurry that makes agitation difficult, interfering with mass transfer from the sample solid to the extracting solvent. Further, the increase in glucoraphanin extractability with an increase in sample to solvent ratio as observed in this study, was also reported by Powell *et al*., (2005). Whereas, the variability in glucoraphanin extraction profile with sample to solvent ratio observed in this study, was contrary to that observed by Campos *et al*., (2013) in maca (*Lepidium meyenii* Walters), who reported that lower solid to solvent ratio provided better yield of glucosinolates at higher extraction time.

4.7.3.4 Effect of pH on Glucoraphanin Extraction

In the present study, pH values varied in a non-proportionate manner, pH 2 and 12 were the lowest and highest possible pH in the food industry, as validated by industry personal. Whereas, pH 7 is in the neutral range and 10 being the midway between the neutral and extreme basic pH. The effect of pH on glucoraphanin extractability, indicated a slight increase in glucoraphanin extractability in florets at higher pH values, but no significant differences were observed for neither of the broccoli by-products **(Table 9)**. Hence in this present study, the pH of the extraction medium was unaltered using the optimized extraction process for broccoli seeds and florets by-products (50% aqueous ethanol, sample to solvent ratio of 1:20, stirred at 400rpm and extracted at 60°C for 60 min for seeds, and for florets 70% aqueous ethanol at room temperature, sample to solvent ratio of 1:20, stirred at 400 rpm for 30 min). The results obtained in this study was contrary to the studies by Powell *et al*., (2005) and Finnigan & Lewis (1988) who reported that acidic extraction conditions enhanced glucoraphanin extractability. Studies by Shahidi & Gabon (1989) and Mahajan & Dua (1994) reported the use of solvents at basic pH to enhance the yield of glucosinolates from canola and rapeseeds respectively, similar to that observed in the present study for florets. The pH, post extraction and evaporation of broccoli seeds and florets using the optimized operation parameters were 5.3 and 5.7 respectively, which was similar to that reported by Powell *et al*., (2005).

4.7.3.5 Multiple extractions and process optimization

The optimized extraction conditions to maximize the yield of glucoraphanin from the discarded lot of broccoli seeds by-products in a single batch extraction process were a solid to solvent ratio of 1:20 extracted with 50% aqueous ethanol at 60°C, for 60 min. Whereas, for lyophilized broccoli florets by-products, the extraction performed with a feed to solvent ratio of 1:20 using 70% aqueous ethanol at room temperature for 30 minutes, maximized the yield of glucoraphanin. The glucoraphanin extraction yield from broccoli seeds and florets by-products using the optimized parameters were 52.1 g glucoraphanin/Kg seeds and 3.8 g glucoraphanin/Kg DW florets respectively. Multiple extractions using the optimized extraction parameters indicated that a single batch extraction process was efficient in extracting glucoraphanin from broccoli seeds and florets by-products. The

glucoraphanin extraction efficiency for multiple batches using fresh solvents per batch showed 91%, 8% and 1%; and 88%, 10% and 2% for the first, second and third extraction from seeds and florets by-products, respectively. Hence, a single staged batch extraction process, recovered at least 88% of the total glucoraphanin present in broccoli by-products. The glucoraphanin recovery results were significantly higher than those reported by Powell *et al*., (2005) for *Cardaria draba*, who reported a glucoraphanin recovery of 60% in the first extraction (optimized extraction parameters were a solid loading of 50 mg/L, aqueous ethanol concentration of 20%, pH 3, extracted at 70°C). Studies by Fauduet *et al*., (1995), reported a recovery of nearly 85% of water extractable material from rapeseed meal after a two-step extraction.

The analysis on the influence of extraction temperature (23°C and 60°C) and time of reaction (0, 15, 30, 60, 120, 240, 360 and 1440 min), on glucoraphanin yield from frozen broccoli florets industrial discards, using the optimized extraction parameters of 70% ethanol and a feed to solvent ratio of 1:2, indicated no significant influence of extraction time and temperature on glucoraphanin extractability. In the present study, similar results were observed for frozen broccoli florets by-products as was reported for lyophilized broccoli samples. The results observed in this study, corresponded to the studies of Fauduet *et al*., (1995), who reported no difference in the concentration of glucosinolates extracted from freeze dried samples and non-freeze dried samples using cold methanol 80%. Therefore, the optimized process parameters that maximized the yield of glucoraphanin from frozen broccoli florets industrial discards were a feed to solvent ratio of 1:2, extracted at room temperature using 70% ethanol for 30 minutes. Hence, this process substantiates the industrial feasibility, scalability and reproducibility of the operation parameters.

4.7.4 Comparison of operation methods and simultaneous characterization and quantification of glucosinolates and polyphenols using UPLC MS/MS

Comparative study on the extractability of glucosinolates and polyphenols from broccoli seeds and florets by-products using the optimized process parameters and conventional extraction technique using methanol, provided similar extractability profiles **(Table 10)**. Characterization and quantification of broccoli industrial discards using the optimized and conventional extraction process, identified 12 glucosinolates and 5 polyphenols, of which

glucoraphanin is the most distinctive. The present study indicated that the optimized extraction process was highly efficient in extracting glucosinolates and polyphenols, with no significant differences in its composition, when compared to the conventional technique involving hot methanol extractions. The optimized extraction technique provided 2.7 and 1.2 times more glucoraphanin extraction yield from broccoli seeds and florets than the conventional technique. Overall, the optimized process provided a 61 and 26% increased yield of total glucosinolates from seeds and florets discards than methanol based extraction technique. Whereas, for the polyphenols extracted from seeds and florets industrial discards, 1.2 and 1.4 times more total polyphenols were extracted using the optimized process than the conventional extraction technique. Greater levels of glucosinolate and polyphenol extractability were observed for broccoli seeds than florets industrial discards using the optimized process. The enhanced extractability of glucosinolates and polyphenols using the optimized extraction process might be due to the enhanced thermal stability of glucosinolates, most predominantly the indolic glucosinolates (Doheny-Adams, Kittipol, Bancroft, & Hartley, 2017) or due to the ease of its extraction using an aqueous solvent than 100% methanol. High solvent concentrations can inactivate myrosinase the glucosinolate degrading enzyme, but extremely high solvent concentrations can coagulate and precipitate proteins and polysaccharides, inhibiting the extraction of glucosinolates (Campos, Chirinos, Barreto, Noratto, & Pedreschi, 2013). Therefore, in the present study, a highly efficient green process for the extraction of glucoraphanin from broccoli industrial discards was developed.

Table 10: Comparative study on the total and individual glucosinolates and polyphenols in broccoli seeds and florets by-products using the optimized (Opt) and conventional (Conv) extraction process

Molecular Compounds	**Seeds**			**Florets**		
(mg/kg)	Opt	Conv	Opt/Conv	Opt	Conv	Opt/Conv
Glucosinolates						
Glucoiberverin[1]	2156.6	956.2	2.3	ND	ND	
Glucoiberin[1]	11961.7	4382.9	2.7	129.7	99.8	1.3
Sinigrin[1]	ND	ND		ND	ND	
Glucoerucin[1]	10579.0	4799.9	2.2	227.2	246.6	0.9
Glucoraphanin[1]	24099.8	8998.7	2.7	1739.5	1403.7	1.2
Glucoraphanin[2]	52146.1	20396.8	2.6	3764.0	2607.1	1.4
Gluconapin[1]	703.0	209.1	3.4	64.5	36.3	1.8
Progoitrin[1]	ND	ND		ND	ND	
Glucoalyssin[1]	141.43	59.3	2.4	12.73	7.8	1.6
Glucobrassicin[1]	65.9	22.6	2.9	991.8	562.8	1.8
Neoglucobrassin[1]	107.3	52.9	2.0	1349.7	1037.6	1.3
4-Hydroxyglucobrassicin[1]	ND	ND		15.0	8.3	1.8
4-Methoxyglucobrassicin[1]	64.6	26.9	2.4	546.1	319.1	1.7
Total glucosinolates[1]	*49879.1*	*19508.6*	*2.6*	*5076.1*	*3722.0*	*1.4*
Polyphenols						
Flavonoids[3]						
Kaempherol-3-o-sophoroside	15.9	14.1	1.1	37.5	22.2	1.7
Quercetin-3-diglucoside-7-glucoside	ND	ND		5.2	3.2	1.6
Phenolic acids						
Hydroxycinnamic acids						
3-Caffeoylquinic acid[4]	ND	ND		119.5	88.7	1.3
5-Caffeoylquinic acid[4]	2.0	1.3	1.5	29.1	21.7	1.3
Sinapic acid[5]	33.3	26.8	1.2	50.4	42.5	1.2
Total Polyphenols	*51.2*	*42.2*	*1.2*	*241.7*	*178.3*	*1.4*

[1] mg sinigrin equivalent/kg; [2] mg glucoraphanin/kg; [3] mg rutin equivalent/kg; [4] mg caffeic acid equivalent/kg; [5] mg sinapic acid equivalent/kg. Values represent the mean of three replicates per cultivar.

4.8 Conclusion

An eco-friendly and industrially feasible, glucosinolates and polyphenols extraction technique from broccoli industrial discards was developed. The optimized process parameters that maximized the yield of glucoraphanin from the discarded lot of broccoli seeds, was an extraction technique using 50% aqueous ethanol with 1:20 feed to solvent ratio, extracted for 60 min at 60°C, whereas, for industrial florets discards the optimized process parameters was an extraction using 70% aqueous ethanol with 1:20 feed to solvent ratio, extracted for 30 min at room temperature (23°C). The optimized parameters provided a glucoraphanin yield of 52.1 g glucoraphanin/Kg seeds and 3.8 g glucoraphanin/Kg DW from broccoli seeds and florets respectively. Multiple batch extractions studies indicated that a single batch process using the optimized process parameters extracted between 88% and 91% of glucoraphanin from the broccoli by-products. Characterization and quantification studies on broccoli seeds and florets by-products, indicated no difference in the glucosinolate and polyphenol composition between the optimized and conventional technique. Comparative study on the extraction processes indicated that the optimized green process, extracted 63 and 19% more glucoraphanin than the conventional technique that involves the use of non-food grade solvent such as methanol. Whereas for the total polyphenols the optimized extraction process provided 18 and 27% increased yield from seeds and florets by-products, compared to the methanol based extraction technique. Therefore, an efficient and environmentally sustainable glucoraphanin extraction process was developed using green technology for industrial discards that is not only economical but also industrially viable for the fabrication of functional food ingredients, providing an alternative route for its valorization.

4.9 Acknowledgement

The authors acknowledge the financial support of the Natural Science and Engineering Research Council of Canada (NSERC), Diana Food Canada Inc. and Productions Maraîchères Mailhot Inc. We would like to express our gratitude to Norseco Inc., Québec, Canada for providing the seed samples.

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Chapter 5

Extraction, Purification and Fabrication of Extracts Rich in Glucoraphanin from Broccoli Seed Discards Using Eco-friendly Solvents and Ion Exchange Resins Through Response Surface Approach (RSM)

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Keywords: Broccoli by-products, glucoraphanin, functional food, nutraceutical industry, cation and anion exchange resins, food supplements, socio-economic sustainability

5.1 Foreword

Ion exchange resins act as an important tool for the purification of glucosinolates. The anionic nature of glucosinolates, facilitates the purification of these charged molecules. In this chapter, we have systematically investigated and optimized the various parameters that influence the purification of glucoraphanin by ion exchange resins and response surface approach. Further, a green process was developed for the purification and fabrication of an extract rich in glucoraphanin from the discarded lot of broccoli seeds, in an industrially feasible and sustainable way.

This chapter is presented as an article entitled: "Extraction, Purification and Fabrication of Extracts Rich in Glucoraphanin from Broccoli Seed Discards Using Eco-friendly Solvents and Ion Exchange Resins Through Response Surface Approach (RSM)" has been revised and we intend to submit it in Food Chemistry.

The authors are Minty Thomas (first author), who participated in the planning, interpretation, discussion and writing of the article. Dr. Paul Angers, director of the thesis, and Dr. André Gosselin, Dr. Yves Desjardins, co-directors of the thesis participated in the planning of the research, the discussion of the results at meetings and the revision of the article presented. Dr. Ashraf Badr, research professional, participated in the discussion of the results and in the revision of the article.

The proposed study provided an efficient, environmental friendly, industrially feasible route towards biorefining and fabrication of health beneficial supplements that can be used in the food and nutraceutical industry.

5.2 Résumé

Les sous-produits de brocoli générés par l'industrie agro-alimentaire sont une source abondante de phytochimiques végétaux favorisant la santé, et plus particulièrement la glucoraphanine, avec un marché important dans l'industrie des aliments fonctionnels et nutraceutiques. Le but de cette étude était d'étudier systématiquement et d'optimiser les facteurs les plus importants qui influencent la purification de la glucoraphanin à partir d'extraits de graines de brocoli, en utilisant des résines échangeuses de cations et d'anions par la méthodologie de surface de réponse. Quatre variables indépendantes, le rapport matière/résine, temps de contact, vitesse d'agitation et concentration des solvants d'élution, ont été étudiées pour les résines cationiques et anioniques en utilisant un modèle Box-Behnken (BBD) à 27 niveaux pour maximiser la pureté et la récupération de la glucoraphanine. Une récupération maximale du processus de 94% et une pureté en glucoraphanin de 14% ont été obtenues pour un rapport matière/résine cationique, temps de contact, vitesse d'agitation et concentration en solvant d'élution de 1:5, 30 min, 80 rpm et 100% d'eau respectivement. Pour les résines anioniques, les variables expérimentales de 1:5, 140 min, 160 rpm et 7% d'hydroxyde d'ammonium dans l'éthanol à 70% ont permis d'obtenir un rendement de 72% et une pureté en glucoraphanine de 37%. Le sel d'ammoniaque de la glucoraphanine a été remplacé par un sel de bicarbonate de potassium plus stable, sans diminution significative de la pureté de la glucoraphanine. L'extraction et la purification de glucoraphanine à l'échelle pilote en utilisant les paramètres optimisés à l'échelle du laboratoire ont été concentrés la glucoraphanin 12 fois, avec une efficacité globale du processus de 93%. La fabrication de suppléments bénéfiques pour la santé à partir de sous-produits pour son utilisation dans l'industrie nutraceutique nous rapproche de la durabilité socio-économique en minimisant le coût des rejets et en générant des revenus à partir des déchets.

5.3 Abstract

Broccoli by-products generated by the agro-food industry is an abundant source of health promoting plant phytochemicals most distinctively glucoraphanin, with a substantial market in the functional food and nutraceutical industry. The purpose of this study was to systematically investigate and optimize the most important factors that influence the purification of glucoraphanin from broccoli seed extracts using cation and anion exchange resins by response surface methodology. Four independent variables, namely feed to resin ratio, contact time, agitation speed and concentration of the eluting solvents, were investigated for cationic and anionic resins by employing a 27 run, three level Box-Behnken design (BBD) to maximize glucoraphanin purity and process recovery. A maximal process recovery of 94% and glucoraphanin purity of 14% were obtained for a feed to cationic resin ratio, contact time, agitation speed and eluting solvent concentration of 1:5, 30 min, 80 rpm and 100% water respectively. For anionic resins, experimental variables of 1:5, 140 min, 160 rpm and 7% ammonium hydroxide in 70% ethanol provided a process efficiency of 72% and a glucoraphanin purity of 37%. The ammonia salt of glucoraphanin was replaced by a more stable potassium bicarbonate salt, without significant decrease in glucoraphanin purity. Pilot scale glucoraphanin extraction and purification technique using the optimized laboratory scale process concentrated glucoraphanin 12 times, with an overall process efficiency of 93%. Fabrication of health beneficial supplements from by-products for its use in nutraceutical industry brings us closer to socio-economic sustainability minimizing the cost of discard and generating revenue from waste.

5.4. Introduction

Nutrition based health promoting and disease preventive strategies along with increased awareness among consumers about food quality and the adverse effects of artificial food additives, have led to a healthier lifestyle, reducing the expenditure on the health sector (Schieber, Stintzing, & Carle, 2001). Rampant food wastage from the agro-food industry generates huge quantities of under-valued by-products that are rich in functional ingredients that can be utilized for the fabrication of functional food ingredients with promising use in food, nutraceutical and pharmaceutical industries (Laufenberg, Kunz, & Nystroem, 2003).

Broccoli (*Brassica oleracea* var. italica) is an economically important vegetable widely consumed and studied for its health beneficial properties. The health promoting and disease preventive properties of broccoli are due to its abundance in plant bioactives such as glucosinolates, polyphenols and other phytochemicals, but most predominantly glucoraphanin and its conversion molecule sulphoraphane (Bellostas, Kachlicki, Sørensen, & Sørensen, 2007). Epidemiological and cohort studies suggest anti-cancerous (Clarke, Hsu, Riedl, Bella, Schwartz, Stevens, et al., 2011), anti-diabetic (Axelsson, Tubbs, Mecham, Chacko, Nenonen, Tang, et al., 2017), anti-inflammatory (Jeffery & Araya, 2009) and anti-microbial properties (Yanaka, Fahey, Fukumoto, Nakayama, Inoue, Zhang, et al., 2009) of plants belonging to the brassica family. While, sulphoraphane has proven beneficial health effects, its extraction is hindered by the instability and volatility of the molecule, restricting its wider applicability in the nutraceutical industry. Hence, nutraceutical supplements are synthesized with glucoraphanin as its main bioactive ingredient (Villarreal-García & Jacobo-Velázquez, 2016), which is subsequently converted to sulphoraphane by the gut microbiota, enhancing its bioavailability inside the body (Clarke, et al., 2011).

The agricultural residues have similar glucosinolate composition as the edible parts and bio-refining of these undervalued products enables the recovery, recycling and sustainability of bioactive ingredients within the food chain. Despite the plethora of information and technological advancements, bio-refining and waste recovery are subjected to difficulties for industrial implementation, due to stringent market and consumer concerns (Otles, Despoudi, Bucatariu, & Kartal, 2015). Bio-refining of industrial residues are

subjected to various restrictions such as seasonal availability of raw materials, microbial spoilage, immediate processing requirements and variations in the concentration of the target compounds. These limitations make the standardization process difficult, restricting the development of green processes that are industrially viable, economical and scalable, whilst retaining the functional properties of the target molecules (Mahro & Timm, 2007; Schieber, Stintzing, & Carle, 2001).

Ion exchange resins are widely and efficiently applied for the purification of glucosinolates. Yet most of the studies involve the use of non-food or non-organic grade solvents. Despite the high recovery and purity of glucoraphanin, its utilizations as food ingredients or additives are restricted, due to the use of solvents that are not easily applicable to the food industry, nor consumer friendly (Toribio, Nuzillard, & Renault, 2007; Wang, Liang, & Yuan, 2012). Limited information is available on the purification of glucosinolates from broccoli by-products, which have variable glucosinolates content, making standardization difficult, especially with the use of green solvents such as ethanol and water. Recently, a patent study described the purification of glucoraphanin from broccoli segments (florets, seeds and sprouts) using non-food grade solvent (methanol) and water, but the process efficiency was very low (Wehrli & Schutz, 2013).

Response surface design (RSD) is a statistically powerful multivariate experimental design described by Box and Wilson (G. Box & Wilson, 1951; G. E. Box & Hunter, 1957; Ferreira, Bruns, Ferreira, Matos, David, Brandao, et al., 2007). The most common types of RSD models used for the extraction and purification studies are the central composite design (CCD) and Box Behnken design (BBD). These experimental models are widely used due to their ease of use compared a full factorial experimentation methods (Tsiaka, Zoumpoulakis, Sinanoglou, Makris, Heropoulos, & Calokerinos, 2015; Wang, Liang, & Yuan, 2012). In RSD, the experimental data generated using the Box-Behnken design (BBD), were fit to a polynomial equation describing the relationships and interactions between the dependent variables (responses) and independent variables (factors), thereby generating an optimal operational set of conditions for a desirable response, with a minimum number of sample runs. Thus, the aim of this study was to obtain a robust technique for the purification of glucoraphanin from unused broccoli seeds by ion exchange

resins using RSD with BBD, and to develop an eco-friendly, economical and industrially feasible method for the fabrication of glucoraphanin–rich extracts, that can not only substitute pesticides in the agricultural industry (Doheny-Adams, Redeker, Kittipol, Bancroft, & Hartley, 2017; Gimsing & Kirkegaard, 2009) and replace synthetic drugs such as metformin with natural ingredients (Axelsson, et al., 2017).

5.5 Materials and Methods

5.5.1 Reagents and Materials

Glucoraphanin potassium salt (>99%) was obtained from Chromadex (Irvine, CA) and ethanol (95%, 100%) from Les Alcools du Commerce (Boucherville, QC, Canada). Sodium hydroxide, potassium hydroxide, ammonium hydroxide, sulfuric acid and methanol were purchased from Fisher Scientific (Ottawa, ON, Canada). The strong cation ion exchange resin (Amberlite FPC23 H) and the weak anionic resin (Amberlite FPA 53) were obtained from (Rohm and Haas, Dow waters and process solution, Philadelphia, PA). Ultrapure water was obtained from Millipore Milli-Q RG system (Billerica, MA). All the solvents used were on the GRAS list.

5.5.2 Plant Material and Sample Treatment

The discarded lot of broccoli seeds cultivar marathon F1 hybrid (MF1), was kindly provided by Norseco Inc. (Laval, QC, Canada). These seeds were non-marketable for different reasons that comprise age and/ or quality of the seed lots, yield parameters that were below export and commercialization standards, poor germination rate, grain quality and size. The seeds were finely ground using a Thermomix (Canada) grinder and stored at -20°C until further use.

5.5.3 Extraction Process

The glucosinolates in broccoli seeds by-product were extracted according to Thomas *et al*., (2018, under revision), using aqueous ethanol (50%, v/v), with a sample to solvent ratio of 1:20 on a magnetic stirrer (VWR international, LLC, Canada) at 400 rpm for 60 min at 60°C. The extract was filtered using a vacuum filter, through Whatman No 1 filter paper. Further, the filtrate was evaporated by rotary evaporator (Buchi, rotavapor R-215, USA) at

40°C, for the removal of the extracting solvent ethanol. Aliquots (300 µL) of standard solution of glucoraphanin (0 to 100 ppm), blank (milli-Q water) and samples were filtered (0.45 µm, nylon filter) and analyzed by HPLC-UV as described by West *et al*., (2002). The dry matter of the extract or the solid content in the extract was estimated by drying a known volume (2 mL) of the extract in a pre-weighed container at 105°C for at least 3 hours, or until constant weight was obtained (Miglio, Chiavaro, Visconti, Fogliano, & Pellegrini, 2008).

5.5.4 Procedures of Ion Exchange Chromatography

5.5.4.1 Cation Ion Exchange Resin

The synthetic strong cation ion exchange resin used in this study was Amberlite FPC23 H, consisting of a macroreticular crosslinked polystyrene matrix with fixed functional sulfonate groups and H+ mobile exchangeable ions. The cationic resin Amberlite FPC32 H was steeped in distilled water, overnight and rinsed with distilled water to remove any adhering dirt. Regeneration of the resin was performed by adding H_2SO_4 (5%, v/v) until the volume equals to thrice the volume of the resin for 30 min, converting the resin into their H+ form. Further, the resin was equilibrated with milli-Q water, until the pH of the solvent in contact with the resin corresponded to the pH of milli-Q water (pH 7). After regeneration and equilibration process, the resin and broccoli seed extracts in known quantities, were brought in contact, in an orbital shaker for a desired speed of agitation as described in **Table 11**. The elution of glucoraphanin from the resin was carried out using a volume of the eluting solvent (water in ethanol) corresponding to thrice the volume of the resin. The cationic extract was separated from the resin using a vacuum filter, by passing it through Whatman No:1 filters. The eluent from all the three washes were combined, mixed well and stored at 4°C, until further use in the anionic resin. Aliquots of 0.5, 3 and 2 mL were utilized to determine the glucoraphanin content, the pH and dry matter, respectively.

Table 11: Response surface Box Behnken design and process responses for purification of glucoraphanin from unused broccoli seeds using cationic resin

Run	$x_1(X_1)$	$x_2(X_2)$	$x_3(X_3)$	$x_4(X_4)$	Y - Response	
	Feed : Resin	Time of contact (min)	Agitation speed (rpm)	Concentration of eluting solvent (water) (%)	Process recovery (%)	Glucoraphanin purity in DM (%)
1	1(1:62)	1(180)	0(80)	0(50)	77.2	11.9
2	1(1:62)	-1(2)	0(80)	0(50)	79.9	12.9
3	-1(1:0.6)	1(180)	0(80)	0(50)	98.3	12.4
4	-1(1:0.6)	-1(2)	0(80)	0(50)	99.9	11.9
5	0(1:31)	0(91)	1(160)	1(100)	94.2	14.8
6	0(1:31)	0(91)	1(160)	-1(0)	79.8	12.4
7	0(1:31)	0(91)	-1(0)	1(100)	94.8	15.4
8	0(1:31)	0(91)	-1(0)	-1(0)	72.5	11.8
9	0(1:31)	0(91)	0(80)	0(50)	83.4	12.8
10	1(1:62)	0(91)	0(80)	1(100)	95.4	14.2
11	1(1:62)	0(91)	0(80)	-1(0)	85.6	13.6
12	-1(1:0.6)	0(91)	0(80)	1(100)	97.1	12.6
13	-1(1:0.6)	0(91)	0(80)	-1(0)	96.7	12.3
14	0(1:31)	1(180)	1(160)	0(50)	79.9	12.9
15	0(1:31)	1(180)	-1(0)	0(50)	74.6	11.6
16	0(1:31)	-1(2)	1(160)	0(50)	83.5	13.0
17	0(1:31)	-1(2)	-1(0)	0(50)	83.7	13.9
18	0(1:31)	0(91)	0(80)	0(50)	76.1	11.3
19	1(1:62)	0(91)	1(160)	0(50)	80.1	13.6
20	1(1:62)	0(91)	-1(0)	0(50)	81.9	13.2
21	-1(1:0.6)	0(91)	1(160)	0(50)	96.9	13.4
22	-1(1:0.6)	0(91)	-1(0)	0(50)	89.3	13.5
23	0(1:31)	1(180)	0(80)	1(100)	98.4	15.1
24	0(1:31)	1(180)	0(80)	-1(0)	75.9	11.5
25	0(1:31)	-1(2)	0(80)	1(100)	95.9	14.3
26	0(1:31)	-1(2)	0(80)	-1(0)	78.8	12.0
27	0(1:31)	0(91)	0(80)	0(50)	82.5	12.1

5.5.4.2 Anion Ion Exchange Resin

The weak anionic resin used was Amberlite FPA 53, that consists of a crosslinked acrylic gel structured matrix with a fixed tertiary amine as its functional group and the mobile exchangeable ion is a free base. The resin was soaked in distilled water overnight and rinsed to remove adhering impurities. The anionic resin was regenerated by adding NaOH (4%, w/v) until the volume equals thrice that of the resin for 30 min, converting it into its OH^- form. The regenerated resin was equilibrated with milli-Q water, until the pH of the solvent in contact with the resin corresponded to the pH of milli-Q water (pH 7). The regenerated and equilibrated resin and the cationic seed extracts in known quantities were brought in contact, in an orbital shaker fixed to a desired speed of agitation as indicated in **Table 12**. The resin loaded with glucosinolates was washed with 3 volumes of milli-Q water to remove water soluble impurities adhering onto the resin. The elution of glucoraphanin from the resin was carried out using a volume of the eluting solvent (ammonium hydroxide in 70% ethanol) corresponding to thrice the volume of the resin. The glucoraphanin enriched extract was separated from the anionic resin using Whatman No:1 filters and further evaporated to remove excess ammonium using rotary evaporator at 40°C. Aliquots of 0.5 and 2 mL were utilized to determine the glucoraphanin content and dry matter of the extract, respectively. The glucoraphanin enriched anionic extract solution was stored at 4°C until further use.

Table 12: Response surface Box Benken design and process responses for purification of glucoraphanin from unused broccoli seeds using anionic resin

Run	$x_1(X_1)$	$x_2(X_2)$	$x_3(X_3)$	$x_4(X_4)$	Y - Response	
	Feed : Resin	Time of contact (min)	Agitation speed (rpm)	Concentration of NH4OH (in 70% ethanol) (%)	Process recovery (%)	Glucoraphanin purity in DM (%)
1	1(1:55.6)	1(180)	0(80)	0(5)	68.5	37.5
2	1(1:55.6)	-1(2)	0(80)	0(5)	20.8	22.8
3	-1(1:0.6)	1(180)	0(80)	0(5)	52.7	44.5
4	-1(1:0.6)	-1(2)	0(80)	0(5)	20.0	36.6
5	0(1:28)	0(91)	1(160)	1(10)	68.3	34.5
6	0(1:28)	0(91)	1(160)	-1(0)	1.3	10.8
7	0(1:28)	0(91)	-1(0)	1(10)	50.1	34.1
8	0(1:28)	0(91)	-1(0)	-1(0)	0.9	12.3
9	0(1:28)	0(91)	0(80)	0(5)	69.7	38.6
10	1(1:55.6)	0(91)	0(80)	1(10)	64.3	22.6
11	1(1:55.6)	0(91)	0(80)	-1(0)	1.3	2.3
12	-1(1:0.6)	0(91)	0(80)	1(10)	54.2	35.2
13	-1(1:0.6)	0(91)	0(80)	-1(0)	0.2	7.6
14	0(1:28)	1(180)	1(160)	0(5)	72.9	43.4
15	0(1:28)	1(180)	-1(0)	0(5)	58.2	31.6
16	0(1:28)	-1(2)	1(160)	0(5)	28.1	33.4
17	0(1:28)	-1(2)	-1(0)	0(5)	18.2	37.8
18	0(1:28)	0(91)	0(80)	0(5)	67.4	35.0
19	1(1:55.6)	0(91)	1(160)	0(5)	70.5	36.4
20	1(1:55.6)	0(91)	-1(0)	0(5)	55.03	45.3
21	-1(1:0.6)	0(91)	1(160)	0(5)	52.6	37.6
22	-1(1:0.6)	0(91)	-1(0)	0(5)	41.1	39.1
23	01:28)	1(180)	0(80)	1(10)	74.5	34.7
24	0(1:28)	1(180)	0(80)	-1(0)	1.2	10.1
25	0(1:28)	-1(2)	0(80)	1(10)	26.9	27.1
26	0(1:28)	-1(2)	0(80)	-1(0)	0.7	2.9
27	0(1:28)	0(91)	0(80)	0(5)	68.9	33.7

5.5.5 Experimental Design and Statistical Analysis for Cationic and Anionic Resin

The process conditions for the purification of glucoraphanin from broccoli seeds discards after solvent extraction were operated according to a Box-Behnken design (BBD) (Ferreira, et al., 2007). After having determined the preliminary ranges of the extraction variables by one-factor-at-a-time experiments, four variables, with three levels each, BBD were employed to determine values that maximized process responses from broccoli seeds extract using cationic and anionic resin. The four independent variables were feed to resin ratio (g:g, X_1), time of contact (min, X_2), agitation speed (rpm, X_3) and concentration of eluting solvent (%, X_4). The latter variable (X_4) was water in ethanol (%) for the cationic resin, and NH_4OH in ethanol (%) for the anionic resin. For statistical analysis, the variables were coded according to

$$xi = \frac{(Xi - Xo)}{\Delta X}, i = 1, 2, 3 \ldots$$

Where, x_i: coded value for the independent variable, X_i: raw value for the independent variable, X_0: raw value for the independent variable at the centre point, ΔX: the step change value of the independent variable. A total of 27 experimental runs were designed using BBD (**Table 11** and **Table 12**). Three runs at the centre of the design were performed to determine the experimental error. The effects of unexplained variability in the process response, due to extraneous factors, were minimized by randomizing the order of experiments.

Regression analysis was performed for the experimental data and was fitted into the empirical second order polynomial model, as shown in the following equation:

$$Y = \beta_0 + \sum_{i=1}^{4} A_i X_i + \sum_{i=1}^{4} A_{ii} Xi^2 + \sum_{ij=1\ (i \neq 1)}^{4} A_{ij} XiXj$$

Where β_0, A_i, A_{ii}, A_{ij} are the regression coefficients in the intercept, linear, quadratic and interaction terms respectively; X_i, X_j and X_i^2 are the independent variables and Y is the dependent variable or the process response, which were; glucoraphanin recovery (Y_1, %) and glucoraphanin purity in the dry matter (DM) of extract (Y_2, %).

5.6 Salt Replacement

The ammonium salt of glucoraphanin obtained subsequent to the anionic glucoraphanin purification process was converted to a more stable glucoraphanin potassium salt, using potassium bicarbonate ($KHCO_3$). An equimolar concentration of potassium bicarbonate ($KHCO_3$) corresponding to the total glucosinolate content in the extract, supplemented with a 20% excess salt concentration, was added into the anionic elute. Further, the mix was evaporated in a rotary evaporator at 40°C, to accelerate ion replacement and to remove the excess eluting solvent ammonium hydroxide. The samples after salt replacement (0.5 and 2 mL) were analyzed for its glucoraphanin and dry matter content using a HPLC-UV and vacuum oven drier respectively, to estimate the responses factors; process recovery (%) and glucoraphanin purity in dry matter of extract (%).

5.7 Extraction, Purification and Fabrication of Extract Rich in Glucoraphanin on a Pilot Scale

The pilot scale extraction of glucosinolates and polyphenols from unused broccoli seeds was adapted from Thomas *et al*. (under revision). The glucosinolates and polyphenols were extracted with aqueous ethanol solution (50%, v/v), using a sample to solvent ration of 1:20, stirred in a 25L stainless steel mixer equipped with a high speed rotor (Tetra Pak Scanima), set at 60°C and extracted for 60 min. The extract was filtered using a vertical top discharge centrifuge CEPA TZ5 (CEPA), and the filtrate was evaporated using a rotary evaporator. The evaporated broccoli seed extract was purified for its glucoraphanin content using the optimized process parameters for strong cation ion exchange resin (Amberlite FPC23 H) and weak anionic resin (Amberlite FPA 53) in series, operated in the batch method, as adapted from the present study. Salt replacement methodology was as described in the present study. The final powered extract fabrication was performed using a spray drier (Niro atomizer) set at an inlet temperature of 140°C and outlet temperature of 81°C, with a sample flow rate at 1.4 L/hr. The powdered extract was collected in an air tight container and further analyzed for its glucosinolates and polyphenols content. Aliquots (300 µL) from the extraction, evaporation, cationic and anionic resin and salt replacement and the final powered extract (0.5 g) dissolved in milli-Q water (5 mL) were filtered (0.22 µm,

nylon filter) and analyzed by UPLC MS/MS, for its glucosinolate and polyphenol content. Further, each samples (2mL), were oven dried at 105°C for at least 3 hours, until a constant weight was obtained, to determine the dry matter content in the extracts.

5.8 Glucoraphanin Determination by HPLC-UV

The chromatographic analysis of glucoraphanin was carried out with a Phenomenex Synergi 4μm hydro-RP 80 A (25 × 0.46 cm, 4 μm particle size) column (HPLC 1100 series, Agilent, Mississauga, Canada) with a security guard for a C-18 (4 x 3 mm) cartridge system (Phenomenex). The system was equipped with a multisolvent delivery system (G1312A), an inlet degasser (G1322A), an autosampler (W717 plus) and a diode array detector (G1329A) (Agilent 1100 series HPLC). The compounds were separated by a linear gradient elution of mobile phase, 100% A (ammonium acetate 50mM w/v) and 0% B (ammonium acetate 50mM w/v in methanol (80:20. v/v)) to 50% A and B at 20 min, to a 100% B at 40 min and finally returning to the initial condition at 43 min. The flow rate was set at 1mL/min, with a sample injection volume of 10 µL, on a column maintained at ambient temperature. Data acquisition was performed using the Chemstation software and the identification of the glucoraphanin was based on the retention time and the UV spectra of the pure compound. The glucoraphanin content in the broccoli seed extracts were expressed as milligrams glucoraphanin (GE) equivalents per kilogram dry matter (mg GE/kg DW).

5.9 Simultaneous Characterization and Quantification of Glucosinolates and Polyphenols Using UPLC MS/MS

The chromatographic analysis was carried out on AQUITY UPLC HSS T3 1.8 µm (18 µm particle 2.1 id X 150mm) column (Waters, Millford, USA). The system is equipped with a Triple Quadrupole Mass Spectrometer (TQD); operated in negative electron-spray ionization mode (ESI^-) and Multiple Reaction Monitoring (MRM). The glucosinolates and polyphenols were separated by a linear gradient elution of mobile phases from 100% A (formic acid 0.1% v/v in 5% v/v acetonitrile) and 0% B (formic acid 0.1% v/v in acetonitrile 60% v/v) to 50% A and 50% B at 15 min and finally returning to the initial condition at 21 min. The flow rate was set at 0.4 mL/min, with a sample injection volume of 2.5 µL. Intact glucosinolates were expressed as sinigrin equivalent (SE), glucoraphanin

as both sinigrin (SE) and glucoraphanin equivalent (GE), flavonoids as rutin equivalent (RE), sinapic acid as sinapic acid equivalent and chlorogenic acid as caffeic acid equivalent. Intact glucosinolates and polyphenols were quantified as milligrams per kilogram dry matter (mg/kg DW) Thomas *et al*., (under revision).

5.10 Data analysis

The statistical analysis for the experimental results of BBD was carried out using SAS (version 9.4, SAS institute Inc, Cary, NC, USA) to obtain the coefficients of the second order polynomial model. The three-dimensional plots from the regression models were obtained using SAS or R (Version 3.4.1). The quality of the fitted model was expressed by the coefficient of determination R^2 and its statistical significance was checked by the F-tests on the regression parameters.

5.11 Results and Discussion

5.11.1 Extraction Process

Extraction of glucoraphanin from broccoli seeds cultivar MF1 hybrid, indicated a glucoraphanin content of 20.8 g/Kg seed. The pH of the extract after extraction and evaporation was 5.3. Drying the broccoli seeds extract under reduced pressure provided 21g of residue per litre sample with a glucoraphanin purity of 12.4%. In the present study, the glucoraphanin content in the seed sample MF1 hybrid, was lower than that reported by Thomas *et al*., (under revision). This may be due to the variability in the content of the bioactive ingredient between the sample lots discarded by the industry. Studies on bio-refining of food waste residues indicated variability in the quality and content of bioactive ingredients in the starting material due to geographic, climatic conditions and food processing parameters (Galanakis, 2015).

5.11.2 Preliminary Experiments

5.11.2.1 Effect of Mode of Operation

Preliminary experiments on the mode of operation for the purification of glucoraphanin from broccoli seed extract using a single anionic resin or a combination of cationic and anionic resins were conducted according, to the results from a sequence of experiments

carried out for the batch (i.e. mixing the resin and the extract in a flask, followed by a vacuum filtration and elution) and continuous (i.e. column chromatography of the extract through the resin) approaches. The operational conditions for the preliminary experiments were: feed to resin ratio of 1:35, contact time of 30 min and elution of glucosinolates from the anionic resin with 5% ammonium hydroxide in methanol. The contact time and concentration of the anionic eluting agent was adapted from the studies of Wehril & Schutz (2013). In the present experiments, high feed to resin ratio was chosen to avoid resin saturation. The maximal process response for the purification of glucoraphanin was achieved by batch method, with cationic and anionic resins in series, providing a process recovery and glucoraphanin purity of 81.4 and 41.9%, respectively. The enhanced process recovery for resins in series, may be due to the displacement of K^+ ion of glucoraphanin with an easily exchangeable H^+ ions, reducing the pH of the cationic elute to 2.3 and correspondingly increasing its affinity towards the anionic resin. The clarification of seed extracts by the removal of impurities such as pigments and oils by the resin enhanced the purity of glucoraphanin. As, observed in the present study, batch separation technique was usually preferred due to its ease of operation and its ability to purify bioactive molecules present in low concentration range in the extracts. The preference of batch over column technique for the purification of bioactives from crude extracts, may be due to the limitations in the operational parameters of the column methods i.e. clogging of the column due to sample impurities, creating severe back pressure disrupting the column procedures (Biosciences, 2002).

5.11.2.2 Effect of pH

The pH of the seed extract prior to the anionic resin experiment is a crucial factor that drastically influenced the affinity of glucosinolates onto the anionic resin. A variety of acids (sulphuric acid, acetic acid and hydrochloric acid (0.1N, v/v)), were utilized to modify the pH of the seed extract to 2.3, prior to the anionic resin experiment, with no significant increase in the affinity of glucoraphanin on to the resin. This may be due to the extracted plant solids, compounds and the anionic nature of the glucosinolates to neutralize the acidic condition (Powell, Hill, Juurlink, & Carrier, 2005), or the inability of the acidic medium to replace the K^+ ion of glucosinolates with an easily replaceable ion, that

enhances the affinity between glucosinolates and the anionic resin. Thus, a combination of cationic and anionic resin in series was identified as a pre-requisite to maximize the process response, as observed by Wehril & Schutz (2013).

5.11.2.3 Effect of Solvent System

The selection of the optimal solvent concentration for the anionic eluting salt (NH_4OH (5%, v/v)) was determined by measuring its ability to extract glucoraphanin from the anionic stationary phase. The eco-friendly nature of this study and the hydrophilicity of glucosinolates necessitated the use of polar solvents such as ethanol and water (0-100%). The results from the preliminary experiments showed an inverse correlation between the process responses (process recovery and glucoraphanin purity) and the solvent concentration. As the concentration of water increased, the process recovery increased from 50 to 89%, with a reduction in glucoraphanin purity from 59 to 32%. This may be due to increased dry matter content in the extract thereby, reducing the purity of glucoraphanin by a dilution process. A 100% ethanol concentration increased the glucoraphanin purity to 59% accompanied by a reduction in process recovery to 50%. The use of aqueous ethanol (70%, v/v) as the eluting solvent, provided a balance between process recovery and glucoraphanin purity in the seed residue of 66% and 45% respectively, hence, chosen as the preferred solvent system. The application of aqueous ethanol for the purification of glucosinolates may be due to its polarity and hydrodynamics, as reported by Toribio *et al*., (2007) for the purification of glucosinolates from broccoli seeds using strong ion exchange resins.

5.11.2.4 Effect of Anionic Eluting Salts

A variety of food grade anionic eluting salts such as sodium hydroxide, potassium hydroxide and ammonium hydroxide (5%, (w/v) / (v/v) in 70% ethanol v/v), were utilized to study its influence in the displacement of glucoraphanin from the anionic stationary phase. The preliminary results showed that ammonium hydroxide (5% v/v in 70% ethanol v/v) provided a process recovery of 73% with a purity of glucoraphanin in the dry matter of the extract of 51%. The efficiency of the eluting salt is based on its affinity for the anionic stationary phase, displacing the already bound glucoraphanin by forming an ion pair.

Similar results were observed by Toribio *et al*., (2007) and Wehril & Schutz (2013), who reported ammonium hydroxide as the best displacer salt for the purification of glucosinolates. This may be due to the inherent ability of alkyl-ammonia to form ion pairs with sulfate groups, mimicking the glucosinolates transportation mechanism in plants.

5.11.3 Ion Exchange Chromatography

5.11.3.1 Cationic Ion Exchange Resin

5.11.3.1.1 Response Surface Analysis and the Model Fitting for Purification of Glucoraphanin from Broccoli Seeds by Cationic Ion Exchange Resin

In the present study, there were a total of 27 experimental runs to optimize the four independent variables required to maximize the responses (process recovery (%) and glucoraphanin purity (%)). Extraction of glucoraphanin from ground broccoli seeds MF1 (1g), using an aqueous ethanol (20 mL, 50%, v/v) at 60°C for 60 min; provided a glucoraphanin purity of 12.4%. Multiple regression analysis on the experimental data indicated that feed to resin ratio and solvent concentration were the statistically significant variables to maximize process responses ($p<0.05$); than shaker speed and contact time. Canonical and ridge analysis on the process variables failed to maximize the process responses simultaneously, as the stationary point is a saddle point. Hence, a new experimental design was created to design a new response variable (Factor1), by applying response surface model to the composite scores of the response variables, i.e. process recovery (%) and glucoraphanin purity (%). The weight associated with each response variables was determined by their loading on the first PCA axis. The composite scores were further calculated from the weighted average of the response variables that had been previously standardized. Multivariate analysis on the experimental data, using PCA **(Table 13)**, indicated that the quadratic model is statistically significant for glucoraphanin productivity ($p<0.05$). The response factor and the test variables were related by the following second order polynomial equation in terms of coded factors:

$$Y = 15.32 - 0.33x_1 - 1.4849x_4 + 0.019x_1^2 + 3.516\,x_4^2 + 0.0793\,x_1\,x_4 \qquad \textbf{(1)}$$

The response surface regression procedure on the experimental data provided a determination co-efficient (R^2 = 0.669), indicating that only 33.1% of the total variation was not explained by the model. The value of the adjusted determination coefficient (Adj. R^2 = 0.59) also confirmed that the model was highly significant. A low value of coefficient of the variation (C.V.) of 5.26% clearly indicated a very high degree of precision and good reliability of the experimental values.

The goodness of fit of the regression model can be determined by residual mean square (MS_{res}) index. Values between 0.5 and 1.5 are considered statistically acceptable, signifying the goodness of fit of the mode. In this study, MS_{res} was 0.63 indicating that the model fits. The regression coefficient values of Equation **(1)** were listed in Table 4. The p-value was utilized to check the significance of each coefficient. The smaller the p-value, the more significant the corresponding coefficient. **Table 13**, shows that linear coefficient and quadratic terms associated with solvent concentration were significant with a very small p-value ($p<0.05$) to maximize process response. The mathematical model that describes the process response as a function of process variables **(1)** was utilized to design a three-dimensional response surface plot (3-D plot) to predict the relationship between independent and dependent variables.

Table 13: Estimated regression coefficient for the quadratic polynomial model and the analysis of variance (ANOVA) for the cationic experimental model for broccoli seeds

Parameter	Estimated coefficient	Standard error	DF	SS	F-value	P-value
Intercept	15.3	0.6				
X_1	-0.3	0.1	1	1.5	2.3	0.2
X_4	-1.5	1.6	1	18.8	29.6	<0.0001**
$X_1 * X_1$	0.02	0.01	1	1.6	2.5	0.1
$X_4 * X_4$	3.5	1.3	1	4.9	7.8	0.01*
$X_1 * X_4$	0.08	0.1	1	0.2	0.3	0.6
Residual						
Lack of fit			3	6.9	6.4	0.004*
Pure error			18	6.4		

*Significant at 5% level
**Significant at 1% level
DF, degree of freedom; SS, sum of squares

5.11.3.1.2 Optimization of the Purification Conditions and Analysis of BBD for Cationic Resin

The response surface plot generated by SAS represents the influence of process variables, feed to resin ratio and solvent concentration on the new response variable (Factor1), fixing the shaker speed and contact time at 80 rpm and 91 min respectively (level 0) **(Figure 13)**. The glucoraphanin productivity increased with an increment in solvent concentration, and reached a peak value at a feed to resin ratio of 1:26 and then dropped with increases in the ratio from 1:26 to 1:62. At lower feed to resin ratio and high solvent concentration, the glucoraphanin yield increased. In the present study, a process recovery and glucoraphanin purity of 93.8% and 14.3% respectively were obtained, with a feed to resin ratio, eluting solvent concentration, contact time and agitation speed of 1:26, 100% water, 91min and 80 rpm, respectively.

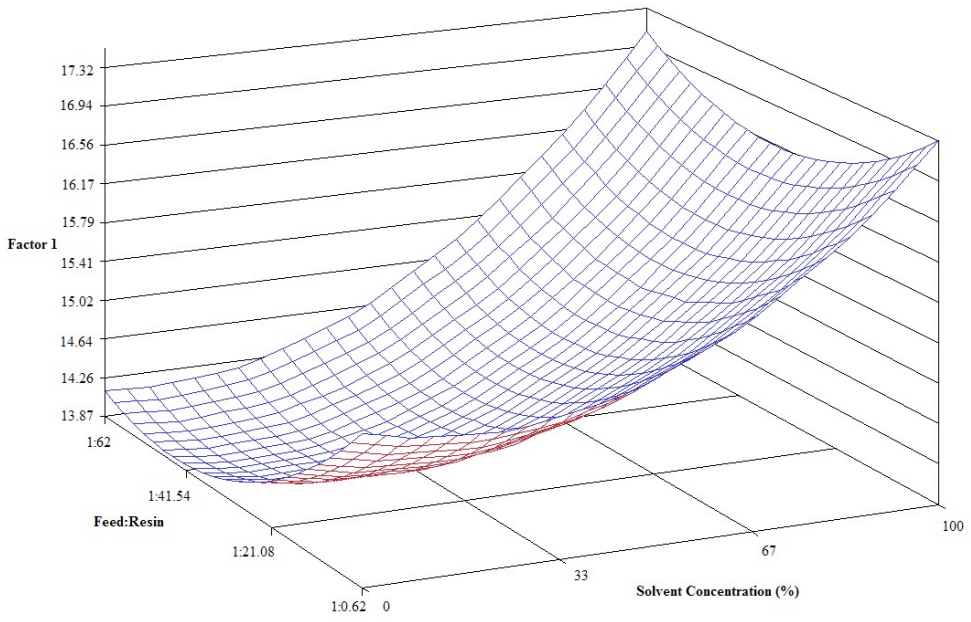

Figure 13: Response surface 3-D plot showing the influence of feed to resin ratio and solvent concentration on glucoraphanin recovery (Factor 1) using cationic resin

5.11.3.1.3 Verification and Optimization of Operational Conditions for Purification of Glucoraphanin from Broccoli Seeds using Cationic Resin

RSM analysis of the experimental model provided an optimized process parameters for feed to resin ratio, solvent concentration, contact time and agitation speed of 1:26, 100% water, 91 min and 80 rpm, respectively, to maximize the process responses. The model predicted a maximal process recovery and glucoraphanin purity of 93.8% and 14.2% respectively. Whereas, an experimental process recovery and glucoraphanin purity of 93.8% and 14.2% was obtained, signifying the validity of the model**.** The influence of time of contact (t = 15 min and 30 min) on the process responses indicated no significant difference in the process responses when compared to the optimized process variable. Contact time of 15 min provided a process recovery of 92% with a glucoraphanin purity of 13.2%, whereas at t = 30 min a process recovery of 93.2% and glucoraphanin purity of 13.5% were obtained. These results corroborate the statistical study, indicating that contact time is a non-significant parameter for the process response.

Studies on the influence of feed to resin ratio on process responses (pH of the eluted extract, process recovery and glucoraphanin purity in the residue), were evaluated, maintaining contact time, solvent concentration and agitation speed at 30 min, 100% water and 80 rpm respectively. Statistical analysis on the experimental model indicated a significant influence of feed to resin ratio on the process responses ($p<0.05$) **(Table 14)**. The pH of the broccoli seeds extract reduced from 5.3 after extraction to 2.2 $\pm$ 0.4 after passing through the cationic resin. The alteration in the pH of the extract may be due to the displacement of the K^+ ions in the glucosinolates with the H^+ ions from the cationic resin or the clarification of the seed extract from plant solids and extracted molecules, that otherwise may neutralize the acidic conditions. The inability to alter the pH of broccoli extract with the addition of an acid or a base was also reported by Thomas *et al*., (2017, under revision) and Powell *et al*., (2005). Hence, cationic resins overcame these limitations, thereby facilitating the downstream processing. Therefore, a feed to resin ratio of 1:5, contact time of 30 min, eluting solvent concentration of 100% water and agitation speed of 80 rpm, maximized the process recovery and purity of glucoraphanin to 98% and

14% respectively. In this study, the process parameters were similar to those reported by Wehril & Schutz (2013) except for the lower feed to resin ratio of 1:2.

Table 14: Optimization of the operation parameters for purification of glucoraphanin from broccoli seeds by-products by cationic resin

		Productivity of glucoraphanin	
Feed : Resin	**pH**	**Process recovery (%)**	**Glucoraphanin purity in DM (%)**
1:0.6	2.6±0.0	97.9	13.1±0.2
1:1	2.3±0.0	96.9	13.2±0.4
1:2	2.3±0.0	97.7	13.7±0.3
1:5	2.2±0.0	97.9	14.1±0.01
1:10	2.3±0.0	92.3	14.1±0.2
1:15	2.4±0.0	93.3	13.9±0.3
1:20	2.4±0.0	94.6	13.8±0.2
1:26	2.5±0.0	93.9	14.3±0.0

5.11.3.2 Anion Ion Exchange Resin

5.11.3.2.1 Response Surface Analysis and the Model Fitting of Box-Behnken Design (BBD) for Anionic Resin

The operational conditions for the purification of glucoraphanin using anionic resin from broccoli seed discards was optimized using a series of experimental conditions in randomized order according to the Box-Behnken design. **Table 12**, presents the experimental BBD and the corresponding process recovery (%) and glucoraphanin purity (%). The regression analysis indicated that all the linear and quadratic parameters, corresponding to the responses, were found to be significant ($p<0.05$), whereas the cross-product interaction parameters were insignificant ($p>0.05$). The regression coefficients of the intercept, linear, quadratic and interaction terms of the model for the response factors are presented in **Table 15**. Multiple regression analysis on the experimental data indicated the statistical significance of independent variables; feed to resin ratio (x_1), contact time (x_2), agitation speed (x_3) and concentration of the eluting solvent (x_4) on process responses (recovery (Y_1) and glucoraphanin purity in the residue (Y_2)) ($p<0.05$). The second-order polynomial model linking the process responses as a function of process variables were described by the following equations:

Y_1 (recovery) = -0.238 + 0.037x_1 + 0.004x_2 + 0.0002x_3 + 0.196x_4 – 0.003x_1^2 – 2.2E-05x_2^2 – 1.13E-07x_3^2 - 0.016x_4^2 + 7.4E-05x_1x_2 + 2.24E-06x_1x_3 + 3.3E-04x_1x_4 + 1.33E-07x_2x_3 +2.7E-04x_2x_4 + 1.07E-05x_3x_4 **(2)**

Y_2 (glucoraphanin purity) = 0.31 – 0.002x_1 + 0.0003x_2 – 1.5E-04x_3 + 0.122x_4 – 3.3E-04x_1^2 – 1.6E-06x_2^2 + 6.9E-08x_3^2 - 0.009x_4^2 + 4E-05x_1x_2 – 4.1E-06x_1x_3 – 1.2E-04x_1x_4 + 5.9E-07x_2x_3 – 3.9E-05x_2x_4 + 1.7E-06x_3x_4 **(3)**

The response surface regression procedure (RSREG) on the experimental data for process recovery (Y_1) **(Table 15)** provided a coefficient of determination (R^2) of 0.986, indicating that the model represents an excellent relation between the parameters chosen. The adjusted determination coefficient (Adj. R^2) value of 0.97 confirms the adequacy of the model. The coefficient of variation (C.V.) value was 9.2%, indicating that the model is reproducible. Similarly, multivariate regression analysis on glucoraphanin purity (%) provided a coefficient of regression (R^2) of the model as 0.95, indicating that 95% of the variability can be explained by the model. The adjusted determination coefficient (Adj. R^2) value of 0.89 confirms the high significance of the model. The coefficient of the variation (C.V.) value was 10%, confirming sufficient reliability of the model.

The response surface methodology (RSM) and ridge analysis on the experimental models provided two sets of operation parameters that maximized the responses. Ridge analysis on maximizing process recovery (Y_1) to 74.5% was obtained with a feed to resin ratio of 1:56, contact time of 180 min, agitation speed of 160 rpm and concentration of the eluting solvent at 10% ammonium hydroxide in 70% ethanol. The optimized process variables were the extreme points set for the variables set for BBD, suggesting its inadequacy on the industrial scale. Whereas, a feed to resin ratio of 1:20, contact time of 136.9 min, agitation speed of 159 rpm and concentration of the eluting solvent at 6.5% ammonium hydroxide in 70% ethanol maximized glucoraphanin purity (Y_2) to 47.7%. Simultaneous maximization of the two responses using PCA proved ineffective due to the lack of co-relation at the extreme points between the process responses (Y_1) and (Y_2).

Table 15: Estimated regression coefficient for the quadratic polynomial model and the analysis of variance (ANOVA) for the experimental results for the purification of glucoraphanin by anionic resin

Parameter	Estimated coefficient		Standard error		DF		SS		F-value		P-value	
	Y_1 (%)	Y_2 (%)	Y_1 (%)	Y_2 (%)	Y_1 (%)	Y_2 (%)	Y_1 (%)	Y_2 (%)	Y_1 (%)	Y_2 (%)	Y_1 (%)	Y_2 (%)
Intercept	-0.23	0.31	0.10	0.09	1	1						
X_1	0.04	-0.002	0.01	0.01	1	1	0.04	0.02	10.3	5.2	0.01*	0.04
X_2	0.004	0.0003	8.7E-04	0.001	1	1	0.43	0.02	118	7.1	<0.0001**	0.02
X_3	0.0002	-1.5E-04	9.6E-05	8.9E-05	1	1	0.05	0.0002	13.1	0.1	0.004*	0.8
X_4	0.19	0.122	0.02	0.01	1	1	1.71	0.31	474.7	101.3	<0.0001**	<0.0001**
X_1* X_1	-0.003	-3.3E-04	7.8E-04	0.0007	1	1	0.01	0.004	1.4	1.5	0.3	0.3
X_2* X_1	7.4E-05	4E-05	5.8E-05	5.4E-05	1	1	0.01	0.001	1.6	0.5	0.2	0.5
X_2* X_2	-2.2E-05	-1.6E-06	3.3E-06	3.04E-06	1	1	0.02	0.01	5.9	1.9	0.03*	0.2
X_3* X_1	2.46E-06	-4.1E-06	6.5E-06	6.01E-06	1	1	0.001	0.001	0.1	0.5	0.71	0.5
X_3* X_2	1.33E-07	5.9E-07	4.2E-07	3.91E-07	1	1	0.0003	0.01	0.1	2.3	0.8	0.2
X_3* X_3	-1.13E-07	6.9E-08	4.07E-08	3.7E-08	1	1	0.02	0.1	5.5	26.1	0.04*	0.0003*
X_4* X_1	3.2E-04	-1.2E-04	0.001	9.6E-04	1	1	0.0003	0.00004	0.1	0.01	0.8	0.9
X_4* X_2	2.7E-04	-3.9E-05	6.8E-05	6..26E-05	1	1	0.1	0.001	15.4	0.4	0.002*	0.5
X_4* X_3	1.07E-05	1.7E-06	7.5E-06	6.96E-06	1	1	0.01	0.0002	2.04	0.1	0.2	0.8
X_4* X_4	-0.02	-0.01	0.001	9.6E-04	1	1	0.81	0.3	222.9	80.6	<0.0001**	<0.0001**
Residual												
Lack of fit					10	10	0.004	0.04	26.9	5.1	0.04*	0.2
Pure error					2	2	0.0001	0.001				

The industrial applicability of the optimized experimental parameters obtained by RSM and ridge analysis were validated by analyzing the influence of feed to resin ratio (1:5, 1:10, 1:20) on the process responses. The process responses contact time, agitation speed and concentration of the eluting solvent were maintained at 140 min, 160 rpm and 7% NH_4OH in 70% ethanol respectively **(Table 16)**. The process variables were fixed based on the results obtained after the RSM and ridge analysis, and the predicted responses calculated from the regression equation **(2)** and **(3).** An inverse correlation was obtained between process responses with respect to feed to resin ratio, indicating that with an increase in feed to resin ratio the process recovery increased with a decrease in glucoraphanin purity. Therefore, the optimized process parameters for maximizing the process response by anionic resin with prior purification using cationic resin were a feed to resin ratio of 1:5, contact time of 140 min, agitation speed of 160 rpm and concentration of the eluting solvent of 7% NH_4OH in 70% ethanol. The optimized parameters offered a process recovery of 70%, providing 8.3g/L of residue with a glucoraphanin purity of 37%. The glucoraphanin recovery in this study was higher than the reported in the patent study of Wehrli & Schutz (2013), where the process recovery was 41% using 5% ammonium hydroxide in methanol and 60% using 5% ammonium hydroxide in water.

Purification of glucoraphanin using anionic resin without prior cationic resin purification provided a process recovery and glucoraphanin purity of 12 and 21%, respectively. The results obtained in this study were less efficient than observed by Wehril & Schutz (2013), who reported a process efficiency of 21.8%, using 5% NH_4OH in a non-food grade solvent methanol and 17% using 5% NH_4OH in water. The results obtained in this study were lower than that reported by Toribio *et al*., (2007), Wang *et al*., (2012) and Cynthia *et al*., (2012), but previously reported methods of glucosinolate purification involved the use of non-food grade solvents, that limited its applicability for its use in producing extracts rich in bioactive glucosinolates used in enriched foods and supplements. Therefore, the optimized green process for the purification of glucoraphanin from broccoli seeds using a combination of cationic and anionic resins in series was more efficient than the previously reported purification processes predominantly involving non-food grade solvents.

Table 16: Optimization of the operation parameters for anionic resin for purification of glucoraphanin from unused broccoli seeds

	Glucoraphanin productivity			
	Measured		Predicted	
Feed : Resin	Process recovery (%)	Glucoraphanin purity in DM (%)	Process recovery (%)	Glucoraphanin purity in DM (%)
1:5	70	37	70	49.9
1:10	72	36.2	74	49.4
1:20	79.2	36.2	79	47.7
1:5*	12	20.5	NA	NA

*purification of glucosinolates using anionic resin, without the cationic resin

5.11.4 Salt Replacement

Salt replacement methodology to substitute ammonia salt of glucoraphanin to a more stable potassium salt with the addition of potassium bicarbonate ($KHCO_3$), an industrially applicable food grade salt, offered a process efficiency of 95% with a glucoraphanin purity of 36%. The chemical decomposition of ammonium bicarbonate in aqueous solution into ammonia, carbon dioxide and water facilitated glucoraphanin salt replacement, without compromising on its purity, with a non-significant increase in dry matter of the extract after drying (Nowak & Skrzypek, 1989). Wehrli & Schutz (2013) reported a process efficiency of 91%, lower than that reported in this study, for broccoli seeds using NaOH (1N). Overall, the proposed optimized extraction and purification process concentrated glucoraphanin 19 times with an overall process efficiency of 67%, thereby proving an efficient and industrially sustainable process using green technology.

5.11.5 Extraction, Purification and Fabrication of Extract Rich in Glucoraphanin on a Pilot Scale

In accordance with the extraction and purification results obtained at the laboratory scale, the pilot batch extraction was performed on 2 kg, ground broccoli seeds. The optimized extraction parameters provided a glucoraphanin yield of 37.8 g/Kg seeds. After the first extraction 562g of residue was removed from the extract with a glucoraphanin purity of 11.2%. Purification of the glucosinolates using the cationic and anionic ion exchange resins, as optimized in the present study, concentrated the glucosinolates 4.5 times, recovering 73 g of extract with a glucoraphanin purity of 48.2%. The optimized purification process was highly efficient with a process efficiency of 97%. Salt replacement

methodology using potassium bicarbonate ($KHCO_3$) offered a process efficiency of 96%, recovering 67 g of residue, with a glucoraphanin purity of 50%. The final extract fabrication process, involved the addition of 50% maltodextrin, and drying the extract using a spray dryer, providing a powered extract with a glucoraphanin purity of 23%. Overall, the optimized process, for the fabrication of a powered extract rich in glucoraphanin from unused broccoli seeds using an industrially feasible solvents and techniques was highly efficient, offering an overall process efficiency of 93%. The process efficiency and glucoraphanin recovery observed in this pilot scale study was higher than that observed for the laboratory scale experiments observed in this study and those reported by Wehrli & Schutz (2013)

Analysing the extracts for its glucosinolates and polyphenols content, quantified 12 glucosinolates and 5 polyphenols, of which glucoraphanin is the most distinctive **(Table 17).** Quantification results show significant increase in the concentration of glucosinolates and polyphenols across the process. The glucosinolates content in the seed extract was concentrated 0.5-10 folds in the final powdered extract after the purification process. Of the 12 glucosinolates quantified, the aliphatic glucosinolate glucoiberverin and glucoerucin showed the least increase in its concentration of 0.4 and 2 times respectively, which is mainly due to the loss of these molecules at the resin purification process. Glucoraphanin, the most predominant glucosinolate present in the seeds is concentrated 12 times during the resin purification process and overall, 7 folds across the extract fabrication process. Whereas, for the indolic glucosinolates there is an increase in its concentration across the process, except 4-hydroxyglucobrassicin, whose concentration drastically reduces after the cationic experiment, and completely lost during the anionic experiment. This may be due to its increased affinity towards the cationic and anionic resins, and its reduced desorption from the resin using water and ammonium hydroxide in ethanol respectively. While for the polyphenolic content, the resin purification process did not enhance its concentration, due to its absence or reduced concentration in the starting material, except for sinapic acid, whose overall concentration increased 13 folds across the process, as observed for the glucosinolate in the present study. There is a lack of literature available on the characterization and quantification of glucosinolates and polyphenols across the extract fabrication process. Hence, this study is the first of its kind, that explains the destiny of

each glucosinolates and polyphenols, upon extraction, purification and extract fabrication process, from unused broccoli seeds. Overall, an efficient, industrially feasible extract fabrication process was developed using green technology from industrial discards.

Table 17: Total and individual glucosinolates (GLS) and polyphenols content in unused broccoli seed cultivar (Brassica oleracea var. italica), across the extract fabrication process on pilot scale

Molecular Compounds (% in DM of extract)		Seed extract fabrication process					
		Extraction	Evaporation	Cationic resin	Anionic resin	Salt replacement	Powered extract
Aliphatic Glucosinolates							
C-3	Glucoiberverin[1]	0.15	0.49	0.20	0.16	0.10	0.06
	Glucoiberin[1]	1.14	3.38	3.76	13.76	13.26	6.14
	Sinigrin[1]	N.D	N.D	N.D	N.D	N.D	N.D
C-4	Glucoerucin[1]	1.14	3.69	3.70	3.90	3.63	1.70
	Glucoraphanin[1]	1.80	4.82	5.74	20.69	21.89	10.85
	Glucoraphanin[2]	3.78	11.15	13.23	48.20	50.43	22.79
	Gluconapin[1]	0.02	0.03	0.05	0.20	0.17	0.09
	Progoitrin[1]	N.D	N.D	N.D	N.D	N.D	N.D
C-5	Glucoalyssin[1]	0.01	0.05	0.06	0.18	0.16	0.08
Indolic Glucosinolates							
	Glucobrassicin[1]	0.01	0.05	0.02	0.11	0.14	0.08
	Neoglucobrassin[1]	0.03	0.08	0.07	0.34	0.36	0.20
	4-Hydroxyglucobrassicin[1]	0.0007	0.001	0.001	N.D	N.D	N.D
	4-Methoxyglucobrassicin[1]	0.01	0.04	0.01	0.12	0.17	0.09
Polyphenols							
Flavonoids[3]							
	Kaempherol-3-o-sophoroside[4]	0.001	0.001	0.001	N.D	N.D	N.D
	Quercetin-3-diglucoside-7 glucoside[4]	N.D	N.D	N.D	N.D	N.D	N.D
Hydroxycinnamic acids							
	3-Caffeoyl quinic acid[5]	N.D	N.D	N.D	N.D	N.D	N.D
	5-Caffeoyl quinic acid[5]	N.D	N.D	N.D	N.D	N.D	N.D

Sinapic acid[6]	0.003	0.007	0.003	0.093	0.074	0.06

[1] mg sinigrin equivalent/kg; [2] mg glucoraphanin/kg; [3] mg rutin equivalent/kg; [4] tentative identification; [5] mg caffeic acid equivalent/kg; [6] mg sinapic acid equivalent/kg.

5.12 Conclusion

This work presents the application of ion-exchange resins in series for the purification of glucoraphanin from the discarded lot of broccoli seed cultivar MF1-hybrid. Glucoraphanin, the key bioactive ingredient in broccoli seeds is present at a concentration of 20.8 g glucoraphanin/kg seeds with a glucoraphanin content of 12% in the dry matter of the extract after extraction. Preliminary studies on glucoraphanin purification using a combination of cationic and anionic resins in series operated under the batch mode maximized the process responses. The RSM and BBD were successfully employed for the optimization of the glucoraphanin purification procedure by ion-exchange resins. Purification of glucoraphanin using a strong cationic resin (Amberlite FPC 23H), indicated that feed to resin ratio is the most important variable that influenced the process responses. Hence, the optimized purification parameters, that maximized the process responses simultaneously using PCA, were a feed to resin ratio of 1:5, contact time of 30 min at an agitation speed of 80 rpm and elution using 100% milli-Q water. The optimized process dropped the pH of the extract from 5.3 to 2.2 providing an optimal process response of 98% process efficiency and 14% glucoraphanin purity in the dried cationic extract. The optimum result obtained for the purification of glucoraphanin using anionic resin indicated that a contact time of 140 min with a 1:5 feed to resin ratio for an agitation speed of 160 rpm and a 7% ammonium hydroxide solution in 70% ethanol as eluting agent, provided a process efficiency of 70% and a 37% glucoraphanin purity in the dried residue. Pilot scale fabrication of extracts using the optimized process parameters concentrated glucosinolates 0.5 to 10 folds, most predominantly glucoraphanin by 7 folds; and polyphenols mainly sinapic acid by 13 folds. Hence, a highly efficient green process for the fabrication of extracts rich in glucoraphanin from industrial discards was developed with an overall process efficiency of 93% and a glucoraphanin content of 23% in the final powdered extract. Overall, the proposed study provided an efficient, environmental friendly, industrially feasible route towards bio-refining of industrial discards and fabrication of health beneficial supplements that can be used in the food and nutraceutical industry.

5.13 Acknowledgement

The authors acknowledge the financial support of the Natural Science and Engineering Research Council of Canada (NSERC), Organic Science Cluster II and Diana Food Canada Inc. We would also like to express our gratitude to Norseco Inc., Québec, Canada for providing the seed samples.

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Chapter 6

Response surface optimization of ion exchange assisted glucoraphanin purification from industrial discards of broccoli florets by-products

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Keywords: Broccoli, industrial discards, glucoraphanin, ion exchange resin, optimization, RSM, BBD, PCA, dietary supplements.

6.1 Foreword

The most widely used technique for the purification of glucosinolates is with the use of ion exchange resins. The inherent anionic nature of the glucosinolates is exploited for its isolation from the crude extracts. In this chapter, an industrially viable green process for the purification and fabrication of extracts rich in glucoraphanin was developed, using the response surface approach.

This chapter is presented as an article entitled: "Response surface optimization of ion exchange assisted glucoraphanin purification from industrial discards of broccoli florets by-products" has been revised and we intend to submit it in Food Chemistry.

The authors are Minty Thomas (first author), who participated in the planning, interpretation, discussion and writing of the article. Dr. Paul Angers, director of the thesis, and Dr. André Gosselin, Yves Desjardins, co-directors of the thesis participated in the planning of the research, the discussion of the results at meetings and the revision of the article presented. Dr. Ashraf Badr, research professional, participated in the discussion of the results and in the revision of the article

An economically viable and industrially applicable green process for the purification of glucoraphanin from industrial discards was developed that brings us closer to sustainability, with the fabrication of bioactive supplements rich in glucoraphanin that can be used in the food and pharmaceutical industry.

6.2 Résumé

Les rejets industriels des fleurons de brocoli sont riches en ingrédient alimentaire fonctionnel notamment la glucoraphanin, qui peut être utilisé pour la fabrication de suppléments Alimentaires. Le but de cette étude était de développer un procédé efficace pour la purification de la glucoraphanine à l'aide d'une résine échangeuse d'ions par l'intermédiaire d'une approche de surface de réponse. La méthodologie de surface de réponse (RSM), basée sur la conception de Box-Behnken (BBD) et l'analyse de composantes principales (PCA) ont été utilisées pour optimiser le processus de purification. Les conditions optimales pour la purification de la glucoraphanin à partir de rejets industriels de fleurons de brocoli en utilisant une résine cationique étaient un rapport matière/résine de 1:1.87, temps de contact de 30 minutes, vitesse d'agitation de 80 rpm et solvant d'élution de 100% d'eau. Dans ces conditions, le pH a été réduit de 5.7 à 2, assurant une récupération du procédé de 95% et une pureté en glucoraphanine de 1.25%. Ensuite, l'extrait cationique contenant de la glucoraphanine a été purifié par la résine anionique. Les paramètres expérimentaux optimisés étaient un rapport matière:résine de 1: 1.3 mis en contact pendant 170 min avec une vitesse d'agitation de 140 rpm et élue en utilisant de l'hydroxyde d'ammonium à 7% dans de l'éthanol à 70%. Une récupération du procédé de 78% et une pureté en glucoraphanine de 5% ont été observées. Le remplacement du sel par le bicarbonate d'ammonium, sous pression réduite, fournit une pureté en glucoraphanine de 4.4%. Les processus optimisés ont augmenté la concentration de glucoraphanine 12 fois, fournissant une efficacité globale du processus de 81%. La fabrication d'extrait en poudre à l'échelle pilote en utilisant la technique optimisée d'extraction et de purification à l'échelle du laboratoire, avec l'addition de 55% de maltodextrine, a augmenté la concentration de glucoraphanine de 4,3 fois, donnant un rendement global de 86%. Ainsi, un procédé vert très efficace pour la fabrication d'extraits enrichis en glucoraphanine a été développé à partir de rejets industriels de fleurons de brocoli, prouvant une voie alternative vers le bioraffinage.

6.3 Abstract

Broccoli florets industrial discards are rich in function food ingredient glucoraphanin that can be utilized for the fabrication of dietary supplements. The purpose of this study was to develop an efficient ion exchange resin assisted glucoraphanin purification process through response surface approach. Response surface methodology (RSM), based on Box-Behnken Design (BBD) and Principle component analysis (PCA) were employed to optimize the purification process. The optimal conditions for the purification of glucoraphanin from industrial discards of broccoli florets using cationic resin were a feed to resin ratio of 1:1.87, contact time of 30 minutes, agitation speed of 80rpm and eluting solvent of 100% water. Under these conditions, the pH was reduced from 5.7 to 2, providing a process recovery of 95% and glucoraphanin purity of 1.25%. Subsequently, the cationic extract containing glucoraphanin was further purified by the anionic resin. The optimized experimental parameters were a feed to resin ratio of 1:1.3 brought in contact for 170 min with an agitation speed of 140 rpm and eluted using 7% ammonium hydroxide in 70% ethanol. Process recovery of 78% and glucoraphanin purity of 5% was observed. Salt replacement using ammonium bicarbonate, under reduced pressure provided a glucoraphanin purity of 4.4%. The optimized processes concentrated glucoraphanin 12 times providing an overall process efficiency of 81%. Pilot scale fabrication of powdered extract using the optimized laboratory scale extraction and purification technique, with the addition of 55% maltodextrin concentrated glucoraphanin 4.3 times providing an overall process efficiency of 86%. Hence, a highly efficient green process for the fabrication of glucoraphanin enriched extracts was developed from broccoli florets industrial discards, proving an alternative route towards biorefining.

6.4 Introduction

Broccoli (*Brassica oleracea* L. var. italica), an economically important crop belonging to the Brassicaceae family is widely known for its health promoting and disease preventive properties. The positive health effects of broccoli are due to its content in plant bioactives such as glucosinolates, polyphenols, carotenoids, vitamins, and minerals, but most distinctively glucoraphanin and its conversion molecule sulphoraphane (Bellostas, Kachlicki, Sørensen, & Sørensen, 2007; Villarreal-García & Jacobo-Velázquez, 2016). Epidemiological and cohort studies indicated a positive correlation between the consumption of broccoli and reduced onset of diseases such as cancer (Clarke, Hsu, Riedl, Bella, Schwartz, Stevens, et al., 2011), diabetes (Axelsson, Tubbs, Mecham, Chacko, Nenonen, Tang, et al., 2017), coronary heart diseases (Ares, Nozal, & Bernal, 2013), neurodegenerative and age related diseases (Lee, Kim, Seo, Choi, Han, Lee, et al., 2014) due to its anti-inflammatory and anti-oxidant properties (Jeffery & Araya, 2009).

Increasing awareness regarding the adverse effects of synthetic food additives and the positive health impact on the consumption of broccoli on health have led to the incorporation of natural ingredients into the diet and its increased consumption (Domínguez-Perles, Martínez-Ballesta, Carvajal, García-Viguera, & Moreno, 2010; Schieber, Stintzing, & Carle, 2001). Worldwide, the annual production of broccoli is 21 million tons. Lack of good agricultural practices, extensive demand for processed foods along with stringent consumer and market requirements have led to an enormous wastage of food (Villarreal-García & Jacobo-Velázquez, 2016). In broccoli, close to 25% of the total produce is discarded (Domínguez-Perles, Martínez-Ballesta, Carvajal, García-Viguera, & Moreno, 2010). The abundance of glucoraphanin in the industrial discards of broccoli florets by-products; make them a suitable raw material for the fabrication of health beneficial nutraceuticals, providing an alternative use for the wasted crop (Thomas *et al*. under revision). The global nutraceutical market is a 140 billion US $ industry, with an annual growth rate of 14.7% (Villarreal-García & Jacobo-Velázquez, 2016). Valorization of vegetable discards for the fabrication of dietary supplements brings us closer to sustainability and have positive socio-economic impact (Villarreal-García & Jacobo-Velázquez, 2016). Despite the technological advancements, bio-refining and waste

recovery is subjected to stringent market and consumer concerns (Charis Michel Galanakis, 2015). Developing an industrially feasible green process for the fabrication of nutraceutical extracts from agro-food residues have varied limitations; such as cost of production, raw material availability and variability, microbial contaminations and marketing regulations (Charis M. Galanakis, 2015; Schieber, Stintzing, & Carle, 2001).

Purification of glucosinolates using ion exchange resins, predominantly anionic resins, have been widely reviewed. Most of these studies, involve the use of non-food or non-organic grade solvents, limiting its utility in the food and nutraceutical industry, despite the high purity of glucosinolates obtained (Gallaher, Gallaher, & Peterson, 2012; Toribio, Nuzillard, & Renault, 2007; Wang, Liang, & Yuan, 2012). Limited information is available on the purification of glucosinolates from industrial discards due to regulatory and health issues and developing a green process with an applicability in the nutraceutical industry is not only laborious but also expensive (Charis Michel Galanakis, 2015; Villarreal-García & Jacobo-Velázquez, 2016). Recently, a patent study described the purification of glucoraphanin from broccoli florets, seeds and sprouts by ion exchange resins using food grade (water) and non-food grade (methanol) solvents. Despite the high purity of glucosinolates, the overall process efficiency is still very low (Wehrli & Schutz, 2013).

Response surface design (RSD), as described by Box and Wilson is a statistically and mathematically powerful multivariate analysis technique (G. Box & Wilson, 1951; G. E. Box & Hunter, 1957). The most commonly used design for the RSD models are the central composite design (CCD) and Box Behnken design (BBD). These techniques optimize process parameters with minimal number of sample runs, compared to a full factorial experimentation. RSD analysis identifies the most significant process parameters and their interactions, enabling the development of an experimental model, optimizing the response (Tsiaka, Zoumpoulakis, Sinanoglou, Makris, Heropoulos, & Calokerinos, 2015; Wang, Liang, & Yuan, 2012). Hence, the objective of the present study is to optimize a process for the purification of glucoraphanin from the industrial discards of broccoli florets by-products by ion exchange resins using RSM and BBD. Moreover, to develop an economically viable, industrially feasible green process for the fabrication of nutraceutical

extracts that can subsequently be utilized as dietary supplements, providing an alternative use for the crop discards.

6.5 Materials and Methods

6.5.1 Sample Preparation

The industrial discards of broccoli florets by-products (cultivar unknown), graciously provided by Productions Maraîcheres Mailhot Inc. (Saint-Alexis, QC, Canada) were lyophilized, ground using Thermomix grinder (Canada), vacuum sealed and stored at -20°C until further use.

6.5.2 Reagents and Materials

Glucoraphanin potassium salt (>99%) was obtained from Chromadex (Irvine, CA) and ethanol (95%, 100%) from Les Alcools du Commerce (Boucherville, QC, Canada). Sodium hydroxide, potassium hydroxide, ammonium hydroxide, sulfuric acid and methanol were purchased from Fisher Scientific (Ottawa, ON, Canada). The strong cation ion exchange resin (Amberlite FPC23 H) and the weak anionic resin (Amberlite FPA 53) were obtained from Rohm and Haas, Dow waters and process solution (Philadelphia, PA, USA). Ultrapure water was obtained from Millipore Milli-Q RG system (Billerica, MA, USA). All the solvents used were on the GRAS list (generally regarded as safe), unless mentioned otherwise.

6.5.3 Extraction of Glucosinolates from Industrial Broccoli Discards

The glucoraphanin in broccoli florets by-products were extracted according to Thomas *et al*. (under revision); from a dry sample (1g) with aqueous ethanol (20mL; 80% v/v) on a magnetic stirrer at 400 rpm for 30 min at room temperature (23°C). Subsequently, the samples were vacuum filtered using Whatman No 1. The filtered solution (10mL) was concentrated in a vacuum evaporator and reconstituted with deionized water (10mL). Aliquots (300µL) of blank (milli-Q water), sample and glucoraphanin standard (0 - 100 ppm) were quantified using HPLC-UV (1100 series, Agilent, Mississauga, Canada) as described by West *et al*., (2002). The purity of glucoraphanin in the extracts were measured by drying a known volume of extract (2 mL) on a pre-weighed container in a pre-weighed

container at 105°C for at least 3 hours, or until constant weight was obtained (Miglio, Chiavaro, Visconti, Fogliano, & Pellegrini, 2008)

6.5.4 Experimental Procedure for Ion Exchange Resins

A strong cationic ion exchange resin (Amberlite FPC23 H) and a weak anionic resin (Amberlite FPA 53) were used for the purification of glucoraphanin from the broccoli florets extracts after evaporation. The cationic and anionic resins were steeped in distilled water overnight and activated with 3 volumes of 5% H_2SO_4 and 4% NaOH respectively (1 volume of the regenerate corresponds to the volume of the resin in the system). Further, the activated resins were equilibrated with milli-Q water, until the pH matched the pH of milli-Q water (pH 7). The activated cationic resin and broccoli florets extract in known quantities, were brought in contact, for a desired contact time and agitation speed in an orbital shaker as described in **Table 18**. The glucoraphanin was eluted from the resin using 3 volumes of milli-Q water and further separated from the resin by vacuum filtration using Whatman No:1 filters. The volume of the elute was recorded and stored at 4°C, until further use in anionic resin. Aliquots of 0.5, 3 and 2 mL were utilized to determine the glucoraphanin content, the pH and dry matter, respectively. Further, a fixed ratio of cationic extract and activated anionic resin were incubated for a desired contact time and agitation speed in an orbital shaker as described in **Table 19**. The glucoraphanin loaded resin was rinsed with 3 volumes of milli-Q water and further, eluted from the anionic resin using 3 volumes of ammonium hydroxide in ethanol. The volume of the anionic extract containing purified glucosinolates was recorded after filtration through a vacuum filter using Whatman no:1. The volume of the glucoraphanin enriched anionic extract was recorded after evaporation using a rotary evaporator (Buchi, rotavapor R-215, USA) at 40°C and stored at 4°C until further use. Aliquots of 0.5 and 2 mL were utilized to estimate the glucoraphanin content and dry matter of the extract respectively, to evaluation the response factors; process recovery (%) and glucoraphanin purity in the dry matter of the extract (%).

6.5.5 Experimental Design and Statistical Analysis

A four variable, three level Box-Behnken design (BBD) (Ferreira, Bruns, Ferreira, Matos, David, Brandao, et al., 2007) was applied to optimizing the purification of glucoraphanin

using ion exchange resins in order to obtain maximal glucoraphanin productivity from the industrial discards of broccoli florets. The four independent variables were feed to resin ratio (g:g, X_1), time of contact (min, X_2), agitation speed (rpm, X_3) and concentration of eluting solvent (%, X_4), and each of the variables were set at three levels (-1, 0 and +1). The variable X_4, concentration of the eluting solvent was water in ethanol for the cationic resin and ammonium hydroxide in ethanol for the anionic resin. For statistical analysis, the variables were coded according to

$$xi = \frac{(Xi - Xo)}{\Delta X}, i = 1, 2, 3 \ldots$$

Where, x_i: coded value for the independent variable, X_i: raw value for the independent variable, X_0: raw value for the independent variable at the center point, ΔX: the step change value of the independent variable. A total of 27 experimental runs were designed using BBD, with three runs at the centre point, to estimate the experimental error **(Table 18** and **Table 19)**.

Regression analysis was performed for the experimental data and was fitted into the empirical second order polynomial model, as shown in the following equation:

$$Y = \beta_0 + \sum_{i=1}^{4} A_i X_i + \sum_{i=1}^{4} A_{ii} Xi^2 + \sum_{ij=1\ (i\neq 1)}^{4} A_{ij} XiXj$$

Where β_0, A_i, A_{ii}, A_{ij} are regression coefficients in the intercept, the linear, quadratic and interaction terms respectively; X_i, X_j and X_i^2 are the independent variables and Y is the dependent variable or the process response which are glucoraphanin recovery (%) and glucoraphanin purity in the dry matter (DM) of extract (%).

Table 18: Response surface Box Behnken design and process responses for purification of glucoraphanin from broccoli florets industrial discards using cationic resin

Run	$x_1(X_1)$	$x_2(X_2)$	$x_3(X_3)$	$x_4(X_4)$	Y - Response	
	Feed : Resin	Time of contact (min)	Agitation speed (rpm)	Concentration of water (%)	Process recovery (%)	Glucoraphanin purity (%)
1	1(1:5)	1(180)	0(80)	0(50)	69.0	1.0
2	1(1:5)	-1(2)	0(80)	0(50)	68.4	0.9
3	-1(1:0.05)	1(180)	0(80)	0(50)	93.2	1.2
4	-1(1:0.05)	-1(2)	0(80)	0(50)	97.2	1.2
5	0(1:2.5)	0(91)	1(160)	1(100)	93.7	1.5
6	0(1:2.5)	0(91)	1(160)	-1(0)	79.0	1.2
7	0(1:2.5)	0(91)	-1(0)	1(100)	93.4	1.5
8	0(1:2.5)	0(91)	-1(0)	-1(0)	76.7	1.2
9	0(1:2.5)	0(91)	0(80)	0(50)	78.6	1.2
10	1(1:5)	0(91)	0(80)	1(100)	63.1	0.9
11	1(1:5)	0(91)	0(80)	-1(0)	51.3	0.7
12	-1(1:0.05)	0(91)	0(80)	1(100)	96.2	1.2
13	-1(1:0.05)	0(91)	0(80)	-1(0)	95.0	1.1
14	0(1:2.5)	1(180)	1(160)	0(50)	66.8	1.0
15	0(1:2.5)	1(180)	-1(0)	0(50)	68.5	1.1
16	0(1:2.5)	-1(2)	1(160)	0(50)	82.3	1.3
17	0(1:2.5)	-1(2)	-1(0)	0(50)	79.9	1.2
18	0(1:2.5)	0(91)	0(80)	0(50)	72.0	0.9
19	1(1:5)	0(91)	1(160)	0(50)	54.8	0.8
20	1(1:5)	0(91)	-1(0)	0(50)	50.4	0.8
21	-1(1:0.05)	0(91)	1(160)	0(50)	86.0	1.1
22	-1(1:0.05)	0(91)	-1(0)	0(50)	96.5	1.2
23	0(1:2.5)	1(180)	0(80)	1(100)	98.2	1.4
24	0(1:2.5)	1(180)	0(80)	-1(0)	78.4	1.1
25	0(1:2.5)	-1(2)	0(80)	1(100)	98.5	1.3
26	0(1:2.5)	-1(2)	0(80)	-1(0)	98.6	1.2
27	0(1:2.5)	0(91)	0(80)	0(50)	76.2	1.0

Table 19: Response surface Box Behnken design and process responses for purification of glucoraphanin from broccoli florets industrial discards using anionic resin

Run	$x_1(X_1)$	$x_2(X_2)$	$x_3(X_3)$	$x_4(X_4)$	Y - Response	
	Feed : Resin	Time of contact (min)	Agitation speed (rpm)	Concentration of NH4OH (in 70% ethanol) (%)	Process recovery (%)	Glucoraphanin purity (%)
1	1(1:6.2)	1(180)	0(80)	0(5)	84.6	3.9
2	1(1:6.2)	-1(2)	0(80)	0(5)	39.9	3.4
3	-1(1:0.62)	1(180)	0(80)	0(5)	32.6	5.2
4	-1(1:0.62)	-1(2)	0(80)	0(5)	10.2	3.6
5	0(1:3.4)	0(91)	1(160)	1(10)	84.3	5.1
6	0(1:3.4)	0(91)	1(160)	-1(0)	0.2	0.2
7	0(1:3.4)	0(91)	-1(0)	1(10)	54.7	3.9
8	0(1:3.4)	0(91)	-1(0)	-1(0)	0.2	0.2
9	0(1:3.4)	0(91)	0(80)	0(5)	71.3	3.9
10	1(1:6.2)	0(91)	0(80)	1(10)	84.1	3.8
11	1(1:6.2)	0(91)	0(80)	-1(0)	0.4	0.2
12	-1(1:0.62)	0(91)	0(80)	1(10)	35.7	4.9
13	-1(1:0.62)	0(91)	0(80)	-1(0)	0.3	0.7
14	0(1:3.4)	1(180)	1(160)	0(5)	77.4	4.9
15	0(1:3.4)	1(180)	-1(0)	0(5)	64.1	3.9
16	0(1:3.4)	-1(2)	1(160)	0(5)	26.4	4.4
17	0(1:3.4)	-1(2)	-1(0)	0(5)	10.5	2.9
18	0(1:3.4)	0(91)	0(80)	0(5)	73.7	3.9
19	1(1:6.2)	0(91)	1(160)	0(5)	79.3	4.7
20	1(1:6.2)	0(91)	-1(0)	0(5)	56.9	3.6
21	-1(1:0.62)	0(91)	1(160)	0(5)	29.6	5.8
22	-1(1:0.62)	0(91)	-1(0)	0(5)	27.9	4.8
23	0(1:3.4)	1(180)	0(80)	1(10)	86.7	4.1
24	0(1:3.4)	1(180)	0(80)	-1(0)	0.2	0.2
25	0(1:3.4)	-1(2)	0(80)	1(10)	38.7	3.7
26	0(1:3.4)	-1(2)	0(80)	-1(0)	0	0
27	0(1:3.4)	0(91)	0(80)	0(50)	74.4	3.8

6.5.6 Salt Replacement

An equimolar concentration of potassium bicarbonate ($KHCO_3$) equivalent to the total

glucosinolates present in the floret extract, with an addition of 20% of the salt was added to the glucosinolates enriched anionic elute. The resulting extract-salt mixture was evaporated using a Rotavapor at 40°C, facilitating ion replacement and removal of excessive eluting solvent ammonium hydroxide (Nowak & Skrzypek, 1989). Aliquots (500 µL and 2 mL) were sampled for glucoraphanin and dry matter content in the extract, to determine the response factors.

6.5.7 Fabrication and Characterization of extract for glucosinolates and polyphenols on a pilot scale

The glucosinolates and polyphenols content in broccoli florets industrial discards were extracted according to Thomas *et al*., (under revision). Glucosinolates and polyphenols were extracted from crushed frozen broccoli discards (15 kg) with ethanol (30 L; 70% v/v) using a stainless-steel mixer (Tetra Pak Scanima), set at room temperature. After 30 min, the sample was filtered using a vertical top discharge centrifuge CEPA TZ5 (CEPA) and further evaporated using a rotary evaporator. The evaporated broccoli floret extract was purified using strong cation ion exchange resin (Amberlite FPC23 H) and weak anionic resin (Amberlite FPA 53) in series, operated in the batch method, as optimized in the present study. Salt replacement methodology was as described in the present study. The glucoraphanin enriched extract was dried using a spray drier (Niro atomizer) set at an inlet temperature of 130°C and outlet temperature of 81°C, with a sample flow rate at 1.26 L/hr. The powdered extract was collected in an air tight container and sample (0.5g) was dissolved in milli-Q water to determine its glucosinolates and polyphenols content. Aliquots (300 µL) from the extraction, evaporation, cationic and anionic resin, salt replacement and the final powered extract were filtered (0.22 µm, nylon filter) and analyzed by UPLC MS/MS, for its glucosinolate and polyphenol content. Further, each sample (2 mL), were oven dried at 105°C for at least 3 hours, until a constant weight was obtained, to determine the dry matter content in the extracts.

6.5.8 HPLC-UV determination of glucoraphanin

HPLC-UV analysis was performed with HPLC 1100 series instrument and an Agilent ChemStation software (Agilent, Mississauga, Canada). A Phenomenex Synergi 4μm hydro-

RP 80 A (25 × 0.46 cm, 4 μm particle size) column (HPLC 1100 series, Agilent, Mississauga, Canada) with a security guard for a C-18 (4 x 3 mm) cartridge system (Phenomenex) was used for the separation. The column was maintained at ambient temperature and compounds were separated by a linear gradient elution of mobile phase, 100% A (ammonium acetate 50mM w/v) and 0% B (ammonium acetate 50mM w/v in methanol (80:20. v/v)) to 50% A and B at 20 min, to a 100% B at 40 min and finally returning to the initial condition at 43 min. The flow rate was set at 1 mL/min, with a sample injection volume of 10µL. Identification of glucoraphanin was based the UV spectrum absorbed by pure glucoraphanin at 235 nm and its retention time at 7.3 min. The glucoraphanin content in the broccoli florets extracts were quantified as milligrams glucoraphanin (GE) equivalents per kilogram dry matter (mg GE/kg DW).

6.5.9 Simultaneous Characterization and Quantification of Glucosinolates and Polyphenols Using UPLC MS/MS

The simultaneous characterization and quantification of glucosinolates and polyphenols was carried out on AQUITY UPLC HSS T3 1.8 µm (18 µm particle 2.1 id X 150mm) column (Waters, Millford, USA), equipped with a Triple Quadrupole Mass Spectrometer (TQD); operated in negative electron-spray ionization mode (ESI^-) and Multiple Reaction Monitoring (MRM). The compounds were separated by a linear gradient elution of mobile phases from 100% A (formic acid 0.1% v/v in 5% v/v acetonitrile) and 0% B (formic acid 0.1% v/v in acetonitrile 60% v/v) to 50% A and 50% B at 15 min and finally returning to the initial condition at 21 min. The flow rate was set at 0.4 mL/min, with a sample injection volume of 2.5 µL, on a column maintained at 40°C. Intact glucosinolates were expressed as sinigrin equivalent (SE), glucoraphanin as both sinigrin (SE) and glucoraphanin equivalent (GE), flavonoids as rutin equivalent (RE), sinapic acid as sinapic acid equivalent and chlorogenic acid as caffeic acid equivalent. Intact glucosinolates and polyphenols were quantified as milligrams per kilogram dry matter (mg/kg DW) Thomas *et al*., (under revision).

6.6 Data analysis

The statistical analysis of the experimental results generated by BBD was carried out using SAS (version 9.4, SAS institute Inc, Cary, NC, USA). Calculations were done at 95% confidence level and p-values less than 0.05 was declared statistically significant. The three-dimensional response surface plots were generated using SAS and R (Version 3.4.1).

6.7 Results and Discussion

6.7.1 Extraction process

The optimized procedure for the extraction of glucosinolates from the industrial discards of broccoli florets provided a glucoraphanin content of 3.9 g glucoraphanin/kg DW florets. The samples after extraction and evaporation measured a pH of 5.8. Drying the broccoli florets extract under reduced pressure, provided 91.8 g of residue per litre sample with a glucoraphanin purity of 1%. Studies by Thomas *et al*., (2017, under revision) reported higher glucoraphanin content in the industrial florets discards than that reported in the present study. Variability in the content of bioactive ingredients inside industrial discards may be due to biotic and abiotic factors such as variability in the soil, climatic changes, pest attack and postharvest losses associated with crop handling, storage, transportation, microbial contaminations and food processing parameters (Domínguez-Perles, Martínez-Ballesta, Carvajal, García-Viguera, & Moreno, 2010; Charis M. Galanakis, 2015).

6.7.2 Preliminary experiments

6.7.2.1 Selection of process parameters

Preliminary experimental studies were preformed to identify the ideal process parameters for Box-Behnken Design (BBD) aimed at the purifying glucoraphanin using the ion-exchange resins. Purification of glucoraphanin by anionic resin or cationic and anionic resins in series were tested in a batch operation mode. The operational conditions for the preliminary experiments were: florets extract to resin ratio of 1:5, contact time of 30 min and elution of glucoraphanin from anionic resin with 5 % ammonium hydroxide in methanol. The contact time and concentration of the anionic eluting agent was adapted from the studies of Wehril & Schutz (2013). Purification of glucoraphanin using anionic

resin provided a process efficiency and glucoraphanin purity of 33 % and 3.5 % respectively. Whereas, for cationic and anionic resin in series offered process efficiency and glucoraphanin purity of 63 % and 4.5 % respectively. The results obtained in this present study confirmed that cationic and anionic resins in series for batch mode of operation are the most appropriate method for the purification of glucoraphanin. The effectiveness of batch technique for the purification of bioactives from crude extracts were also reported Thomas *et al*., (2017, under revision) and Biosciences (2002). The green nature of the study replaced the solvent for the anionic eluting salt (NH_4OH (5%, v/v) in methanol) to a food grade solvent (aqueous ethanol (70%; (v/v)) as adapted from Thomas *et al*., (2017, under revision), who reported the effectiveness of aqueous ethanol for the elution of glucoraphanin form weak anionic resin Amberlite FPA 53. The applicability of aqueous ethanol for the purification of glucosinolates may be due to its polarity and hydrodynamics as reported by Toribio *et al*., (2007). Hence, the experimental process parameters for the investigational study were a feed (dried florets extract) to resin ratio of 1:5, contact time of 30 min and elution of glucoraphanin from anionic resin with 5% ammonium hydroxide in 70% ethanol. The response factors associated with these process variables were a process recovery of 67 % and glucoraphanin purity in the dry matter of the extract at 4 %. Therefore, feed to resin ratio (x_1), contact time (x_2; min), agitation speed (x_3; rpm) and concentration of the eluting solvents (x_4; %) were chosen as the process variables used for the purification of glucoraphanin by ion exchange resins in series operated in the batch mode using BBD.

6.7.3 Ion Exchange chromatography

6.7.3.1 Cationic Ion Exchange Resin

6.7.3.1.1 Response surface analysis and the model fitting for purification of glucoraphanin by cationic ion exchange resin

The 27 runs Box-Behnken Design matrix and the corresponding responses of the RSD analysis were shown in **Table 18**. Multiple regression analysis on the experimental data indicated that feed to resin ratio (x_1) and concentration of the eluting solvent (x_4; %) were the statistically significant independent variables than contact time (x_2; min) and agitation

speed (x_3; rpm) for maximizing the response factors (recovery (%) and glucoraphanin purity (%)). Canonical and ridge analysis on the experimental variables failed to maximize the process responses simultaneously, as the stationary point was a saddle point. Hence, a new experimental design was generated using PCA (Principle Component Analysis), connecting the process variables feed to resin ratio (x_1) and solvent concentration (x_4), to maximize the process responses simultaneously. The new process response (Factor1) for the experimental design was generated by developing a correlation matrix between the responses i.e. recovery (%) and glucoraphanin purity (%). The composite scores were calculated from the weighted average of the process responses, on the first PCA axis, that were previously standardized. Multivariate analysis on the experimental data using PCA **(Table 20)**, indicated that the model was statistically significant ($p<0.05$). The mathematical model describing the process response as a function of process variables was given by the following equation:

$$Y = 8.8 + 0.1x_1 - 2.2x_4 - 0.2x_1^2 + 0.3\,x_4^2 + 3.02\,x_1\,x_4 \qquad \textbf{(1)}$$

The response surface regression procedure on the experimental data provided a determination co-efficient ($R^2 = 0.765$), that explains 77 % of the total variations of the model. The value of the adjusted determination coefficient (Adj. $R^2 = 0.709$) also confirmed that the model was significant. A very low value 9.1 % of coefficient of the variation (C.V.) clearly indicated a very high degree of precision and reliability of the experimental value.

The residual mean square (MS_{res}) provided another index for the goodness of fit of the polynomial regression model. Values between 0.5 and 1.5 are considered statistically acceptable, providing evidences for the goodness of fit for the experimental model. In this study MS_{res} was 0.52 indicating that the model had an excellent fit. The regression coefficient values of Equation (**1)** are listed in **Table 20** . The significance of each coefficient was determined using the p-value, indicating that the linear (x_1, x_4) and quadratic (x_1^2, x_4^2) interactions between the process variables feed to resin load (x_1) and solvent concentration (x_4) were significant ($p<0.05$).

Table 20: Estimated regression coefficient for the quadratic polynomial model and the analysis of variance (ANOVA) for the cationic experimental model

Parameter	Estimated coefficient	Standard error	DF	Sum of squares	F value	Prob > F
Intercept	8.8	0.6				
X_1	0.1	0.3	1	19.6	37.4	<0.0001**
X_4	-2.2	1.4	1	5.8	11.2	0.003**
$X_1 * X_1$	-0.2	0.1	1	6.4	12.3	0.002**
$X_4 * X_4$	3.02	1.1	1	3.6	6.9	0.02*
$X_1 * X_4$	0.3	0.4	1	0.3	0.6	0.4
Residual						
Lack of fit			3	4.7	4.5	0.02*
Pure error			18	6.3		

Significant at 5% level

**Significant at 1% level

DF, degree of freedom; SS, sum of squares

The graphical representation of the regression equation **(1)**, obtained using SAS are presented in **Figure 14**. In the 3-D response surface plot, two significant variables, feed to resin ratio and solvent concentration were plotted against the new response factor (Factor1), while the other variables were kept constant at zero level i.e. fixing the agitation speed and contact time at 80 rpm and 91 min respectively. As shown in **Figure 14**, the process response (Factor 1) increased with augmentation in feed to resin ratio (x_1) reaching a maximum value at 1:1.87 and beyond 1:1.87 the process response slightly dropped. When feed to resin load (x_1) was set at 1:1.87, process response increased with solvent concentration (x_4) from 0 to 98% water. The maximum process response of 94.75 % process recovery and 1.38 % glucoraphanin purity were achieved for a feed to resin ratio, contact time, agitation speed and solvent concentration of 1:1.87, 91 min, 80 rpm and 98 % water in ethanol respectively.

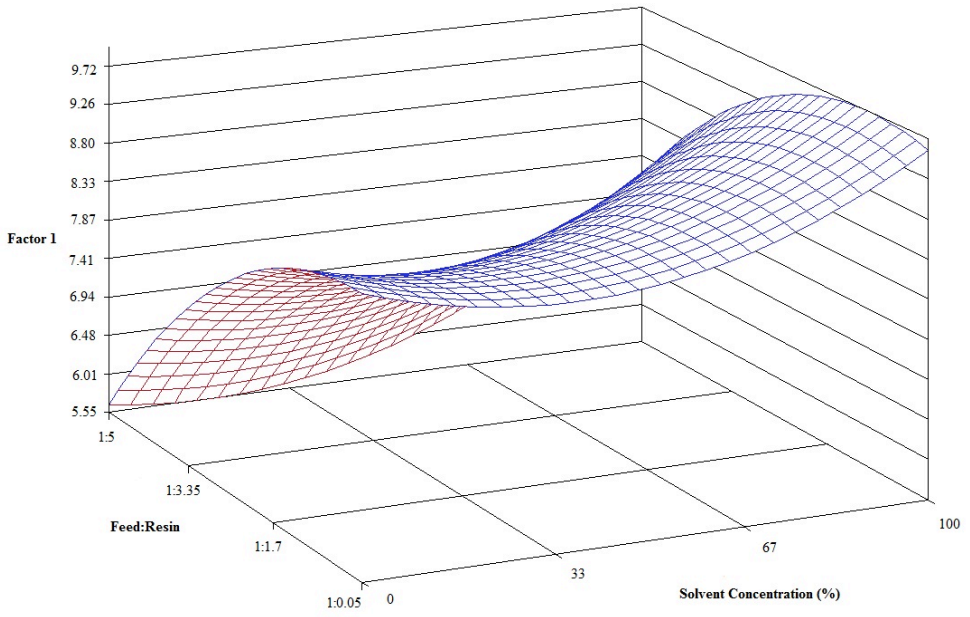

Figure 14: Response surface 3-D plot showing the influence of feed to resin ratio and solvent concentration on glucoraphanin recovery (Factor 1) using cationic resin

6.7.3.1.2 Verification and optimization of operational conditions for purification of glucoraphanin from broccoli florets by-products using cationic resin

The optimized parameters for the purification of glucoraphanin using cationic resin by RSM analysis were a feed to resin ratio of 1:1.87, contact time of 91 min, agitation speed of 80 rpm and concentration of eluting solvent of 98 % (water in ethanol; v/v). The optimized model predicted a maximal process recovery and glucoraphanin purity of 94.8 % and 1.4 % respectively. An experimental process recovery of 97.9 % and glucoraphanin purity of 1.3 %, was obtained using the optimized process parameters, indicating the validity of the predicted model. The influence of eluting solvent concentration on process responses indicated that 98% water and 100% water, offered no significantly variation in the process recovery. Hence, 100% water was optimized as eluting solvent concentration, providing a process recovery of 96.6% and glucoraphanin purity of 1.27 %. Similarly, the impact of contact time (t = 15 min and 30 min) on the optimized experimental model, provided a recovery of 94.7 % and glucoraphanin purity of 1.26 % at t = 15 min and a recovery of

91.5 % with a glucoraphanin purity of 1.15 % in the residue at t = 30 min. Multiple regression analysis on the experimental data indicated that time of contact was not a statistically significant process variable. Therefore, a contact time of 30 min was identified as the optimized process variable (x_3) for maximizing the process responses, instead of a contact time of 91 min, thereby, accelerating the industrial process.

The influence of feed to resin ratio on process responses (pH of the eluted extract, process recovery and glucoraphanin purity in the residue) were analyzed for the experimental model were contact time, agitation speed and concentration of the eluting solvent were 30 min, 80 rpm and 100% water respectively. Statistical analysis on the experimental model indicated a significant influence of feed to resin ratio on the process responses ($p<0.05$) **(Table 21)**. The pH of the broccoli florets extract decreased with increase in feed to resin ratio. The pH of the florets extract was 5.75 prior to contact with cationic experiment, and gradually decreased to 2.00 ± 0.01 for a feed to resin ratio of 1:1.87. The decrease in the pH of the broccoli florets extract might be due to the elimination of the floret extracts from plant solids and extracted molecules, that interferes with the alteration of the pH of the extract or due to the displacement of the K^+ ions in the glucosinolates with the H^+ ions from the cationic resin (Powell, Hill, Juurlink, & Carrier, 2005). Hence, the optimization studies identified that feed to resin ratio of 1:1.87, contact time of 30 min, agitation speed of 80 rpm and concentration of the eluting solvent at 100 % water provided an economical process that reduced the pH of the cationic extract to 2.0, with a process recovery of 94.7 % and glucoraphanin purity of 1.258 %. Similar, operational parameters were reported by Wehril & Schutz (2013) and Thomas *et al*., (2017, under revision); with a slight modification in the feed to resin. Studies by Wehril & Schutz (2013) utilized a feed to resin ratio of 1:2 and Thomas *et al*. (under revision) utilized a ratio of 1:5; for the purification of glucoraphanin form broccoli seed extract. The present study, utilized lesser quantity of the resin for the purification of glucoraphanin, than the patented protocol of Wehril & Schutz (2013), thereby, economizing the process without compromising on glucoraphanin extractability.

Table 21: Optimization of the operation parameters for purification of glucoraphanin from broccoli florets by-products by cationic resin

		Productivity of glucoraphanin	
Feed : Resin	**pH**	**Process recovery (%)**	**Glucoraphanin purity (%)**
1:0.05	5.3±0.1	91.5	1.1±0.0
1:0.1	5.0±0.0	94.1	1.1±0.0
1:0.2	4.8±0.0	99.2	1.1±0.1
1:0.45	3.7±0.0	97.3	1.2±0.1
1:0.9	2.7±0.0	93.9	1.2±0.0
1:1.4	2.2±0.0	97.0	1.3±0.1
1:1.87	2.0±0.0	94.7	1.3±0.0

6.7.3.2 Anion Ion Exchange Resin

6.7.3.2.1 Response surface analysis and the model fitting of Box-Behnken design (BBD) for anionic resin

The operational conditions for the purification of glucoraphanin using anionic resin from industrial broccoli florets by-products was optimized by a 27 run Box-Behnken Design (BBD). **Table 19**, represents the experimental design matrix and the corresponding process responses generated by the Response surface method analysis (RSM). The regression analysis on the experimental data indicated that the linear and quadratic interactions of the process variables were significant ($p<0.05$); whereas, the cross-product parameters were insignificant ($p>0.05$). The regression co-efficient for the intercept, linear, quadratic and cross-product terms of the variables for the experimental model are represented in **Table 22**. Multiple regression analysis indicated that the independent variables resin load (x_1), contact time (x_2), agitation speed (x_3) and concentration of the eluting solvent (x_4) have a significant influence ($p<0.05$) on the process responses i.e. process recovery (Y_1) and glucoraphanin purity in the residue (Y_2). The second order polynomial model describing the process responses as a function of process variables are given by the following equations and the regression coefficient values for equation **(2)** and **(3)** were listed in **Table 22**:

$$Y_1 = -0.38 + 0.16x_1 + 0.01x_2 + 0.0002x_3 + 0.17x_4 - 0.04x_1^2 - 2.2E\text{-}05x_2^2 - 1.9E\text{-}07x_3^2 - 0.02x_4^2 + 0.0002x_1x_2 + 3.4E\text{-}05x_1x_3 + 0.01x_1x_4 - 2.2E\text{-}07x_2x_3 + 0.0003x_2x_4 + 2.04E\text{-}05x_3x_4 \quad \textbf{(2)}$$

$$Y_2 = 0.04 - 0.02x_1 + 0.001x_2 - 8.6E\text{-}06x_3 + 0.05x_4 - 0.003x_1^2 - 1.2E\text{-}06x_2^2 + 1.4E\text{-}08x_3^2 - 3.1E\text{-}03x_4^2 - 3.2E\text{-}02x_1x_2 + 6.1E\text{-}07x_1x_3 - 1.6E\text{-}04x_1x_4 - 7.04E\text{-}08x_2x_3 - 1.8E\text{-}05x_2x_4 + 1.3E\text{-}06x_3x_4 \quad \textbf{(3)}$$

The response surface regression procedure (RSREG) on the experimental data for process response (Y_1) had a determination co-efficient (R^2) of 0.983, indicating the adequacy of the model. The adjusted determination co-efficient (Adj. R^2) value of 0.965, explains 97 % of the total variation of the model. The co-efficient of variation (C.V.) value of 11.8 %, indicates that the model is reproducible. Similarly, the multiple regression analysis on the experimental data for the process response glucoraphanin purity (Y_2), provided a co-efficient of regression (R^2) of 0.993, indicating that 99 % of the variability can be explained by the model. The adjusted determination co-efficient (Adj. R^2) value of 0.985, confirmed the significance of the model. The low value of co-efficient of variation (C.V.) of 5 %, indicated the reliability of the model.

Response surface regression analysis on the experimental data along with canonical and ridge analysis provided two different sets of point maximum for the process responses (Y_1 and Y_2). Also, Principle component analysis (PCA), on the experimental data, failed to maximize the process responses simultaneously, due to the lack of co-relation between the process responses at the extreme points. According to Ridge analysis a maximized process recovery (Y_1) of 99 % was obtained for a feed to resin ratio of 1:5.2, contact time of 169 min, agitation speed of 135 rpm and concentration of the eluting solvent at 9.5 % ammonium hydroxide (NH_4OH) in 70 % ethanol. Whereas, a feed to resin ratio of 1:0.6, contact time of 109 min, agitation speed of 127 rpm and concentration of the eluting solvent of 7.1 % NH_4OH in 70 % ethanol, maximized the glucoraphanin purity (Y_2) to 6.3 %. The suitability of the experimental model was verified by confirming experiments providing a process recovery of 88 % and glucoraphanin purity of 5 % using the predicted process parameters for Y_1 and Y_2. These data proved that the model designed in this study was valid.

Table 22: Estimated regression coefficient for the quadratic polynomial model and the analysis of variance (ANOVA) for the anionic experimental model

Parameter	Estimated coefficient		Standard error		DF		SS		F value		P-value	
	Y_1 (%)	Y_2 (%)	Y_1 (%)	Y_2 (%)	Y_1 (%)	Y_2 (%)	Y_1 (%)	Y_2 (%)	Y_1 (%)	Y_2 (%)	Y_1 (%)	Y_2 (%)
Intercept	-0.38	0.04	0.14	0.02	1	1						
X_1	0.16	-0.02	0.05	0.01	1	1	0.43	0.002	70.1	23.3	<0.0001**	0.0004**
X_2	0.01	0.001	0.001	0.0001	1	1	0.51	0.002	83.7	25.1	<0.0001**	0.0003**
X_3	0.0002	-8.6E-06	0.0001	1.4E-05	1	1	0.08	0.002	12.4	22.5	0.004**	0.0005**
X_4	0.17	0.05	0.02	0.002	1	1	2.45	0.08	403.1	1070.1	<0.0001**	<0.0001**
X_1* X_1	-0.04	0.003	0.01	8.8E-04	1	1	0.001	0.005	0.2	70.7	0.7	<0.0001**
X_2* X_1	0.0002	-3.2E-05	0.0002	2.4E-05	1	1	0.01	0.0001	1.7	1.8	0.2	0.2
X_2* X_2	-2.2E-05	-1.2E-06	4.1E-06	4.7E-07	1	1	0.02	0.0004	2.7	5.1	0.1	0.04*
X_3* X_1	3.4E-05	6.1E-07	2.47E-05	2.6E-06	1	1	0.01	3.9E-06	2.1	0.1	0.2	0.8
X_3* X_2	-2.2E-07	-7.04E-08	5.5E-07	6.1E-08	1	1	0.001	0.0001	0.2	1.3	0.7	0.3
X_3* X_3	-1.9E-07	1.4E-08	5.3E-08	5.9E-09	1	1	0.001	0.01	0.1	94.7	0.8	<0.0001**
X_4* X_1	0.01	1.6E-04	0.004	4.2E-04	1	1	0.07	1.06E-05	10.8	0.1	0.007*	0.7
X_4* X_2	0.0003	-1.8E-05	8.8E-05	9.7E-06	1	1	0.06	0.0002	9.4	3.4	0.01	0.09
X_4* X_3	2.04E-05	1.3E-06	9.8E-06	1.1E-06	1	1	0.03	0.0001	4.4	1.4	0.06	0.3
X_4* X_4	-0.02	-3.1E-03	0.001	1.5E-04	1	1	0.79	0.03	130.1	420.8	<0.0001**	<0.0001**
Residual												
Lack of fit					10	10	0.07	0.001	21.7	39.2	0.04*	0.03*
Pure error					2	2	0.0006	4.5E-06				

*Significant at 5% level

**Significant at 1% level

DF, degree of freedom; SS, sum of squares; R^2, coefficient of determination; Y_1, process recovery; Y_2, glucoraphanin purity

The feasibility and industrial applicability of the experimental model were validated by analyzing the influence of feed to resin ratio (x_1) on the process responses. The process variables contact time, agitation speed and solvent concentration were set at 170 min, 140 rpm and 7 % NH_4OH in 70 % ethanol respectively **(Table 23)**. An inverse co-relation between the process responses was obtained with reference to feed to resin ratio (x_1). As the feed to resin ratio increased, the glucoraphanin purity decreased with an increase in process recovery**.** Therefore, the optimized parameters for the purification of glucoraphanin using anionic resin were a feed to resin ratio of 1:1.3, contact time of 170 min, agitation speed of 140 rpm and concentration of the eluting solvent of 7 % NH_4OH in 70 % ethanol. The predicted process recovery and glucoraphanin purity for the process parameters were 68 % and 6 %, respectively. Validation experiments on the experimental parameters provided a process recovery of 78 % and glucoraphanin purity of 5 % respectively. The minimal variations in the observed and predicted responses specified the validity of the model. The process recovery observed in the present study was significantly higher than reported in a patented protocol (Wehrli & Schutz, 2013), where the glucoraphanin recovery from broccoli (florets, seeds or sprouts) was 41 % using 5 % ammonium hydroxide in methanol and 60 % using 5 % ammonium hydroxide in water. The results observed in this study also provided a better glucoraphanin recovery than observed by Thomas *et al*., (2017, under revision), reporting a 70% glucoraphanin recovery using a feed to resin ratio of 1:5, contact time of 140 min, agitation speed of 160 rpm and concentration of the eluting solvent of 7 % NH_4OH in 70 % ethanol, from broccoli seeds discards.

Table 23: Optimization of the operation parameters for purification of glucoraphanin from broccoli florets by-products by anionic resin

	Glucoraphanin productivity			
	Observed		Predicted	
Feed : Resin	Process recovery (%)	Glucoraphanin purity (%)	Process recovery (%)	Glucoraphanin purity (%)
1:0.6	50	4.9	52	6.5
1:1.3	78	4.6	68	6
1:2.6	86	4.1	88	5.3
1:5.2	97	4.2	48	5.2
1:1.3*	6.3	1.3	NA	NA

*purification of glucosinolates using anionic resin, without the cationic resin

The optimized process parameters for the purification of glucoraphanin without prior treatment with the cationic resin gave a low process response of 6.3 % and glucoraphanin purity of 1.25 %, confirming that cationic resin purification step prior to the anion process is mandatory to enhance the process responses. The glucoraphanin recovery observed in this study was comparable to that reported by Thomas *et al*., (2017, under revision). Studies by Wehril & Schutz (2013), reported higher process recoveries than the present study, where elution of glucoraphanin from anionic resin using 5 % NH_4OH in methanol provided a process recovery of 21.8 % and 17 % recovery using 5 % NH_4OH in water. Many studies have reported using anionic resin for the purification of glucosinolates (Gallaher, Gallaher, & Peterson, 2012; Guo, Yuan, Chen, & Guo, 2005; Mohn, Cutting, Ernst, & Hamburger, 2007), but, these studies involve the use of non-food grade solvents limiting its applicability and use in the manufacture of fortified food supplements, despite its high process efficiency. Studies by Fahey *et al*., (2003) and Toribio *et al*., (2007) provided highly efficient glucoraphanin purification techniques such as strong ion-exchange displacement centrifugal partition chromatography (SIX-CPC) and high speed counter current chromatography (HSCCC). These techniques offered glucoraphanin with high purity, but the use of highly salted, highly polar biphasic solvent system limited its applicability in the food and pharmaceutical industry.

6.7.4 Salt replacement

The ammonia salt of glucoraphanin was substituted to a more stable potassium salt with the addition of potassium bicarbonate ($KHCO_3$). In this study, a process recovery of 96 % and glucoraphanin purity of 4.4 % was obtained. Thermal decomposition of ammonium bicarbonate under reduced pressure to ammonia, carbon dioxide and water, facilitated the forward reaction of salt replacement, thereby, minimizing the residue formation from the salts without compromising the glucoraphanin purity (Nowak & Skrzypek, 1989). The process efficiency observed in this study was comparable to that reported by Thomas *et al*., (2017, under revision), who indicated an efficiency of 95 % and higher than reported by Wehril & Schutz (2013), who obtained a process efficiency of 91 % using sodium hydroxide (1N). Hence, the optimized extraction and purification of glucoraphanin from industrial discards of broccoli using resins in series gave an overall process efficiency of 81

% increasing the glucoraphanin concentration 12 times. Therefore, a green process for the purification and formulation of food grade broccoli extracts was developed for the industrial discards of broccoli florets.

6.7.5 Fabrication and characterization of extract for glucosinolates and polyphenols on a pilot scale

The extraction and purification parameters optimized in the present study for laboratory scale was extrapolated on a pilot scale for crushed frozen broccoli discards. Extraction of glucoraphanin on a pilot scale from crushed frozen broccoli discards provided a glucoraphanin yield of 0.7 g/Kg. The preliminary extraction and evaporation process provided 687 g residue with a glucoraphanin purity of 1.5%. Purification of glucoraphanin using cationic and anionic resin in series, was highly efficient, with a process efficiency of 99%, concentrated glucoraphanin 7 times, extracting 55.7 g residue with a glucoraphanin purity of 8%. Salt replacement methodology using potassium bicarbonate ($KHCO_3$), recovered 56 g residue with a glucoraphanin purity of 6.8%, offering a process efficiency of 88%. The fabricated powdered extract had a glucoraphanin purity of 3.2%, with the addition of 55% maltodextrin into the glucoraphanin enriched extract. Hence a highly efficient process for the fabrication of powdered extract rich in glucoraphanin was developed form industrial broccoli discard was developed with an overall process efficiency of 87%. The process efficiency observed on the pilot scale was higher than that observed for the laboratory scale process and that reported by Wehrli & Schutz (2013).

Analysis the extracts for its glucosinolates and polyphenols content identified and quantified 12 glucosinolates and 5 polyphenols, of which glucoraphanin was the most predominant **(Table 24)**. The glucosinolates content in broccoli florets industrial discards was concentrated 0.2-4 times across the powdered extract fabrication process and most distinctly glucoraphanin by 4.3 times. Of the 12 glucosinolates analyzed, aliphatic and indolic glucosinolates were concentrated uniformly between 2 - 4 times its initial concentration, except, 4-hydroxyglucobrassicin that was only concentrated 0.2 times its initial concentration. The reduced concentration across the process, might be due to its increased affinity towards the anionic resin and its low desorption rate from the anionic resin using ammonium hydroxide in ethanol. The polyphenolic content in the sample

increased 0.2 - 6 folds across the powdered extract fabrication process, of which 5-caffeoylquinnic acid was concentrated the highest. Hence, the present study provides a through insight on the variation in the glucosinolates and polyphenols content, across the various process involved in the fabrication of an extract from broccoli florets industrial discards.

Table 24: Total and individual glucosinolates (GLS) and polyphenols content in broccoli florets industrial discards (Brassica oleracea var. italica), across the extract fabrication process on pilot scale

Molecular Compounds (% in DM of extract)		Florets extract fabrication process					
		Extraction	Evaporation	Cationic resin	Anionic resin	Salt replacement	Powered extract
Aliphatic Glucosinolates							
C-3	Glucoiberverin[1]	N.D	N.D	N.D	N.D	N.D	N.D
	Glucoiberin[1]	0.04	0.06	0.07	0.23	0.21	0.17
	Sinigrin[1]	N.D	N.D	N.D	N.D	N.D	N.D
C-4	Glucoerucin[1]	0.0004	0.003	N.D	N.D	N.D	N.D
	Glucoraphanin[1]	0.32	0.56	0.83	3.65	2.96	1.35
	Glucoraphanin[2]	0.66	1.54	1.93	7.92	6.89	3.19
	Gluconapin[1]	0.002	0.004	0.006	0.024	0.016	0.007
	Progoitrin[1]	N.D	N.D	N.D	N.D	N.D	N.D
C-5	Glucoalyssin[1]	0.003	0.005	0.007	0.026	0.027	0.01
Indolic Glucosinolates							
	Glucobrassicin[1]	0.11	0.25	0.31	1.18	0.88	0.44
	Neoglucobrassin[1]	0.54	1.21	1.49	6.27	4.84	2.26
	4-Hydroxyglucobrassicin[1]	0.001	0.003	0.004	0.003	0.001	0.0002
	4-Methoxyglucobrassicin[1]	0.02	0.03	0.01	0.07	0.07	0.04
Polyphenols							
Flavonoids[3]							
	Kaempherol-3-o-sophoroside[4]	0.002	0.004	0.003	0.0004	0.0002	0.0003
	Quercetin-3-diglucoside-7-glucoside[4]	0.004	0.005	0.007	0.003	0.003	0.001
Hydroxycinnamic acids							
	3-Caffeoyl quinic acid[5]	0.004	0.009	0.01	0.01	0.01	0.003
	5-Caffeoyl quinic acid[5]	0.001	0.002	0.003	0.02	0.02	0.006

Sinapic acid[6]	N.D	N.D	N.D	0.02	0.02	0.01

[1] mg sinigrin equivalent/kg; [2] mg glucoraphanin/kg; [3] mg rutin equivalent/kg; [4] tentative identification; [5] mg caffeic acid equivalent/kg; [6] mg sinapic acid equivalent/kg.

6.8 Conclusion

The present work described an optimized, efficient and green glucoraphanin purification process using ion-exchange resins in series from industrial discards of broccoli florets. RSM and BBD were used to maximize the recovery and purity of glucoraphanin in an industrially feasible and sustainable process. The optimized parameters for the purification of glucoraphanin using cationic resin were a resin to feed ratio of 1:1.87, contact time of 30 min, shaker speed of 80 rpm and pure water as eluting solvent. RSM analysis indicated that resin to feed ratio and solvent concentration are the most significant factors that influenced process responses. The optimized process parameters reduced the pH of the extract from 5.75 to 2 after cationic purification process with a process recovery of 95 % and glucoraphanin purity of 1.25 %. Subsequently, purification of glucoraphanin by anionic resin using the optimized process parameters i.e. 1:1.3 cationic extract to resin ratio, 170 min contact time, shaker speed of 140 rpm and an eluting solvent of 7 % NH_4OH in 70 % ethanol provided a process recovery of 78 % and glucoraphanin purity of 5 % respectively. Replacement of the ammonia salt of glucoraphanin with potassium salt using $KHCO_3$ gave 96 % efficiency, providing a final glucoraphanin purity of 4.4 %, thereby concentrating glucoraphanin 12 times. The pilot scale fabrication of powdered extract using the lab optimized extraction and purification process concentrated glucoraphanin by 4.3 times and polyphenols by 0.2 to 6 folds. Hence a highly efficient technique for the fabrication of an extract rich in glucoraphanin from broccoli florets industrial discards was developed, with an overall process efficiency of 86%, providing a final powered extract with a glucoraphanin purity of 3.2%. Overall, an industrially applicable green process for the purification of glucoraphanin from industrial discards was developed that brings us closer to sustainability, with the fabrication of bioactive supplements rich in glucoraphanin that can be used in the food and pharmaceutical industry.

6.9 Acknowledgement

The authors acknowledge the financial support of the Natural Science and Engineering Research Council of Canada (NSERC), Organic Science Cluster II and Diana Food Canada Inc. We would also like to express our gratitude to Diana Food Canada Inc., Productions Maraîchères Mailhot Inc., and Norseco Inc., Québec, Canada.

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Chapter 7

Discussion, Conclusion and Future Perspective

7.1 Discussion

Increase in population and improper food handling have led to rampant food wastage. The residues generated by the agro-food industry are discarded at great expense that are otherwise used as animal feeds and green manures. The rise in environmental concerns and limitations in natural resources, have led to the evolution of a revolutionary concept of food waste recovery. The waste generated by the agro-food industry are a potent source of bioactive molecules that can find its application in food, pharmaceutical, cosmetic and nutraceutical industry. It is widely known that plants belonging to the brassica family are distinctively rich in glucosinolates and most predominantly glucoraphanin in broccoli. Transforming broccoli industrial discards into valuable extracts rich in bioactive molecules can be beneficial from both the economic and environmental point of view.

This project was carried out in collaboration with our industrial partner Diana foods (previously Nutra Canada), who are specialized in the fabrication of extracts rich in bioactive molecules, that have wide applicability in the nutraceutical market and as functional food ingredients. The present study, was designed to find solutions, to the persisting research problems related to biorefining, whilst meeting the requirements of the company. The nutraceutical applicability of the extracts implied the use of solvents and chemicals belonging to the GRAS list, and at the same time, providing high yield of the compound of interest i.e. glucoraphanin from broccoli industrial discards. Hence, this project was designed to link science with the industry, providing a real-life applicability.

In the first part of this study, the industrial discards of broccoli (florets, stalk, mix and 10 discarded lots of broccoli seeds) were simultaneously characterized and quantified for its glucosinolates and polyphenol content using UPLC MS/MS. A standardized process for glucosinolates and polyphenol extraction using methanol was used in this study. In this present work, 12 glucosinolates and 5 polyphenols were simultaneously identified and quantified. The results obtained from this study add to the existing literature, since scarce information is available with respect to industrial discards. In this study, the operation

parameters for the UPLC MS/MS, was optimized, for the simultaneous identification of the glucosinolates and polyphenols. Characterization and quantification results indicate the relative abundance of glucosinolates most predominantly glucoraphanin in broccoli industrial discards. Relatively low levels of polyphenols were present in the industrial discards. The glucosinolate content in broccoli discards varied from 0.2-2% dry weight of sample, whereas, the polyphenol content was less than 0.02% dry weight of the sample. Glucoraphanin, corresponds to 45-65% of the total glucosinolates and hydroxycinnamic acids corresponds to 40-70% of the total polyphenols. The glucosinolates and polyphenols content in broccoli industrial discards were at par with the fresh material, previously used for industrial purpose. There is also a huge variability in the content of the bioactive ingredients within and between the cultivars of broccoli that may be due to the influence of genetic and eco-physiological factors. From the analysis, we were able to identify that broccoli florets and the seed cultivar MF1 hybrid had the highest glucoraphanin content, and can inevitably be used as a promising starting material for the fabrication of extracts rich in glucosinolates. This study, also stress the importance on the alternative use of broccoli industrial discards, creating a positive socio-economic impact, and providing more fresh materials for consumption.

The second objective was to optimize a glucoraphanin extraction process from broccoli seeds and florets obtained from industrial discards, using green solvents. In this study, the process parameters such as time-temperature, feed to solvent ratio, ethanol concentration and pH of the extracting media, were optimized to maximize the yield of glucoraphanin. The optimized process parameters, for maximizing the yield of glucoraphanin from broccoli seeds and florets by-products were found to be 50% and 70% aqueous ethanol, extracted for 60 and 30 minutes, at 60°C and 23°C, for a feed to solvent ratio of 1:20 respectively. Extraction using the optimized green process parameters provided a high glucoraphanin yield of 55.5 g/Kg seeds and 4.3 g/kg DW florets by-products. Analysis of the process parameters indicated that solvent concentration is the most important parameter for glucoraphanin stability and extractability. This may be due to the inactivation of the glucosinolate degrading enzyme myrosinase, enhancing its stability. The hydrophilic nature of glucosinolates facilitated its extraction using protic solvents such as ethanol and water, providing greater than 88% glucoraphanin extractability. This was obtained by means of a

single batch extraction process using the optimized extraction parameters. Comparing the optimized glucoraphanin extraction procedure, with a standardized extraction process, with methanol as its solvent, indicated greater efficiency for the optimized process. This extraction process is interesting in the industrial point of view, as it is highly simple, involving less expensive equipment for operation, simultaneously enhancing the concentration of glucoraphanin 6 and 3 times in seeds and florets respectively. This study, signifies the applicability of industrial discards as raw materials for bioactive extract fabrication, which is at par with industrialized crops, that is currently used as the raw material for extract formulation.

Despite the high efficiency and recovery of glucoraphanin, obtained during the extraction process, the concentration of glucoraphanin obtained is not significantly high enough to obtain profitability in the nutraceutical market. The most commonly used glucosinolate purification technique, involves the use of an anionic ion exchange resin. These purification processes, mainly encompasses the use of non-food grade solvents like methanol, acetonitrile, hexane and high salt concentrations. Hence, the third and fourth objective of our study was to isolate and purify glucoraphanin from the crude broccoli seeds and florets extracts, using an eco-innovative technique. The industrial requirement of this study was in fact the major technical constrain encountered in this research, i.e. development of an industrially feasible, green process, that can simultaneously maximize the process responses such as glucoraphanin recovery and purity.

The glucoraphanin isolation and purification process was optimized by the response surface approach (RSM), using the Box Behnken design (BBD) and principle component analysis (PCA). These experimental models are widely used statistical technique to study the interactions between the process parameters, and its influence on the process responses. RSM analysis is simple and highly efficient technique, with minimal sample runs, providing a set of operational parameters to obtain the desired process response. Further, these operational parameters were utilized to design an experimental procedure, that are economical and feasible at the industrial scale.

Studies on glucoraphanin purification indicated that a strong cationic (Amberlite FPC23 H) and a weak anionic (Amberlite FPA 53) resins in series, operated under the batch mode,

enhanced the process responses. The optimized operational parameters for the purification of glucoraphanin from broccoli seeds using cationic resin were a feed to resin ratio of 1:5, contact time of 91 minutes, shaker speed of 80 rpm and an eluting solvent concentration of 100% water, providing a process recovery of 94% and glucoraphanin purity of 14%. Further purification using the anionic resin, involved the use of a resin to feed ratio, contact time, shaker speed and concentration of the eluting solvent of 1:5, 140 min, 160 rpm and 7% ammonium hydroxide in 70% ethanol provided a process efficiency of 72% and a glucoraphanin purity of 37%. Whereas, for broccoli florets industrial discards, the operational parameters as determined by the response surface approach for the cationic resin were a feed to resin ratio of 1:1.87, contact time of 30 minutes, shaker speed of 80rpm and eluting solvent of 100% water, provided a process recovery of 95% and glucoraphanin purity of 1.25%. Subsequent purification with the anionic resin provided a process recovery of 78% and glucoraphanin purity of 5%, using optimized experimental parameter of feed to resin ratio of 1:1.3 brought in contact for 170 min with a shaker speed of 140 rpm and eluted using 7% ammonium hydroxide in 70% ethanol. Overall, the glucoraphanin content in the seeds and florets discards were concentrated 9 and 12 times respectively, using the optimized extraction and purification technique. The initial purification of crude broccoli extracts using cationic resin, clarifies the extract from impurities such as chlorophyll and oils, that interfere with the glucoraphanin purification process. The anionic nature of the glucosinolates is exploited in the second level of glucoraphanin concentration process. The negatively charged glucosinolates is bound to the positively charged stationary phase of the anion exchange resin, that can be easily displaced using a strong displacing ion (like ammonia) in the eluting agent, purifying the glucosinolates in the extract.

The optimized extraction and purification process is highly beneficial in the industrial point of view, as it provides a highly efficient glucoraphanin enrichment process, from industrial discards that are low in concentration of biomolecules, using an eco-friendly technique. The glucoraphanin enrichment process, meets the research and industrial objectives, generating high value products, from zero value starting materials, bringing us closer to food security and sustainability.

7.2 Conclusion

The present study, provides a thorough insight on the possible route of valorization of broccoli industrial discards, which are abundantly rich in bioactive molecules such as glucosinolates, predominantly glucoraphanin. In this study, an eco-innovative glucoraphanin extraction and purification technique using green technology was developed to meet the research and industrial objectives. The optimized green process for the fabrication of extracts were highly efficient, with an overall efficiency comparable to those obtained using non-food grade solvents. Finally, an industrially feasible and scalable process, for the formulation of bioactive extracts from agro-food waste was developed, to reduce the negative impact from food wastage, providing a socio-economic sustainability, whilst meeting the future demands of the ever-growing population.

7.3 Future Perspective

The future of this study can take a very interesting and promising route, where the industrial implementation of the process developed and commercialization of the bioactive ingredients for consumer needs can be studied. Marketing, products derived from agro-food discards, can be challenging. This is mainly due to the high quality and regulatory standards for supplements derived from industrial discards, despite the positive health and environmental benefits.

Another interesting perspective for this study is to valorize industrial discards of broccoli for the formulation of health beneficial supplements rich in other bioactive ingredients such as folic acid, dietary fibers, vitamins and minerals. These environmental friendly bioactive extracts can capture a huge market for multivitamin tablets and dietary fibers among the aged, immunocompromised individuals and expecting mothers, as they prefer natural ingredients over the synthetic additives.

Finally, it would be noteworthy to study the synergistic effects of a combination of bioactive molecules, such as glucosinolates and polyphenols on the gut health. Significant number of studies, on the influence of single bioactive ingredients on health has been widely studied. Whereas, in a healthy diet, the combination of these bioactive molecules provides the desired health effect. Therefore, extrapolating this concept for the fabrication

of health supplements, tailored to meet personal needs and wellbeing will be highly beneficial.

Chapter 8

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