MUSTARD: PROTEIN, MUCILAGE AND BIOACTIVES

Research & Commercialization

Final report for

The Saskatchewan Mustard Development Commission

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THE PATHFINDERS RESEARCH & MANAGEMENT LTD.

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EXECUTIVE SUMMARY

Mustard is one of the oldest recorded spices recognized both for its therapeutic and condiment value. It was historically used to treat scorpion bites, entomb kings and as a flavouring agent to disguise spoiled food.

Globally, three types of mustard seeds are used as condiments: pale yellow or white mustard (*Sinapis alba* syn. *Brassica hirta* or *B. alba*); brown or oriental mustard (*Brassica juncea*) and black or dark brown mustard (*Brassica nigra*). Mustard is also widely used as a salad crop (its green leaves), as an oilseed crop in India, for green manure, as a fodder crop or for industrial oil purposes

Canada is one of the major contributors to the world trade market of mustard, supplying both yellow (*Sinapis alba)* and brown/oriental (*B. juncea*) mustard seeds. Canada produced 123,000 tonnes of mustard seed in 2015. According to the most current statistics from FAO, Canada was the highest producer of mustard seed in the world in 2013 with 154,500 tonnes, followed by Nepal, Russian Federation, Myanmar and Ukraine. India is not included in the FAO statistics for pure mustard seed

Mustard is used as a condiment, as a flavouring agent or spice and as an emulsifying/binding agent/thickener in meats, sauces, dressings and bakery products. Commercially, mustard is available in many forms---whole or cracked seeds, flours, deheated flours, de-oiled ground flours, mustard bran and oils.

Beyond its use as a whole seed or flour, there is interest in mustard components—protein, fiber (mucilage), oil and the bioactive components such as glucosinolates, isothiocyanates (ITC) and phenolic acids-- in food, industrial and pharmaceutical applications, particularly in light of their functional properties or antimicrobial, antioxidant and therapeutic properties.

Consumer interest in health and wellness and the desire for “natural” ingredients and “clean labels” on food products are prime factors driving the industry to investigate new plant-proteins as a healthy protein alternative to animal proteins and natural alternatives to chemical preservatives, including antimicrobial and antioxidant agents. These trends spur the research community to provide the evidence required to incorporate new ingredients into foods.

From the research literature search, it appears Canada is the leader in isolating and characterizing mustard protein and yellow mustard mucilage and investigating the antimicrobial activity of mustard glucosinolates and isothiocyanates (ITC) for use in foods and antimicrobial packaging. Researchers at Agriculture and Agri-Food Canada (AAFC) (Drs. Wanasundera and Aluko

(now at University of Manitoba (UofM)) and the University of Toronto (Dr. Diosady) have done significant research on mustard protein characterization, extraction and purification. Dr. Wanasundera has been instrumental in determining some of the genes responsible for the mustard protein allergenicity. Dr. Cui (AAFC) and Dr. Eskin (UofM) have lead programs in isolating and characterizing yellow mustard mucilage. The antimicrobial activity of yellow and brown mustards has been extensively studied by Dr. Holley and his graduate students at the University of Manitoba investigating the effect of mustard and ITCs on microorganisms in fermented and processed meats and chicken, evaluating methods of delivery and studying ITC mechanism of actions in bacteria. Dr. Lim's research program at the University of Guelph has investigated how these ITCs can be incorporated into antimicrobial packaging films.

Research opportunities for mustard proteins and yellow mustard mucilage identified in a report for Mustard 21 (2009) remain valid in 2016. These include functionality characteristics and applications in foods, in-depth nutritional profiles, dietary exposure evaluations, optimization of processes to extract and concentrate the proteins or mucilage, safety assessments and defining health benefits for human consumption.

Glucosinolates from the Brassicaceae family (Brussel sprouts, turnip, radish, mustard etc) are of interest to many groups globally. Research groups are investigating the antimicrobial, antioxidant, anticancer, anti-inflammatory and wound healing properties of the isothiocyanates in *in vitro* and *in vivo* experimental models.

For the food industry, the two main uses for the isothiocyanates from mustard—allyl isothiocyanate (AITC) from brown/oriental mustard and *para*-hydroxybenzyl isothiocyanate (*p*-HBITC) from yellow/white mustard — will be as antimicrobial agents and antioxidants to be added to foods directly or incorporated into packaging films. The primary challenge for AITC and p-HBITC use in foods will be the impact on the sensory profile of the foods. While these ITCs are effective against pathogenic microorganisms, they impart strong flavours and aromas which negatively affect consumer acceptance. It is important to find delivery mechanisms that will permit effective use without affecting taste and smell. Other factors to be considered include regulatory oversight, enhancing antimicrobial efficacy, determining validation methods to confirm antimicrobial activity, effective delivery mechanisms and keeping the antimicrobial form as close as possible to the natural source form (e.g. white mustard essential oil versus the purified ITC, *p*-HBITC).

In the last two decades there has been considerable interest in learning about phenolics in commodity crops (e.g. pulses, cereals, oilseeds) and the associated positive benefits. Research into mustard phenolics is very much in its infancy with a limited number of studies investigating the extraction, identification and quantification of phenolics and determining the antioxidant activities, including how they work in different food systems.

Mustard meals are being used as natural pest control agents, also called biofumigants, in the control of nematodes and fungi in horticultural crops and turf grass.

Regardless of the depth of research conducted on mustard proteins, mucilage, or bioactive components, the key to successful commercialization for any application will require engaging and partnering with large multinational companies with the interest, experience and financial resources to commercialize the product. Critical factors for success will include regulatory approvals (if needed), sound scientific evidence of efficacy, consumer acceptance, cost, and value. For the food industry part of the challenge will be the recognition that the use of ITC in food will be as a component of an overall food safety hurdle strategy and not as a single “magic bullet”.

Until industry champions emerge, the challenge for mustard ingredient companies and, indirectly mustard producers, will reaching out to new users and describing the functional benefits and advantages of using mustard in food products beyond condiments.

1.0 PROJECT OBJECTIVE

To provide an overview of the current state of research and commercialization opportunities for mustard ingredients (flour and bran) and components (protein, mucilage and bioactives) in *Sinapsis alba* and *Brassica juncea* for food and non-food applications

2.0 PROJECT COMPONENTS

This project was comprised of two parts:

Part 1: A technical analysis of mustard ingredients, components and bioactives which form this particular report.

Part 2: A competitive intelligence mission to the Institute of Food Technologists (IFT) Annual Meeting and Conference in Chicago, IL, July 2016. The results of the competitive intelligence mission to IFT are provided in a separate report.

Part 1: The technical analysis of mustard ingredients was composed of the following steps:

1. A scientific literature review describing the current state of research on the following value-added mustard components: mustard flour, ground mustard and mustard bran in food and non-food applications and bioactive components including such compounds as the essential oils, glucosinolates, isothiocyanates, phytin (phytate) and sulfur containing compounds
2. Protein and mucilage with particular emphasis on updating the Mustard 21 (2009) report “An evaluation of the potential for value addition to mustard protein and mucilage”
3. Summary of health benefits of mustard components & bioactives
4. Identification of current and potential commercialization opportunities for mustard components (protein/mucilage) and bioactives
5. Challenges to commercialization (e.g. extraction & processing; regulatory; allergenicity, competitors etc)
6. Identification of research gaps and recommendations to SaskMustard to support commercialization of mustard bioactives, protein or mucilage for food or non-food use

3.0 PROJECT METHODOLOGY

The project was conducted in the following manner:

1. The scientific literature review was based upon a search of the publically available scientific databases including, but not limited to, AGRICOLA, CABI, PUBMED and others.Key words, alone or in combination, used in the search included the following:

Genus species	Common Name	Ingredient/component	Activity
Sinapis alba *Brassica juncea*	Mustard Yellow mustard Brown mustard Oriental mustard	Seed Flour, powder, ground, deordorized Deheated Bran Mucilage Essential oils Erucic acid Hydrocolloid Protein + concentrate + isolate Hydrolyzed protein Glucosinolate(s)-sinalbin, sinigrin Isothiocyanates-allyl, hydroxybenzyl Phenolics Phytate (phytin)	Antimicrobial Antioxidant Anticarcinogenic Nutrient composition Functionality Fumigation Packaging Pest control

2. A search of patent databases in Canada (Canadian Intellectual Property Office, http://www.ic.gc.ca/eic/site/cipointernet-internetopic.nsf/eng/Home) and the United States Patent and Trademark Office (http://www.uspto.gov/) was conducted to identify new research and potential commercialization opportunities for mustard.
3. Internet search for market studies, media articles etc
4. Discussions with research scientists and industry representatives to gain additional information on challenges and market opportunities

4.0 RESULTS

4.1 MUSTARD OVERVIEW

Mustard is one of the oldest recorded spices according to records dating back to 3000 BC. It was recognized both for its therapeutic value and condiment value, historically being used to treat scorpion bites, entomb kings and as a flavouring agent to disguise degraded food (Wanasundera, 2011).

Globally, three types of mustard seeds are used as condiments: pale yellow or white mustard (*Sinapis alba* syn. *Brassica hirta* or *B. alba*); brown or oriental mustard (*B. juncea*) and black or dark brown mustard (*B. nigra*). Mustard is also widely used as a salad crop (its green leaves), as an oilseed crop in India, for green manure or as a fodder crop or for industrial oil purposes. In Canada, *Sinapis alba* is also listed as a traditional Chinese medicine (Bai Jie Zi) in the Natural and Non-Prescription Health Products Directorate database[1].

Canada is one of the major contributors to the world trade market of mustard, supplying both yellow (*Sinapis alba)* and brown/oriental (*B. juncea*) mustards. Canada produced 123,000 tonnes of mustard seed in 2015 (Statistics Canada, 2015). According to the most current statistics from FAO, Canada was the highest producer of mustard seed in the world in 2013 with 154,500 tonnes (FAOSTAT, 2016).

Figure 1[2] Canadian Mustard

Sinapis alba (yellow) | *Brassica juncea* (brown) | *Brassica juncea* (oriental)

[1] Natural and Non-Prescription Health Products Directorate. Natural Health Products Ingredients Database, http://webprod.hc-sc.gc.ca/nhpid-bdipsn/search-rechercheReq.do

[2] Canada Grain Commission, Quality of western Canadian mustard. 2015 https://www.grainscanada.gc.ca/mustard-moutarde/harvest-recolte/2015/hqm15-qrm15-1-en.htm Accessed June 2016.

4.1.1 MUSTARD COMPOSITION

Mustards seeds are composed of protein (23-30%), fixed oil (29-40%), carbohydrate (12-18%) as well as minor constituents including minerals (4%), essential oils (glucosinolates; 0.8-2.3%), phytate (phytin) (2-3%), phenolic compounds and dithiolthiones. Table 1 compares the macronutrient composition and isothiocyanate composition of mustard seed and mustard seed products.

Table 1 Chemical composition of mustard seed and its products (Cui & Eskin, 1999)[23]

Mustard product	Isothiocyanates (ITC)		Composition					
	Allyl-ITC	ρ-Hydroxy benzyl ITC	Fixed Oil	Protein	Phytate (phytin)	Crude Fibre	Moisture	Ash
	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)
Whole seed, yellow		2.3	29	30	2-3	9	6	4
Whole seed, brown	0.80	ND[1]	32	26	2-3	7	6	4
Whole seed, oriental	0.78	ND	36	23	2-3	6	6	4
Yellow flour	-[2]	0.0	30	35	-	3.5	6	4
Brown flour	0.95	-	40	35	-	3.5	6	4
Oriental flour	0.90	-	42	30	-	3.5	6	4
Yellow bran	-	-	7	16	-	15	10	4
Brown bran	0.20	-	7	13	-	15	10	4
Oriental bran	0.35	-	7	15	-	15	10	4

[1]ND-not detected; [2] – data not provided;

The variability in mustard composition is shown by the harvest data collected for the Canadian Grain Commission's (CGC) quality program for western Canadian mustard where the CGC monitors harvest samples of mustard for protein, oil and glucosinolate content in Canadian grown mustards. Figures 2, 3 and 4 show the variation in protein and oil content for yellow mustard, brown mustard and oriental mustard respectively and Figure 5, the range of glucosinolate content in brown/oriental mustard over a ten year period (2005-2015). For yellow mustards, protein ranged from 29% to 34% and oil from 27% to 32% in the past decade; brown mustard protein and oil ranged from ~25% to 27.5% and 36% to 40% respectively; with protein ranges of 24% to 28% and oil content of 37% to 45% in oriental mustards.

Figure 2 Oil and protein content of yellow mustard (No. 1 Canada) harvest samples, 2005-2015. [3]

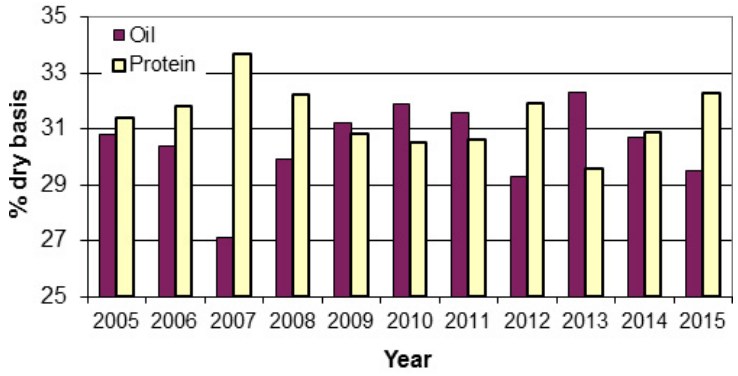


Figure 3 Oil and protein content of brown mustard (No. 1 Canada) harvest samples, 2005-2015.[3]

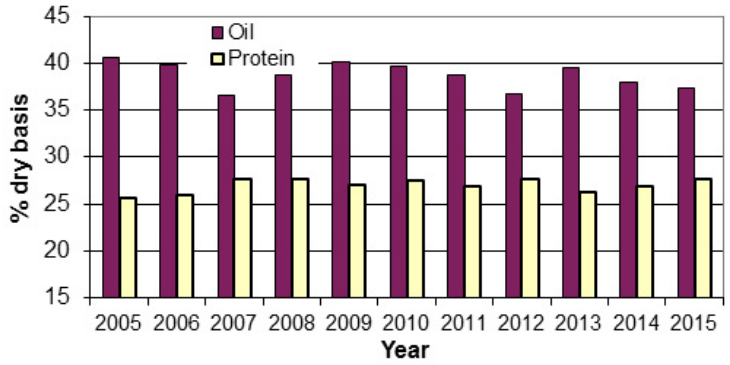
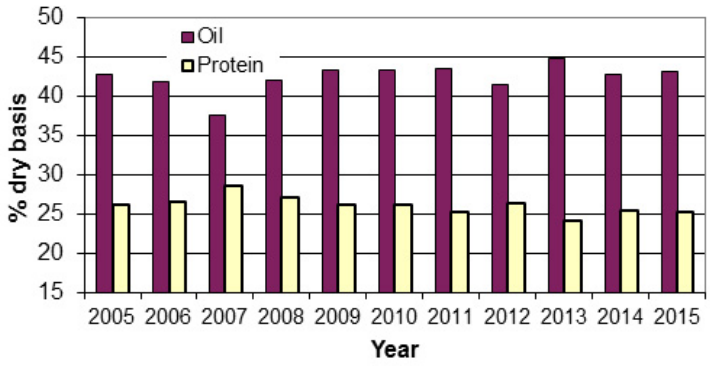


Figure 4 Oil and protein content of oriental mustard (No.1 Canada) harvest samples, 2005-2015.[3]



[3] Canada Grain Commission, Quality of western Canadian mustard. 2015 https://www.grainscanada.gc.ca/mustard-moutarde/harvest-recolte/2015/hqm15-qrm15-1-en.htm Accessed June 2016

The fatty acid composition of mustard oils differs significantly from canola and soybean. Mustard seed oils are high in erucic acid (19%-36%) compared to virtually no erucic acid in canola or soybean. Canola oil is high in oleic (C18:1; ~61%) while soybean is high in linoleic (C18:2; ~56%) (Table 2). Mustard is low in saturated fatty acids and high in mono- and polyunsaturated fatty acids.

Table 2 Fatty acid composition (%) of Canadian grown canola, mustard and soybeans(Canada Grain Commission, 2015)[4]

Nutrient	*Brassica napus* (canola)	*Brassica juncea* (Oriental)	*Brassica juncea* (brown)	*Sinapis alba* (yellow)	Glycine max (soybean)
Palmitic (16:0)	3.9	2.5-3.0	2.7-2.8	2.4-2.5	9.5-10.0
Palmitoleic (C16:1)	0.3	0.1	0.2	0.2	
Stearic (C18:0)	1.9	1.3-1.5	1.3-1.5	0.9-1.0	3.5-4.2
Oleic (C18:1)	61.3	19.6-23.3	19.1-21.0	21.6-25.3	20.2-22.2
Linoleic (C18:2)	18.4	20.2-21.2	20.4-20.8	8.9-9.6	53.3-56.7
Linolenic (C18:3)	9.9	10.6-12.5	13.1-14.6	10.2-10.7	8.0-10.2
Arachidic (C20:0)	0.7	0.9	0.9-1.0	0.6-0.7	0.1-0.6
Eicosenic (C20:1)	1.2	12.2-13.3	12.1-12.3	10.6-11.4	trace
Eicosadienoic (C20:2)	0.1	1.0-1.2	1.0-1.2	0.3	
Behenic (C22:0)	0.3	0.5	1.4-0.5	0.5-0.6	0.7
Erucic (C22:1)	0.00	19.0-24.9	22.4-24.1	35.4-36.6	
Docosadienoic (C22:2)	0.2	0.4-0.5	0.4-0.5	0.3	
Lignoceric (C24:0)	0.2	0.3	0.3-0.4	0.3	0.5
Nervonic (C24:1)	0.2	1.3-1.6	1.2-1.5	2.2-2.4	ND[1]
Minor Fatty Acids					1.4-2.0
Total Unsaturated (C18)	89	57	58.1	36.8	89
Total Saturated (C16, C18)	7	6.2	5.2	5.1	15

[1]ND, Not detected

Researchers from Agriculture and Agri-Food Canada examined the protein content and composition of *Brassica napus* (canola), *Sinapis alba* (yellow mustard) and *Brassica juncea* (brown/oriental mustard). In *Brassica* plants, two classes of seed storage proteins are predominant: legumin-type **globulins** (11S or 12 S or cruciferin) and napin-type **albumins** (2S or napins). Globulins are soluble in dilute salt solutions and albumins are soluble in water (Wanasundera, 2011). The 11S cruciferins and 2S napins in mustard have different molecular

[4] Canada Grain Commission, Quality of western Canadian mustard. 2015 https://www.grainscanada.gc.ca/mustard-moutarde/harvest-recolte/2015/hqm15-qrm15-1-en.htm Accessed June 2016

assemblies, molecular masses, biochemical properties and biological activities. The seed storage proteins in mustard are similar with other crucifer proteins such as canola (Wanasundera, 2009). Table 3 shows the % protein and % oil composition of whole seed, cotyledon and hull of mustard and canola. While the seed coats of the *Brassica* species do contain proteins, they are not the seed storage proteins that are contained in the cotyledon (Wanasundera et al, 2012).

Table 3 Protein, ash and lipid content in Canadian mustard varieties, *Brassica juncea* (brown) and *Sinapis alba* (yellow) compared to *Brassica napus* (canola) (data extracted from Wanasundera et al., 2012)

	Ash (%)	**Crude Protein (%) (%N x 6.25)**	**Lipids (%)**
Brassica juncea			
AC Vulcan			
Whole seed	4.1	26.9	42.5
Cotyledon	4.1	28.0	48.3
Seed coat	4.3	17.3	15.6
Duchess			
Whole seed	3.5	26.9	43.5
Cotyledon	3.5	29.9	44.5
Seed coat	4.3	14.7	9.2
Sinapis alba			
AC Pennant			
Whole seed	4.4	32.2	33.5
Cotyledon	4.3	33.5	29.9
Seed coat	4.6	16.9	9.4
Andante			
Whole seed	3.9	36.7	30.4
Cotyledon	3.9	39.0	31.8
Seed coat	4.4	17.5	7.4
Brassica napus			
AC Excel			
Whole seed	4.0	23.9	49.2
Cotyledon	3.7	25.2	55.2
Seed coat	6.2	14.7	14.3

Mustard protein has an excellent nutritional profile being rich in lysine with adequate amounts of sulphur-containing amino acids (methionine & cysteine) which are limiting amino acids in most cereals and oilseed proteins. Values for each amino acid may vary widely most likely due to differences in cultivars examined, growing environment, flour and meal processing parameters and analytical method used to quantify the amino acids (Table 4).

Table 4 Comparison of the amino acid profile of yellow mustard (YM) and brown mustard (BM) flours and meals to canola and soybean meal

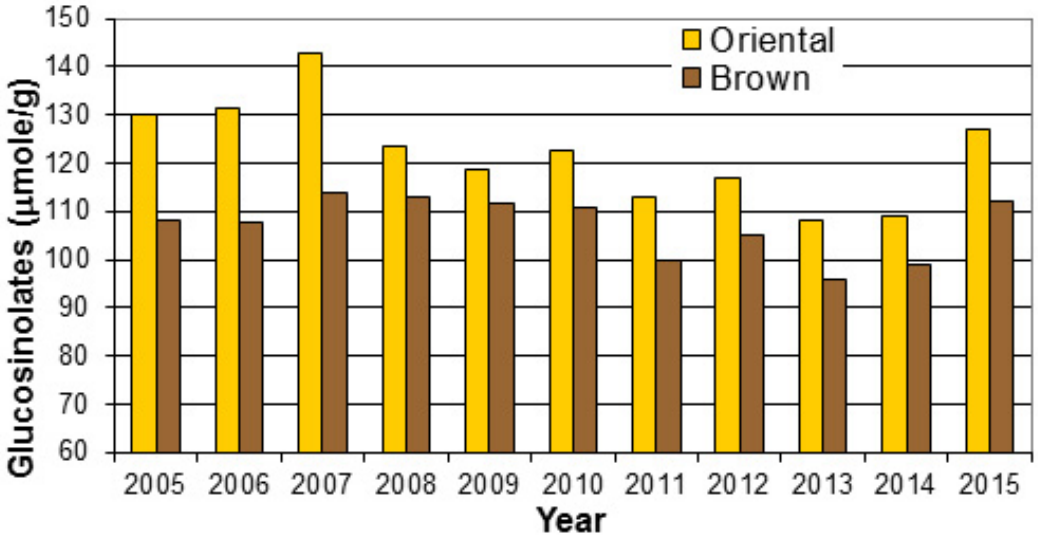
	Cserhalmi et al., 2001	Abul-Fadl et al, 2011		Sarwar et al, 1981	Wanasundera 2016	Wanasundera 2016	Aluko et al, 2005a	Wanasundera 2016	Cavins et al, 1971
	YM flour	YM flour	BM flour	YM meal (defatted)	YM meal (AC Pennant)	Commercial defatted YM flour	YM defatted seed meal	Canola meal	Soybean meal
Amino Acid	g AA/100 g protein								
Essential Amino Acids									
Histidine	37	25	26	23	12	18	19	16	24
Isoleucine	40	43	45	32	29	19	31	18	50
Leucine	74	79	80	56	38	32	57	33	80
Lysine	61	65	55	87	22	38	48	25	61
Methionine	37[1]	43[1]	59[1]	10	11	9	16	11	12
Phenylalanine	45	76[2]	84[2]	34	21	17	32	19	50
Threonine	47	44	40	71	21	20	39	20	40
Tryptophan	-[3]	-	-	5	8	7	15	7	-
Valine	31	67	57	60	22	22	43	20	50
Non-essential Amino Acids									
Alanine	42	40	45	38	27	23	34	21	44
Arginine	62	47	77	33	26	34	51	33	72
Aspartic acid	78	90	87	101	54	22	69[4]	39	123
Glutamic acid	191	198	202	133	93	139	133[5]	85	197
Glycine	55	45	50	66	36	27	44	26	42
Proline	111	81	42	95	32	48	48	26	53
Serine	51	56	49	69	26	22	37	20	51
Tyrosine	29			55	14	10	29	14	36
Cysteine + cystine	-	-	-	-	19	17	16	22	12

[1]Met + Cys; [2] Phenylalanine (Phe) + Tyrosine (Tyr); [3]"-" not determined; [4]Aspartic acid + asparagine; [5]Glutamic acid + glutamine

The total glucosinolate content of mustard varies from year to year and is cultivar dependent. Figure 5 shows the variation in total glucosinolates in Canadian grown oriental and brown mustards for the period of 2005 to 2015, as analyzed by the Canadian Grain Commission. The total glucosinolate content in brown mustard ranged from ~95 to ~112 umol/g mustard seed and from ~109 to ~142 umol/g oriental mustard seed. While the GCC does not report glucosinolates content in yellow mustard, literature values indicate the glucosinolate content can range from 100-200 umol/g (Rakow et al, 2009).

The wide range of glucosinolate content in brown mustards was also shown in a French study (Merah, 2015) which evaluated the genetic variability of glucosinolates in 190 genotypes of *Brassica juncea* seeds. It was found that total glucosinolates varied two fold (70 to140 umol/g) between extreme genotypes and the predominant glucosinolate, sinigrin (allyl glucosinolate), content varied from 0 to more than 134 umol/g seed.

Figure 5 Glucosinolates (µmole/g) in Oriental and brown mustard seed harvest samples, 2005-2015.[3]



4.1.2 COMMERCIAL MUSTARD INGREDIENTS

A number of mustard products are derived from yellow, brown & oriental mustards (Figure 6) and added to foods as seasonings or for their functional properties. These mustard products provide emulsification, stability, water and fat binding, preservative and nutritive properties to foods. Whole mustard seeds, mustard flour, ground mustard, de-oiled mustards, heated deactivated mustards, mustard brans and mustard oils are used in baked goods, meat products, condiments, and emulsion type dressings (Table 5).

In North America, a number of companies process and sell mustard ingredients to the food industry.

Canada

a) GS Dunn (Hamilton, ON)
b) Besco Grain (Brunkild, MB)
c) Sakai Spice (Calgary, AB)

United States

a) Wisconsin Spice (Berlin, WI)
b) Minn-Dak Growers Ltd (Grand Forks, ND)
c) Montana Specialty Mills (Great Falls, MT)

The ultimate goal of mustard processing is to maximize the pungency and taste and extend mustard shelf life; however there are challenges to doing this. The quality of the final mustard product is dependent upon the generation of heat during processing, the oil and moisture content, and enzyme activity (particularly myrosinase).

Conventionally, prepared mustard was manufactured by crushing the mustard seed, expelling the crude oil from the crushed seed, then soaking the mustard flour in a liquid to allow development of flavor and pungency. Conventional processing methods of producing mustard flours involved soaking the dehulled, ground flours in hot water to activate the myrosinase enzyme and then re-drying the wet flour. Flours of varying degrees of hotness were obtained; however shelf life was limited as residual isothiocyanate were degraded to other products.

In the 1960-1970s new thermal treatments were used to better control the pungency and shelf life of mustard ingredients. Rather than hot water, saturated steam was used to completely or partially inactivate the myrosinase enzyme in cracked yellow mustard seeds resulting in a mustard product with a controlled degree of “bite” (US Patent 3574640). In the 1970’s McCormicks introduced a spray dried mustard flour that was interchangeable with conventional dry ground

mustard flour in mayonnaise formulations. It was possible to reduce the mustard level in a standard mayonnaise recipe by 25% without adversely affecting flavor or viscosity. Also due to enhanced functional properties of the spray dried mustard, the egg yolk content in the formulation could also be reduced by 7.5%. This resulted in reduced ingredient costs (US Patent 4062979). Shiro Sakai (established Sakai Spice Canada Corp in 1993) developed a mustard process that thermally inactivated the myrosinase enzyme in de-oiled cracked seeds prior to being ground into flour resulting in a product with reduced pungency, good flavor, enhanced protein content and extended shelf life (US Patent 4496598)

Mustard's use as a food ingredient expanded considerably in the 1990s. It was at that time that Canadian-based UFL Foods (working with POS Biosciences) introduced a thermal process for inactivation of the enzyme myrosinase in yellow mustard seeds. Inactivation of the enzyme prevented the glucosinolates in mustard from being converted to isothiocyanates, compounds responsible for mustard's "hot" flavour.

After grinding, the mustard powder yielded an ingredient with excellent emulsifying, binding, stabilizing and thickening properties, but which lacked the intense hot flavour (also known as cold or deodorized powder) normally caused in yellow mustard by para-hydroxylbenzyl ITC (p-HBITC) (Cui and Eskin, 1999). Because of its high protein content and low cost, deodorized mustard powder became a popular ingredient in cooked cured meats. Deheated ground mustard is now sold in more than 50 countries in meat products, condiments, salad dressing, cheese spreads, gravies and mayonnaise and can be a substitute for egg and guar ingredients in food formulations.[5] [Note UFL Foods was acquired by Newly Weds Foods in 1998.]

Whole ground mustard and deactivated mustard can also be used to replace sodium phosphate or soy protein isolate (SPI) in marinated chicken products. Sodium phosphate or SPI are used as water binding agents that increase the water holding capacity of meat proteins. The mustard substitution (~3% of formulation) is expected to result in significant cost savings, higher yields and increased shelf life (GS Dunn, 2016).

[5] POS Biosciences website, http://www.pos.ca/opportunites/industries/

Figure 6 Processing of Mustard and Mustard Products
(Cui & Eskin, 1999)

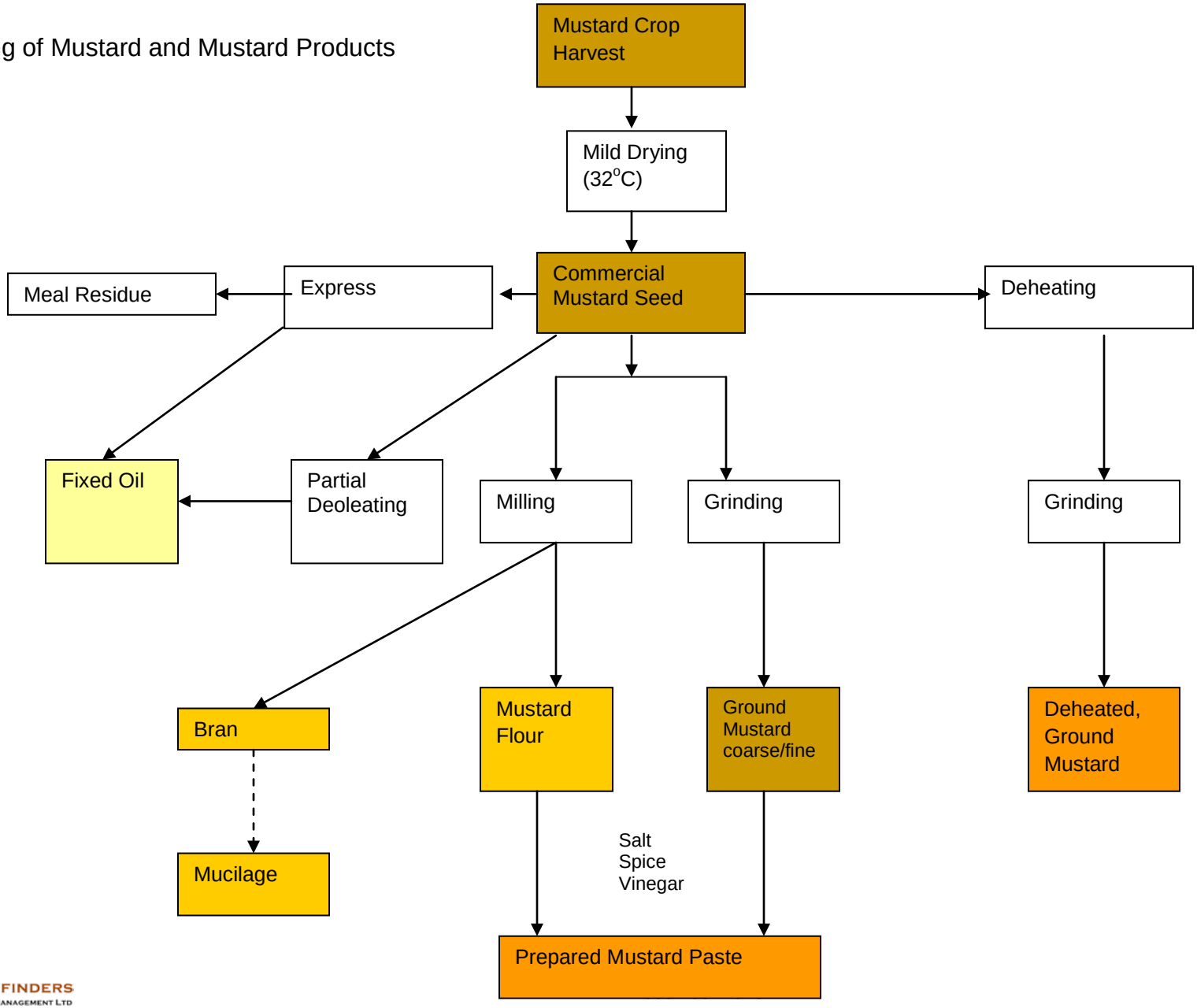


Table 5 Commercial Mustard Products

Product Form	Seed type	Application	Reason for use
Oil	*Brassica juncea* (brown)	Edible oil (India) Industrial uses	As an edible oil, pungency and taste High erucic acid content makes for an excellent lubricant
	Sinapis alba (yellow)	Lubricant, illuminator Mayonnaise (in Sweden) White mustard oil extract (WMEO)	Lubricant Potential antimicrobial agent
Mustard flour (bran (hull) removed)	Yellow mustard flours Yellow/oriental blends	Retailed as flour Ingredient in salad dressings, mayonnaise, BBQ sauce, pickles, processed meats	Flavour Emulsifier Various flavor profiles offered (from mild to hot & pungent)
Ground Mustard (ground seeds, bran included)	Yellow mustard Oriental mustard Yellow/Oriental blends Brown mustard	Meat products Seasoning for frankfurters, bologna, salamis, luncheon meats Salad dressings, pickled products, condiments Brown mustard used primarily for preparation of hot, spicy table mustards (e.g. Dijon)	Flavour Emulsion stability for oil/water emulsions Water binder Bulking agent Reduces product shrinkage during cooking Low cost vegetable protein in meat products Preservative (isothiocyanates inhibit microbial growth)-preserves colour and freshness in fruit pies, tarts and quiches.
Cracked mustard seeds	Yellow mustard Brown mustard	Mustards, salad dressings Seasonings and topical blends	Specific granulations achieved Emulsification Thickener
Deheated	Yellow mustard	Finely ground: Myrosinase enzyme	Bland tasting functional ingredient

mustard		deactivated Processed cheese slices, bakery products and beverages Meat products Sauces Mayonnaise (can partially replace egg yolk) Tomato-based products (e.g. ketchup)	High protein source Water binder Antioxidant-retards lipid oxidation Stabilizer Thickener Potential as reducing agent in bakery mixes to break down gluten matrix, relaxing the dough and improving stretchability
De-oiled ground mustard		Meat products (hot dogs, sausages, bratwurst, and processed deli meats) Creamy dressings Mayonnaise Applications include hot Chinese mustard, wasabi paste/powder, and Asian cuisine	Fixed oil component is cold-expelled concentrating the protein (minimum 40%), mucilage, phospholipids and fiber.
Mustard Bran	Yellow mustard Yellow/Oriental blends (50/50)	Coarse flakes or ground to fine powder Natural thickener in sauces	Water binding/holding capacity Thickener Low cost filler Oriental mustard bran has hotter flavour, only small amount of mucilage present.

Other mustard flour processing methods are also being examined. Cserhalmi and co-workers (2001) used radio frequency (RF) treatment (a cold processing method) as an alternative to conventional heat treatments to inactivate the myrosinase enzyme and produce mustard flour without pungency. RF treated mustard flour lost its pungency and had similar functionalities to conventional heated mustard flours (Table 6). RF-treated mustard seed flour replaced 25% of egg yolk powder and resulted in mayonnaise with better sensory properties, emulsion stability and consistency than the control with no mustard flour.

Table 6 Protein content and functional attributes of conventional and radio frequency (RF) treated yellow mustard flour (Cserhalmi et al, 2001)

Sample	Protein (%)	Water binding ability (%)	Fat binding Ability (%)	EAI (%)	ES (%)	Nitrogen Solubility Index (%)
Yellow mustard seed flour	34.7	130.0	96.87	53.9	55.4	9.1
Yellow mustard seed flour (RF treated)	34.7	131.0	83.9	53.4	65.8	7.0

From a product development perspective, mustard ingredient companies at the Institute of Food Technologists show in July 2016 stated they either did the majority of product development in-house, contracted the work to food development centres or supported university research for their own proprietary use. The companies also indicated that the major mustard users (e.g. Kraft or French's, McCormick's spice) did similar in-house development.

The challenge for mustard ingredient companies is reaching out to new users and describing the functional benefits of using mustard in food products beyond condiments. There does appear to be renewed interest in studying applications of mustard ingredients in food products, particularly in light of its antimicrobial and antioxidant properties. These applications will be discussed later in the report in the respective sections.

4.1.3 REGULATORY STATUS OF MUSTARD INGREDIENTS

Under the *Food & Drug Act & Regulations* (Division 7 Spices, Dressings and Seasonings) (Department of Justice, 2016) some mustard products are identified as standardized products because the regulations designate the composition (B.07.024-025). Other mustard ingredients on the market place are not standardized (e.g. mustard bran) and thus, their composition does not need to be defined.

B.07.024 [S]. Mustard Seed shall be the seed of *Sinapis alba*, *Brassica hirta* Moench, *Brassica juncea* (L) Cosson or *Brassica nigra* and shall contain (a) not more than (i) seven per cent total ash, (ii) one per cent ash insoluble in hydrochloric acid, and (iii) 11 per cent moisture; and (b) not less than 25 per cent non-volatile ether extract.
B.07.025 [S]. Mustard, Mustard Flour or Ground Mustard shall be powdered mustard seed (a) made from mustard seed from which (i) most of the hulls have been removed, and (ii) a portion of the fixed oil may have been removed; and (b) that contains not more than (i) 1.5 per cent starch, and (ii) eight per cent total ash, on an oil-free basis.

The use level (e.g. 0.1% to 5%) in processed food products is limited to practical applications (functionality or sensory). Deheated mustard flours can be used up to 5%. Condiment mustards can contain up to 50% mustard ingredients depending upon the formulation.

Mustard bran (fiber) is the outer seed layer that traditionally was removed prior to milling for flour production. Approximately, 20% of the seed weight was lost to the bran. Now with the emphasis on fibers and health, there is renewed interest in its use as a food ingredient. Currently mustard bran is used at levels of 2-4% in condiments as a filler, emulsifier, and stabilizer (due to the mucilage factor) on a non-flavor contributing basis.

However, mustard bran does not have dietary fibre status (Health Canada, 2013) and thus cannot be included in the dietary fibre calculations in the Nutrition Facts table on a food package. Its physiological efficacy must be established before it can claim to be a source of dietary fibre.

To be classified as a dietary fibre, mustard bran must show at least one of the following physiological effects (Health Canada, 2012):

- Improves laxation or regularity by increasing stool bulk
- Reduces blood total and/or low-density lipoprotein cholesterol levels
- Reduces post-prandial blood glucose and/or insulin level
- Provides energy-yielding metabolites through colonic fermentation

There could be industry interest in pursuing Health Canada approval of mustard bran as a dietary fiber.

4.1.4 ALLERGENICITY OF MUSTARD

Mustard is now considered a priority allergen in Canada and according to the new allergen labelling regulations (came into force August 4, 2012) (Canada Gazette, 2011) must be identified as a food allergen on the food label in the ingredient list. Mustard is also a priority allergen in Europe (EU Directive 2003/89/EC) and must be declared when present in foods. Previously, when mustard was used as a flavor compound at less than 2% of the formula or as a component of a spice mixture it did not need to be identified. With these regulations, mustard must be listed separately as an ingredient, regardless of its use level in the food.

Allergenicity in yellow mustard is due to four different allergenic proteins [*Sin* a 1, *Sin* a 2, *Sin* a 3 and *Sin* a 4] which have been identified and characterized (Marambe et al., 2014). *Sin* a 1, a napin protein and a 2S albumin storage protein, is considered to be the most important allergen with respect to allergenic potential and does not appear to lose its allergenicity during the thermal process to inactivate myrosinase (Marambe et al, 2015). *Brassica juncea* also contains allergenic proteins in the 2S fraction (*Bra j*1E). Allergic reactions to mustard proteins are considered to be severe and include anaphylactic shock that requires clinical intervention. Commercial mustard protein detection kits (ELIZAS) recognize cruciferin, napin and other proteins within mustards and are used by the food industry as part of quality assurance programs to identify potential cross contamination of foods with mustard.

A Health Canada consumer information document (Health Canada, 2016) provides information about mustard as a priority allergen. The document recommends consumers not consume any protein derived from the seeds of the Brassicicacea family including canola protein or cold-pressed oils of canola which are less refined and may contain residual protein. Highly refined canola oils do not contain protein residues.

4.1.5 MUSTARD COMPONENTS

Globally, there is increasing interest in finding value-added opportunities, beyond whole seed applications, for crop components—macro components such as protein, starch, oils, and fiber and the minor bioactive compounds such as phenolics, glucosinolates or phytates. Interestingly, these minor compounds, once considered to be solely antinutritional factors, are being thoroughly investigated for their potential health benefits.

To realize the full potential of a crop, a use must be found for each fraction that is isolated and concentrated. As much as possible all fractions must be considered co-products, and not just by-products of one particular fraction.

Yellow mustard is a rich source of three valuable macro-components in food processing: proteins, polysaccharide gums, and oil. These components provide a variety of technologically important functions which bear further investigation. In addition, mustard bioactives such as the glucosinolates, phenolics, thiols and their derivatives, and phytate could also be valuable co-products of mustard processing.

Table 7 Macro and micro mustard components: food and health applications

Mustard component	Food Applications	Health
Protein (native or modified)	Food (regular and functional foods)- beverages, dessert type, energy bars, flavour enhancers, extruded products, meat products	Source of high quality protein Allergen
Proteins-hydrolyzed	Foods as flavour enhancer Functional foods Pet foods	Hydrolyzed proteins with bioactivities such as antioxidative, antihypertensive, calcium-absorbing, antithrombotic, etc
Mucilage (from *Sinapis alba*)	Binder Emulsifier with unique oil/water properties	Anticarcinogenic effects
Glucosinolates as source of Isothiocyanates	Antimicrobial agents for application in foods or food packaging	Anticarcinogenic effects Antiinflammatory Wound healing Antimicrobial
Phenolics	Antioxidant activity Antimicrobial activity	Antioxidant activity Anticancer activity Antimicrobial activity

4.2 PROTEIN

4.2.1 PROTEIN MARKET OVERVIEW

To better understand the growing interest in mustard proteins, a summary of protein market trends and drivers provides market context.

Global protein consumption exceeded 250 billion tons in 2014 and is expected to reach 280 billion tons in 2022. This growth is driven by the growing demand for protein-fortified foods, the growing popularity of meat substitutes, increasing concerns towards lactose intolerance, and the increasing use of proteins in non-food applications such as pharmaceuticals, cosmetics, personal care products and animal feeds. Rising consumer awareness about health and wellness and understanding the positive role of protein-rich foods in weight management is also spurring consumption[6].

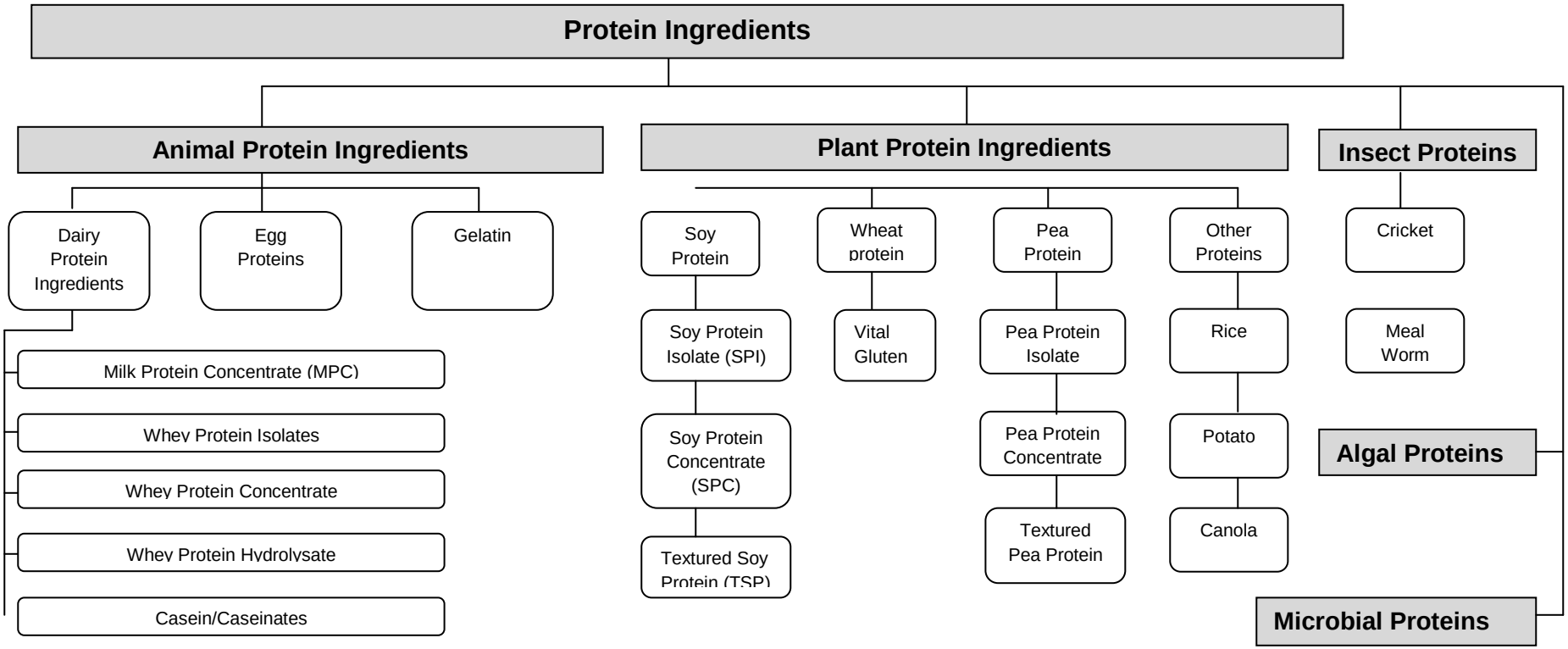
Frost & Sullivan[7] identified the top three growth factors for proteins and amino acids as being the following:

1. The food industry looking for improved functionality from specialty protein ingredients
2. Emerging regions creating new opportunities for all protein and amino acid suppliers
3. Food industry's drive to promote potential health benefits

Animal protein ingredients, derived primarily from fish, meat, eggs and milk, dominate the market and hold about 61% of the total protein and amino acids market. However, proteins derived from plants (e.g. soy, wheat, pulses, oilseeds, and other cereals), microorganisms and even insects are becoming an integral part of the food & beverage industry and are providing intense competition to animal proteins (Figure 7) . In 2013 plant proteins made up 13.8 % of the total protein and amino acid market.[8] This drive for plant proteins is also partially driven by environmental concerns regarding the ability to sustain meat and dairy production to feed a growing world population.

[6] Global Industry Analysts Inc. 2016. High nutritional value and functional health benefits to fuel growth in the global protein ingredients market. April 2016.
[7] Frost & Sullivan presentation: 2013 Protein Trends & Technologies Seminar http://www.globalfoodforums.com/wp-content/uploads/2013PTT-Strategic-Insights-into-the-Global-Protein-Ingredient-Market-C.Shanahan.pdf
[8] ibid

Figure 7 Protein Ingredients



Modified from Frost & Sullivan presentation: 2013 Protein Trends & Technologies Seminar http://www.globalfoodforums.com/wp-content/uploads/2013PTT-Strategic-Insights-into-the-Global-Protein-Ingredient-Market-C.Shanahan.pdf

Figures 8 & 9 show the differences between the animal and protein ingredients market as a percentage of revenue by category.

Figure 8 Animal protein ingredients market: % of revenue by category, Global 2013[9]

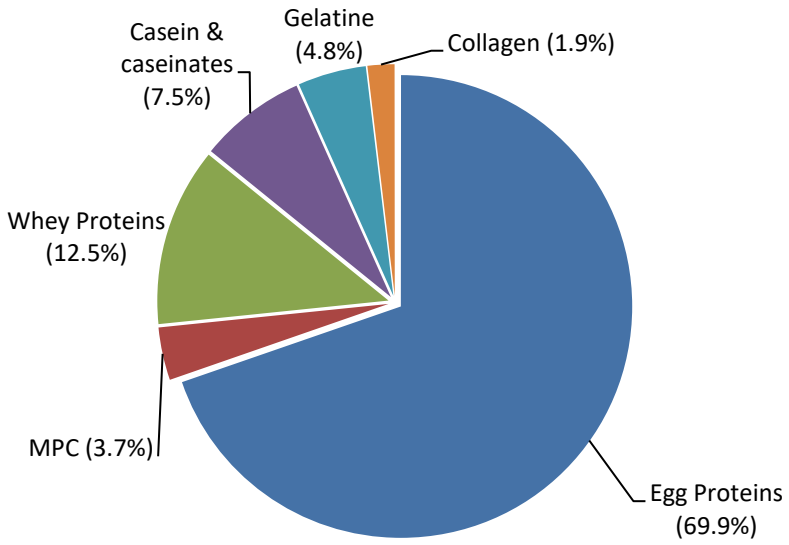
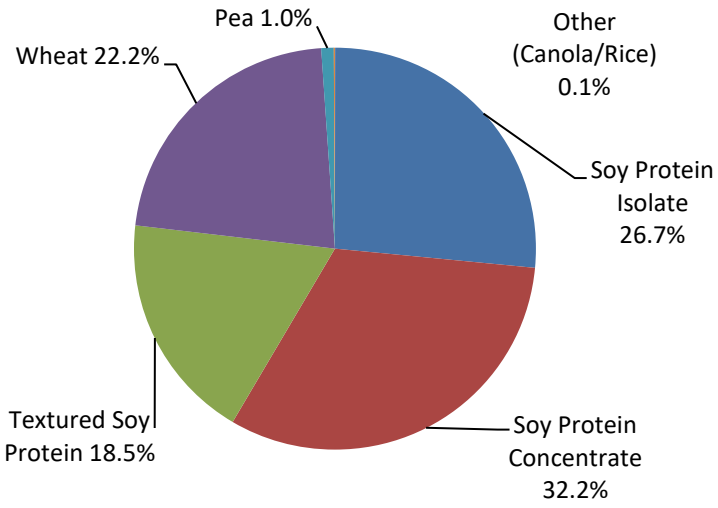


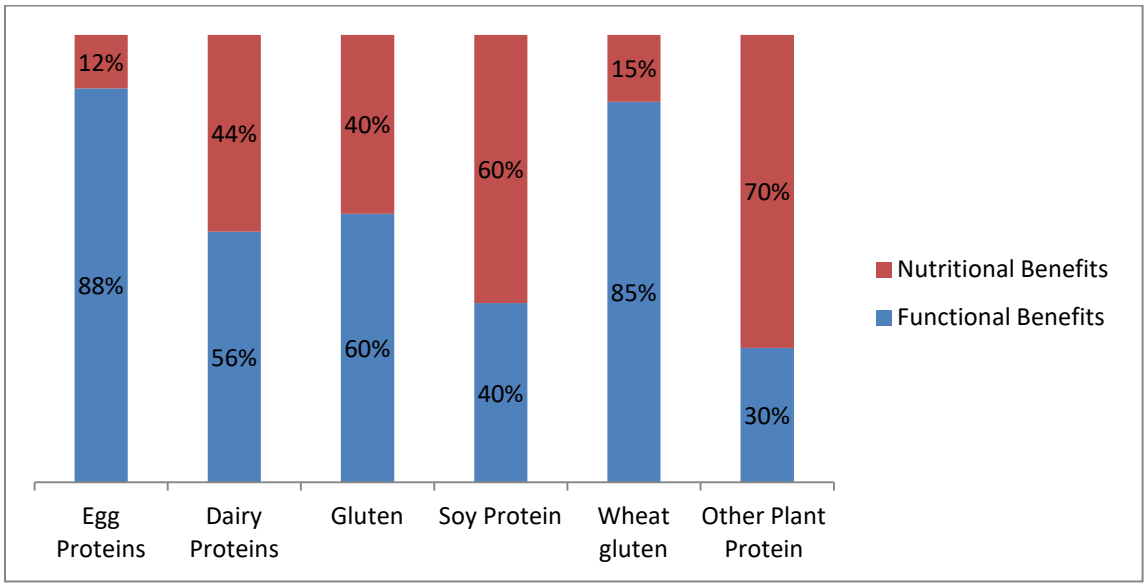
Figure 9 Plant Protein Ingredients Market: % of Revenue by Category, Global, 2013[7]



[9] Frost & Sullivan presentation: 2013 Protein Trends & Technologies Seminar http://www.globalfoodforums.com/wp-content/uploads/2013PTT-Strategic-Insights-into-the-Global-Protein-Ingredient-Market-C.Shanahan.pdf

Egg proteins hold the largest segment as it is preferred by food formulators for its emulsifier, sensory and textural properties. Dairy proteins are preferred in higher valued applications such as sports and infant nutrition formulas. For plant proteins, soy is the leader as it is a cost-effective way to reduce fat and match or increase the protein content of dairy products. Pea protein, however, is beginning to make inroads due to its nutritional and functional benefits and is forecast to exceed USD 200 million by 2023.[10] Regardless of which protein is used, the main reason proteins are incorporated into foods is for their functional attributes (59.8% of usage), enhanced by nutritional benefits.

Figure 10 Protein Ingredient Usage Trends[11]



[10] Global Market Insights. 2016. Pea Protein Market. https://www.gminsights.com/pressrelease/pea-protein-market Accessed May 2016

[11] Frost & Sullivan presentation: 2013 Protein Trends & Technologies Seminar http://www.globalfoodforums.com/wp-content/uploads/2013PTT-Strategic-Insights-into-the-Global-Protein-Ingredient-Market-C.Shanahan.pdf

Proteins are a volatile commodity with plant proteins tending to be more stably priced than animal derived proteins. Typically, plant proteins cost 40-50% less than animal proteins (Table 8).

Table 8 Protein market landscape (2013): cost hierarchy[12]

Protein	Product	US$/Tonne
Soy	Highly functional soy isolate	$9000
	Soy isolate	$7000
Whey	Premium whey isolate	$13,000
	Commodity whey isolate	$11,000
Casein	Caseinate milk protein	$11,000
Pea	Pea protein isolate	$7000
Egg-white	Spray-dried egg white	$13,000

Frost & Sullivan's report identified some success factors for those companies in the protein market:

- Global protein and amino acids ingredients market is relatively fragmented and very competitive, meaning price matters
- Protein consumption patterns vary across geographical areas and by income. Higher valued concentrated ingredients are more prominent in the west than in the east
- Vertical integration is a critical success factor
- The market is becoming more technology intensive, hence increases in R&D spending serves as the competitive edge
- Best practices such as customer service, product differentiation and value addition boost company growth.

[12] Frost & Sullivan presentation: 2013 Protein Trends & Technologies Seminar http://www.globalfoodforums.com/wp-content/uploads/2013PTT-Strategic-Insights-into-the-Global-Protein-Ingredient-Market-C.Shanahan.pdf

4.2.2 MUSTARD PROTEIN

The relatively high protein content of mustard seed makes the crop an attractive potential source of food-grade vegetable protein. The balance of amino acids found within the seeds also compares favourably with that required for human nutrition.

As described previously, the proteins of most interest in mustard are the seed storage proteins, cruciferin and napin found within the cotyledon. During the past two decades, there has been considerable work on developing processes to extract and concentrate or isolate mustard proteins, characterize the composition and functionality and find applications in human nutrition.

In 2008, Wanasundara reviewed the *Brassica* protein extraction methods in the literature. The report for Mustard 21 included those procedures relevant to mustard protein extraction and in this report has been updated to reflect additions since 2009 (Table 9).

Protein recovery methods include alkali extraction and isoelectric precipitation, protein micelle formation, ultrafiltration, and fractionation to produce protein isolates (>90% protein) and concentrates (>60% protein). The majority of methods isolate heterogeneous protein fractions that may or may not contain non-nutritive bioactive factors of health concern (glucosinolates, phytates, phenolics). Berot's chromatographic separation method (2005) and Wanasundara's patented aqueous protein extraction process (2012/2013) separate the proteins into napin and cruciferin fractions.

Most of the mustard protein research has been conducted in Canada through Agriculture and Agri-Food Canada (Dr. J. Wanasundera, & Dr. R. Rotimi, Saskatoon) and the University of Toronto (Dr. L. Diosady and graduate students) Of particular note is the interest in mustard proteins by researchers in South East Asia (India, Bangledesh, Pakistan) and the Middle East (Algeria). The interest arises out of a need to find a market for the mustard meals from mustard oil production. The extraction processes used by Indian researchers Das (2011) and Sarkar (2015) are based upon those of Diosady's Canadian laboratory whereas Alireza-Sadeghi (2006, 2009) investigated steam injection, isoelectric precipitation and ultrafiltration as protein recovery methods.

Diosady and colleagues evaluated aqueous or enzyme-assisted aqueous extraction processes (AEPs/EAEPs) for the production of food-grade proteins and industrial oils from dehulled yellow mustard flour. These are technologies where water is used as an extraction medium to extract protein and other soluble

components in oilseeds. The process eliminates the dangers associated with solvent (e.g. hexane) extractions and allows the simultaneous recovery of high-quality protein products and vegetable oils. A two stage alkaline extraction followed by protease assisted demulsification appears to be a viable process (Tabtabaei and Diosady, 2013).

Diosady's laboratory is also investigating ways to efficiently extract the oil from yellow mustard. Vegetable oils are typically extracted with hexane; however there are health and environmental concerns over its use which is prompting the search for alternative solvents. Yellow mustard contains about 30% oil, of which 33-51% is erucic acid, making this oil illegal for human consumption in Europe and North America. The oil has industrial uses as a lubricant and release agent and due to its high erucic acid content could become a feedstock for biodiesel production. Tabtabaei and Diosady (2012, 2014) investigated how the aqueous extraction method could be used to recover yellow mustard oil for industrial applications, specifically as a potential biofuel. From another approach, isopropyl alcohol (IPA) was used in a multistage extraction process to produce an IPA-oil miscella suitable for industrial application; however, issues with water content >5% in the oil and reuse of IPA need to be resolved before it can be considered for commercial application (Sinichi and Diosady, 2012).

No commercial technologies are yet available for protein recovery from *Sinapis alba* or *Brassica juncea* mustard seeds. This could soon change as a Canadian company has indicated it is commercializing a diafiltration/ultrafiltration extraction process developed and patented by Dr. Levanti Diosady of the University of Toronto. The company is currently working through scale-up issues and hopes to have mustard proteins available for sale in the United States , Asia or Latin America by 2018 (Personal communication, 2016).

Burcon Nutrascience developed a mustard protein product using a protein micellar mass (PMM) protein technology similar to that used to produce two canola proteins (Supertein and Puratein). The company was issued a US patent (US 8128974) in 2012 for the extraction of mustard protein. Burcon confirms it has not commercialized the mustard protein (Personal communication, 2016).

Table 9 Protein Extraction Methodologies for *Brassica* spp.

Brassica	Method	Products	Author/Country
Ground yellow mustard seed (*Sinapis alba*-defatted)	Alkali extraction, acid precipitation, ultrafiltration and diafiltration	86% protein enrichment, 71% nitrogen recovery Precipitated protein isolate, Soluble protein isolate Method removes glucosinolates and phytates	Xu et al., 2003 (Diosady's lab, Canada)
Defatted Brassica oil seeds –including mustard seeds	Protein solubilization, separation via diafiltration, isoelectric precipitation	Precipitated protein isolate and soluble protein isolate	Diosady et al, 2002 Patent filed 2002 CA2449007 (Canada)
Dehulled defatted yellow mustard flour	Alkali extraction (pH12), microfiltration, pH lowered to 5.0	Permeate and retentate gave protein products, with high protein enrichment in permeate	Prapakornwiriya and Diosady (2004) (Canada)
Five mustard varieties: 1-*Sinapis alba* 3-*Brassica juncea*-brown, oriental, Ethiopian 1-Brassica carinata	Alkali extraction, acid precipitation, Addition of calcium chloride to the alkali extract generated Ca-soluble protein and Ca-precipitated protein isolates.	Ca-soluble protein Ca-precipitated protein isolates 75% to 85% protein recovery	Aluko and Tosh, 2004 (Canada)
Defatted Brassica	Chromatographic separation of 2S and 11S proteins. Nanofiltration eliminated the major phenolic compounds.	Pilot scale process yielding 200g cruciferin (40%) 42 g napin (18%) and 5g of lipid transfer proteins (LTP) from 3.5 kg defatted meal	Berot et al, 2005 (France)
Brassica juncea-defatted meal	Alkali extraction, activated carbon treatment, heat coagulation by steam injection	95% protein isolate obtained with minimal levels of glucosinolate, phytate or phenolics. Minimal functionality due to heat treatment	Alireza-Sadeghi et al., 2006 (India)
Brassica juncea defatted meal (oriental mustard)	Alkali extraction, membrane filtration, precipitation at pH5	96% protein containing precipitated protein isolate and 72% protein containing soluble protein isolate	Marnoch and Diosady (2008) (Canada)

Brassica juncea	Alkali extraction, centrifuged, ultra/diafiltration, freeze-dried (based upon method of Marnoch & Diosady, 2008)	96.0% mustard protein isolate	Das et al., 2009 (India)
Brassica juncea- defatted meal	Alkali extraction, activated carbon treatment, then 3 methods of further treatment: steam injection; isoelectric precipation and ultrafiltration	Studied effects of 3 methods of protein recovery and removal of antinutritional factors. Steam injection resulted in good yield with removal of ANF but reduced functionalities. Enzyme hydrolysis of steam injected proteins re-established functionality	Alizera-Sadeghi & Bhagya, 2009 (India)
Brassica sp (including mustard seed) Protein Isolation and Fractionation	Low pH extraction (pH3-4) resulted in insoluble cruciferin and soluble napin proteins. Further recovery of cruciferin attained by alkali extraction of solubilized cruciferin or removing cell wall polysaccharides via enzyme –assisted degradation at low pH concentrates cruciferin without solubilization	Separation of cruciferin and napa proteins Napin-rich (90% N based protein) and cruciferin-rich (80-90% fractions)	Wanasundara and McIntosh (2014) (Canada) Canadian Patent 2688464 2014. US Patent 85579632013
Sinapis alba *Brassica juncea*	Protein micelle formation by solubilising seed meal protein in presence of salt, changing the ionic strength and precipitating the proteins with water to produce an amorphous, glutaneous mass.	Mustard seed: protein micellar mass (PMM) with protein content of >90%. Same process as was used to produce Burcon's canola proteins.	Burcon Nutrascience (Canada) US Patent 8128974 Issued 2012
Sinapus alba- dehulled YM flour	Two stage alkaline extraction followed by protease assisted demulsification	Enzyme assisted aqueous extraction gave higher protein yields (83.4%) than the aqueous extraction alone (79.4%)	Tabtabaei & Diosady, 2013 (Canada)
Brassica juncea *Sinapis alba*	Alkali extraction, acid precipitation, and centrifugation (based upon Marnoch & Diosady, 2008)	Protein extraction of 89.13% and 87.76% from black and yellow mustard meal cakes, respectively	Sarkar et al, 2015 (India)

To be useful as a food ingredient, an isolate must have good food functionality, a well-balanced amino acid composition, and acceptable organoleptic properties.

Unfortunately, seeds from the Brassicacea contain several antinutritional and flavour components that tend to bind to the protein and are carried to the isolate. These components include glucosinolates and their toxic breakdown products, and phenolics and phytates which hinder bioavailability of amino acids and minerals. These components are largely responsible for the dark colour and the strong astringent flavour of the products; therefore they must be substantially removed

4.2.3 MUSTARD PROTEIN FUNCTIONALITY

As was the case in 2009, there is still relatively little information in the scientific literature about the functional, nutritional and safety properties of *Brassica* (canola/rapeseed) proteins in general and *Brassica juncea* and *Sinapis alba* proteins in particular.

Compared to the depth of knowledge known about soy and whey proteins (see Appendix 1) a significant body of information will be needed before mustard protein can reach any type of commercial success. Table 10 outlines the functional properties commonly evaluated for proteins.

Table 10 Physicochemical and functional properties of proteins (Sikorski, 2001)

Hydration Properties (Protein-water interactions)	Protein-Protein Interaction Properties	Surface Properties
Water absorption Water retention Wettability Swelling Extrudability Adhesion Dispersability Solubility Viscosity	Precipitation Gelation Texturization Dough forming ability Fiber formation	Surface tension Emulsification (formation and stabilization) Foaming characteristics (aeration/whipping, formation and stabilization) Protein-lipid interactions Lipid and flavour binding

Evaluating protein functionality is quite an extensive undertaking and functional characteristics are dependent upon the protein source, its manufacturing process and the methods of assessment. While it is difficult to compare data available in the literature due to these differences, the data suggest mustard proteins with different functionalities could be developed for very specific applications.

Among the functional properties, nitrogen solubility is probably the most critical as it affects other properties such as emulsification, foaming and gelation. Water and oil absorption is the ability of a meal or protein to absorb and retain water or oil to improve binding, enhance flavour retention, improve mouth feel and reduce moisture and fat losses of food products (Kinsella, 1976; Sreerama et al, 2008). Oil adsorption of a meal relies on the physical entrapment of the oil and acts as a flavour retainer and enhancer of mouthfeel. Emulsifying capacity (EC) and stability (ES) are important functional properties of food proteins. They vary with the type of protein, its concentration, pH, ionic strength and viscosity of the system. Foaming is the ability of the protein to whip and hold its form in specific applications.

To demonstrate how mustard variety and protein processing methods affect functionalities, data from various researchers is provided in Tables 11, 12 and 13.

Aluko et al (2005a) compared the polypeptide profiles and functional characteristics of seven defatted mustard meals (Table 11) (six *Sinapis alba* ascensions and one *Brassica juncea* cultivar) to soy flour to show the differences in functionality between yellow and brown mustards.

Table 11 Protein content and functional properties at pH 7 of defatted seed meals of *Sinapis alba* and *Brassica juncea* compared to soybean flour (Aluko et al, 2005a)

Sample	Protein (%)	Protein (N) solubility (%)	Heat coagulability	Emulsifying Activity Index m/g)	Emulsion Stability (%)	Foaming capacity (%)	Foaming Stability (%)
Sinapis alba (range of 6 ascensions)	40.0-47.16	42.30-55.66	28.95-37.95	29.79-38.61	5.0-30.45	223.20-238.33	45.31-77.97
Brassica juncea	47.52	55.08	43.16	47.30	6.12	249.68	57.58
Soy flour	52.42	67.70	2.48	58.17	58.59	196.72	51.92

The following differences were noted:

- Polypeptide profiles between the yellow and brown mustards differed. *Sinapis alba* had 50kDa and 135kDa polypeptide bands as well as a range of polypeptides in the 29-48 kDa range that were not present in *B. juncea*. A 55kDa band present in both species was of a higher intensity in *S. alba*.

- Meals from the brown mustard had a slightly higher protein content than the yellow seeded varieties
- The *S. alba* varieties had significantly lower emulsifying activity than the *Brassica* sp which may be due to the higher concentration of high molecular weight polypeptide chains in *S.alba*. The lower EAI are indicative of lower emulsion formation ability.
- When acid precipitated and calcium precipitated protein isolates were prepared from defatted mustard meals of *Sinapis alba* and *Brassica juncea* differences were also noted in functionalities (Table 12) (Aluko et al., 2004)
- Calcium prepared protein precipitates had lower protein content than acid prepared precipitates
- Calcium induced protein concentrates from B. juncea and soy bean protein isolates had significantly higher EAI values compared to the calcium induced protein from S.alba.
- Foaming capacity (FC) and foam stability (FC) between the seed varieties either as protein concentrate or meal did not significantly differ indicating that the mechanisms involved in foam formation differ from those of emulsion activity.
- Foams and emulsions stabilized by acid precipitation were relatively more stable than those stabilized by calcium precipitation, with meals producing the most stable foams.

Table 12 Protein content and functional properties of defatted mustard meal, acid-precipitated protein concentrates and calcium-precipitated protein concentrates prepared from the meals (Aluko et al, 2004, 2005a)

Sample	Protein (%)	EAI (m/g)	ES (%)	FC (%)	FS (%)
Mustard Meal					
Yellow mustard (*S. alba*)	47.1	28.0	7.9	226.9	63.3
Oriental-type yellow seed (*B. juncea*)	46.7	51.6	3.9	245.9	61.5
Commercial brown mustard (*B. juncea*)	52.4	52.3	4.1	230.7	60.6
Soybean flour (commercial)	52.4	58.1	58.6	196.7	51.9
Acid-Precipitated Protein Concentrate					
Yellow mustard (*S. alba*)	81.8	21.4	23.8	200.5	37.7
Oriental-type yellow seed (*B. juncea*)	82.0	39.9	38.7	200.5	33.9
Commercial brown mustard (*B. juncea*)	79.3	44.5	47.7	196.7	36.5
Calcium-Precipitated Protein Concentrate					
Yellow mustard (*S. alba*)	75.2	23.7	9.9	189.1	14.0
Oriental-type yellow seed (*B. juncea*)	79.8	40.2	24.5	170.2	13.3
Commercial brown mustard (*B. juncea*)	77.0	34.9	23.9	174.0	13.0
Soybean Protein Isolate					
Soybean protein Isolate (commercial)	87.7	64.9	39.6	253.4	41.8

In another study Aluko et al. (2005b) characterized the calcium-soluble protein fraction and showed this fraction was mainly comprised of cruciferin proteins with some contribution from napins (the major allergenic proteins of *S. alba*). The protein solubility of this fraction from *S. alba* was significantly higher (~95%) at all protein concentrations (5-30%) than calcium-soluble protein from *B. juncea* or soybean concentrates which steadily decreased from 95 to ~80% for *B. juncea* and 85% to ~65% for soybean concentrates as protein concentration increased. It is possible that *S. alba* calcium soluble protein isolates could be used to formulate functional beverages that contain high levels of calcium.

Alizera-Sadeghi and Bhagya (2009) prepared mustard protein isolates (MPI) by steam injection heating and protein hydrolysates from these MPIs by treating with alcalase enzyme. The functionalities of these preparations were compared to the source material, dehulled defatted meal (Table 13). Heat treating by protein coagulation significantly reduced the functional values of the protein isolate compared to the meal and protein hydrolysate. Functionality of the protein hydrolysates was better than the protein isolates. Protein isolates were also prepared by isoelectric precipitation and by ultrafiltration; however, no functionality data was reported for these extracted proteins. While the steam injection heating method successfully reduced the content of isothiocyanates, phytate and phenolics by more than 98%, 90% and 78% respectively, it also reduced the solubility of the protein isolate to 20-40% at the different pHs tested.

Table 13 Functional properties of mustard meal, protein isolate[1] and protein hydrolysates (Alizera-Sadeghi and Bhagya, 2009)

Sample (*Brassica juncea*)	Water absorption capacity (g/100g)	Fat Absorption capacity (ml/100g)	Emulsion capacity (mL/100g)	Foam Capacity (%)	Foam stability at 30 min (%)
Dehulled defatted mustard meal	275	180	62	110	100
Protein isolate	21	90	31	60	50
Protein hydrolysates (DH =9.4)[2]	260	130	47	90	45

[1]Protein prepared by steam injection (93°C); [2]DH: degree of hydrolysis (protein is 9.4% hydrolyzed)

The functionalities of mustard protein isolates prepared according to the method of Alizera-Sedeghi above were evaluated under conditions simulating food formulations by changing the pH, salt (NaCl) and sucrose concentrations. Aider and colleagues (2012) found that the presence of salt enhanced the foaming capacity and that the emulsifying properties were pH and concentration dependent.

Functional properties of protein isolates derived from the patented process of Diosady et al (US Patent 8048463, 2011) have been determined but the information is not in the public domain as it is part of the intellectual property of the technology. The patent identifies the use of isolated mustard protein in processed meat products (2% level), bakery products, nutritional bars, beverages (1% level), infant formula and as a nutritional supplement.

There are only a few examples of mustard protein food applications in the literature. For instance,

- The functionality of oriental mustard meal and derived protein isolates in meat emulsions (wiener and bologna) was found to be similar to that of soy protein isolate in terms of colour, texture and flavor, as ranked by a sensory panel (Marnoch & Diosady, 2006).
- Mustard protein isolate, prepared by steam injection, was used to fortify pasta with increased protein content and protein digestibility (Alireza-Sadeghi & Bhagya, 2008).
- A calcium soluble protein fraction from yellow mustard seed meal has potential application as an additive to prepare calcium-fortified high protein liquid products (Aluko et al, 2005b).

4.2.4 Research Gaps: Mustard Protein

The nutritional, functional, and safety properties of mustard proteins must be investigated prior to commercialization. These are the same issues identified in the Mustard 21 report.

The items identified in the lists below, while not exhaustive, provide some direction for research.

Functional Characteristics

- Characterization of proteins, and understanding protein molecular properties, modes of action and their relation to food functionality
- Structural changes, through genetic manipulations, to improve protein functionality in specific food systems
- Determining the safety, functionality and nutritional value of isolated proteins
- Determining the safety, functionality, health implications and efficacy of hydrolysed proteins (bioactive peptides)
- Modification of proteins, chemically or enzymatically, to improve protein functionality and the underlying impact on the structural characteristics

- Determining the interactions between mustard proteins and other food ingredients components (other proteins, carbohydrates, fibre, minerals, vitamins etc)
- Comprehensive studies comparing properties of competing products (i.e soy protein) with the properties of specific mustard protein fractions.
- Use of proteins in specific applications
- Identification and evaluation of proteins for specific food applications
- Detailed product specifications for all types of protein products developed
- Acceptable sensory characteristics of developed food products

Nutritional Characteristics

- Determining protein quality for human nutrition-amino acid profile (level, distribution and bioavailability)
- Investigating and understanding protein digestibility and bioavailability
- Understanding how mustard proteins will complement or supplement the nutritional value of other protein sources
- Determination of the effect of proteins on the absorption of physiologically beneficial components in the human diet

Processing

- Development and optimization of extraction, isolation and purification procedures for large scale production of mustard proteins, both native and modified
- Optimization of processes that could enhance the functionality of proteins (i.e. extrusion, infrared heating, high pressure processing)
- Identification and isolation of valuable components in the by-product material of the processing extraction of proteins.
- Effect of processing on the non-nutritive bioactives (antinutritional factors) present in mustard protein

Safety Considerations

- Development of food safety and quality programs-hence the importance of working with well established companies
- Microbiological safety
- Allergenicity of mustard proteins
- Presence of organic and non-organic chemical contaminants (i.e. heavy metals, mycotoxins etc)
- Effects of non-nutritive bioactives
- Toxicological safety of ingesting mustard proteins
- Dietary exposure evaluations

- Possible reproductive, teratogenic, and mutagenic studies

Health Benefits

- Identification and evidence to support the nutritional and health benefits associated with mustard protein or bioactive components

4.2.5 Regulatory Approvals of Brassica proteins

Since the Mustard 21 report of 2009, there have been regulatory approvals of *Brassica* proteins, specifically *Brassica napus* (canola). Under a commercialization agreement with Archer Daniels Midland, ADM has attained GRAS status for Burcon's two canola protein isolates (GRAS 327, 2010) for its cruciferin-rich (Puratein) and napin rich (Supertein) canola/rapeseed protein isolates. The intended use for these proteins is in dairy products, grain products, fruit and vegetable juices and beverages, salad dressings, meal replacements and nutritional bars. Production of the proteins is targeted for late 2016.

BioExx (while still operating in Canada) obtained GRAS approval (GRAS 386, 2011) for its canola protein isolate and hydrolyzed protein isolate. The company, unfortunately, is no longer operating in Canada. However, under a new company name and now operating in Europe, TeuTexx acquired European Food Safety Authority (EFSA) approval for the BioExx "Isolexx" protein (EFSA, 2013) for use in Europe. These proteins are derived from non-GMO canola varieties (*B. napus*)

The intended use of "Isolexx" proteins are as a replacement for soy protein isolate in meal replacements, protein drinks, nutrition bars, soups and soup mixes, breakfast cereals, plant protein products (e.g. meat analogues); for improving the texture of bakery products, chilled or frozen processed meat products, pasta, desserts and other foods or in food supplements. It is not intended for use in infant formulae. The products has a relatively high protein quality value (PDCAAS ranging from 0.92 to 1.0) similar to that of soy and casein. Lysine is the main limiting amino acid in contrast to soy which is relatively high in lysine.

The question remains as to whether mustard proteins for human consumption will require regulatory approval in Canada or the United States. Unlike canola, mustard is not genetically modified and is considered an "ancient" food having been consumed as whole seed and flour for many centuries. It is possible a case could be made to the regulatory authorities that mustard proteins could be grandfathered under the Canadian Novel Food Regulations, similar to pea and wheat proteins. However, pea and wheat proteins were already being used in the

food industry prior to the implementation of the Novel Food Regulations (1994). The introduction of mustard protein concentrates or isolates into the Canadian food system could be deemed “novel”. If mustard proteins are to be sold in Canada, it is recommended Health Canada be consulted for further guidance. A similar consultation with the United States Food & Drug Administration (FDA) might be needed to determine if mustard proteins would be considered GRAS (generally recognized as safe) for sale into the United States.

A number of factors to be considered when commercializing mustard fractions include the following:

- A company willing to champion the development, sale and marketing of a new plant protein
- Availability of raw material to support both a condiment and niche fraction market
- Fractionation and markets for co-products as production of protein concentrates and isolates will require a commercial use for mustard oils (human food or industrial) and for any bioactives isolated and recovered from extraction processes (e.g. phytate, glucosinolate, phenolics)
- Development of economical extraction techniques that minimize antinutritional factors in mustard meal, protein products or mucilage products
- Cost competitiveness with commercial products
- Market volumes
- Production to market mechanisms
- Regulatory approvals for animal feed and human food use, if required, will require significant effort (money, time, research, personnel)
- Product safety: toxicology and allergenicity issues associated with *Brassica* proteins

4.3 HYDROCOLLOIDS

Similar to the scenario in 2009, this updated review of the mustard literature clearly indicates that yellow mustard mucilage is still being investigated for its functional properties as a hydrocolloid with very little attention paid to its role, or mustard brans' role, as a potential dietary fibre. Yellow mustard mucilage is a food polysaccharide with characteristics very similar to xanthan gum, a microbial-derived hydrocolloid.

Of all the food polysaccharides, starch-derived products dominant the world market and are the chief source of food carbohydrate. Other polysaccharides, also known as non-starch polysaccharides (NSP), are being used in increasing quantities, although usually at lower levels of incorporation. The NSPs play important roles in food product functionality and human nutrition (i.e. as dietary fibres). Water soluble polysaccharides such as starch, galactomannans and xanthan gum, are commonly called hydrocolloids. Hydrocolloids are incorporated into foods for the emulsifying, gelling, stabilizing, and thickening properties.

4.3.1 HYDROCOLLOID MARKET OVERVIEW

A brief overview of the hydrocolloid market is provided here.

BCC Research[13] in a 2016 market report estimated the global hydrocolloid market was valued at $6.6 billion in 2015 (1.66 million metric tons). Growing at an estimated five year (2012-2020) compound annual growth rate (CAGR) of 4.4%, the market could reach 2.1 million MT in 2020 with a value of almost $8.2 billion.

Growth was expected across Europe, the Asia-Pacific and North American regions due to the increasing demand for hydrocolloids in the end-user industries such as food & beverage, drilling, pharmaceutical, nutraceutical, cosmetic, textile printing and paper treatment.

The growth in the food and beverage industry is primarily due to increased consumer awareness of health, diet and nutrition and the desire for natural products.

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[13] BCC Research. 2016 Hydrocolloids: Technologies and Global markets http://www.bccresearch.com/market-research/advanced-materials/hydrocolloidS-tech-markets-report-avm131a.html Accessed June 2016

The hydrocolloid market (except starch) is broadly divided into five major sources: plant, seaweed, microbial, animal and synthetic hydrocolloids (as listed in Table 14).

Of the hydrocolloids being used in the market place, three are projected to have high growth in the next five years: a) gelatin, derived from bones and skin of swine and cattle, accounts for 99% of the animal hydrocolloid market and its use will continue to grow (CAGR 5.2%) b) guar gum, a plant hydrocolloid, continues to be fastest growing hydrocolloid across all market segments (CAGR 5.3%) and c) xanthan gum accounts for over 90% of the overall microbial hydrocolloid market.

Outside the food industry, xanthan gum is also widely used in industrial applications such as oil field drilling, agrochemicals formulations, toothpastes, cosmetics, toilet cleaners, ceramics, paints, and construction materials.

Table14 Examples of Common Hydrocolloids (Stephen et al, 2006)

Polysaccharide Source				
Animal	Microbial	Plant	Seaweed	Synthetic
Glycosamino glycans: Chitin, chitosan Chondroitin Hyaluronate	Curdlan Gellan Pullulan Xanthan	Arabinoxylans β-glucans Cellulose Galactomannans Oligosaccharides Pectin Starch Modified starches Xyloglucans Inulins Gum arabic Gum tragacanth	Agar Alginates Carrageenan Ulvan	Chemically modified such as sodium alginate and derivatives of other hydrocolloids

The leading players in the food hydrocolloids market include CP Kelco (U.S.), Cargill Inc. (U.S.), Ashland Inc. (U.S.), Kerry Group (Ireland), Cargill (U.S.) DuPont (U.S.), and Royal DSM (The Netherlands). These market players have been focusing on expansion of new facilities and launching new products.

4.3.2 YELLOW MUSTARD MUCILAGE

The following information is extracted from the 2009 Mustard 21 report[14] to provide background information on yellow mustard mucilage research. New information will be identified when incorporated into this section.

The majority of work on yellow mustard mucilage has been conducted by Dr. Steven Cui at Agriculture and Agri-Food Canada's Guelph Research & Development Centre and Dr. Michael Eskin, University of Manitoba.

*Extract*_____

All mustard seeds contain mucilage but only yellow mustard is significant because of its high yield and functionality. *Sinapis alba* contains about 5% mucilage compared to less than 1% in the brown, oriental or black mustards.

The crude mucilage of *Sinapis alba* is approximately 5% of the total seed weight and contains 80-94% carbohydrates mainly comprised of glucose (22-35%), galactose (11-15%), mannose (6.0- 6.4%), rhamnose, (1.6 – 4.0%) arabinose (2.8-3.2%) and xylose (1.8% to 2.0%) (Cui et al, 2006).

Figure 11 briefly outlines the types of commercial mustard products available for the food and non-food sectors. It also shows the optimum extraction conditions for yellow mustard mucilage extraction from bran based upon the Canadian patent CA 2270750 (US patent 6,194,016, 2001)(Cui et al, 2003).

Update: Dr. Cui's patent is based upon extraction of mucilage from mustard bran unlike that of Sharafabadi (1990, 2005) which was from whole seed. A patent search in 2016 noted that both Sharafabadi 's patents have been abandoned.

Based upon the extensive research conducted by Dr. Cui and colleagues since the early 1990's, yellow mustard mucilage has been characterized and many of its unique functionalities explored and compared to other hydrocolloids. This has been accomplished through fractionation and identification of the polysaccharide components contributing to its functionality.

[14] Mustard 21. 2009. An evaluation of the potential for value addition to mustard protein and mucilage. Extracted with permission.

Figure 11 Yellow Mustard Products

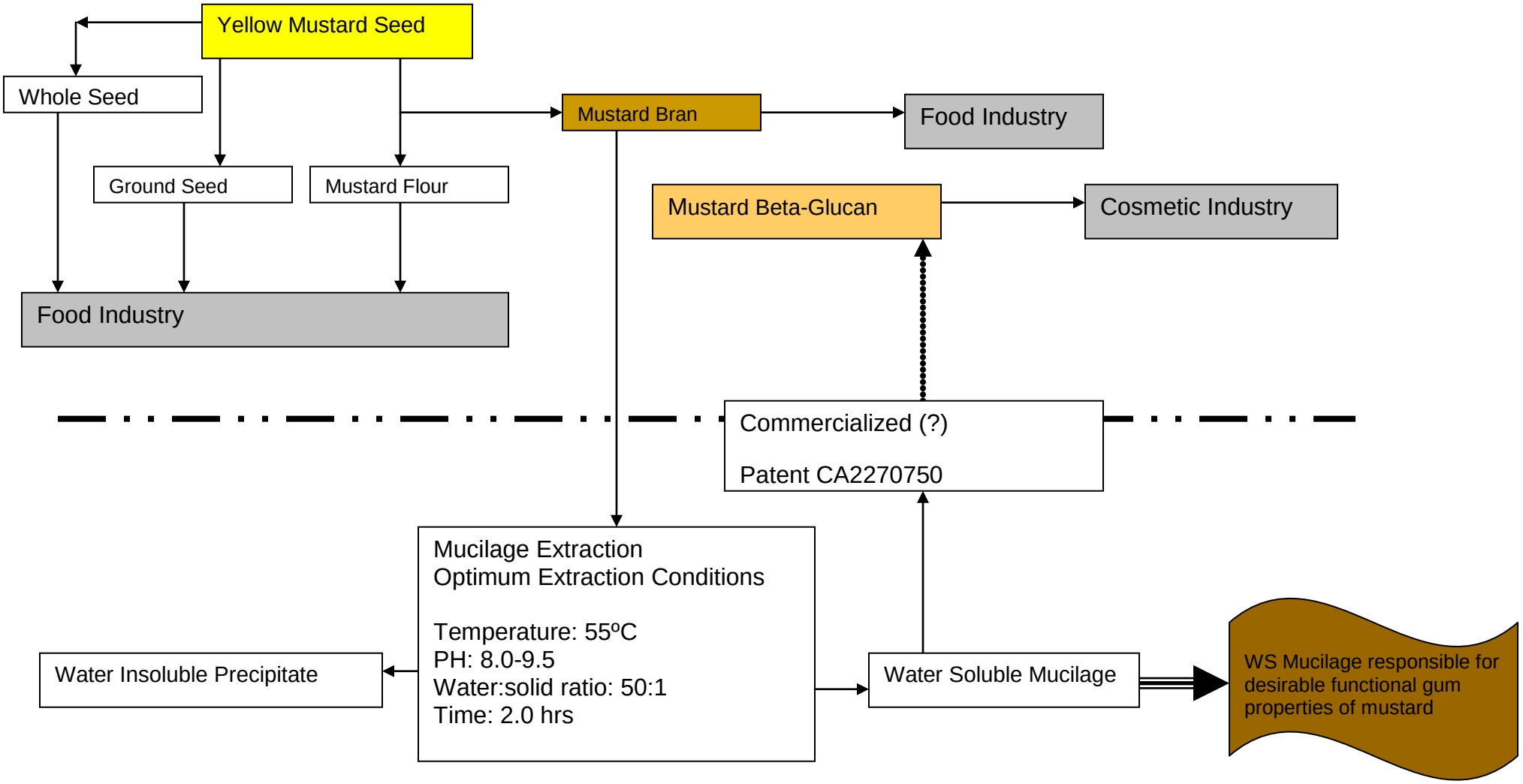
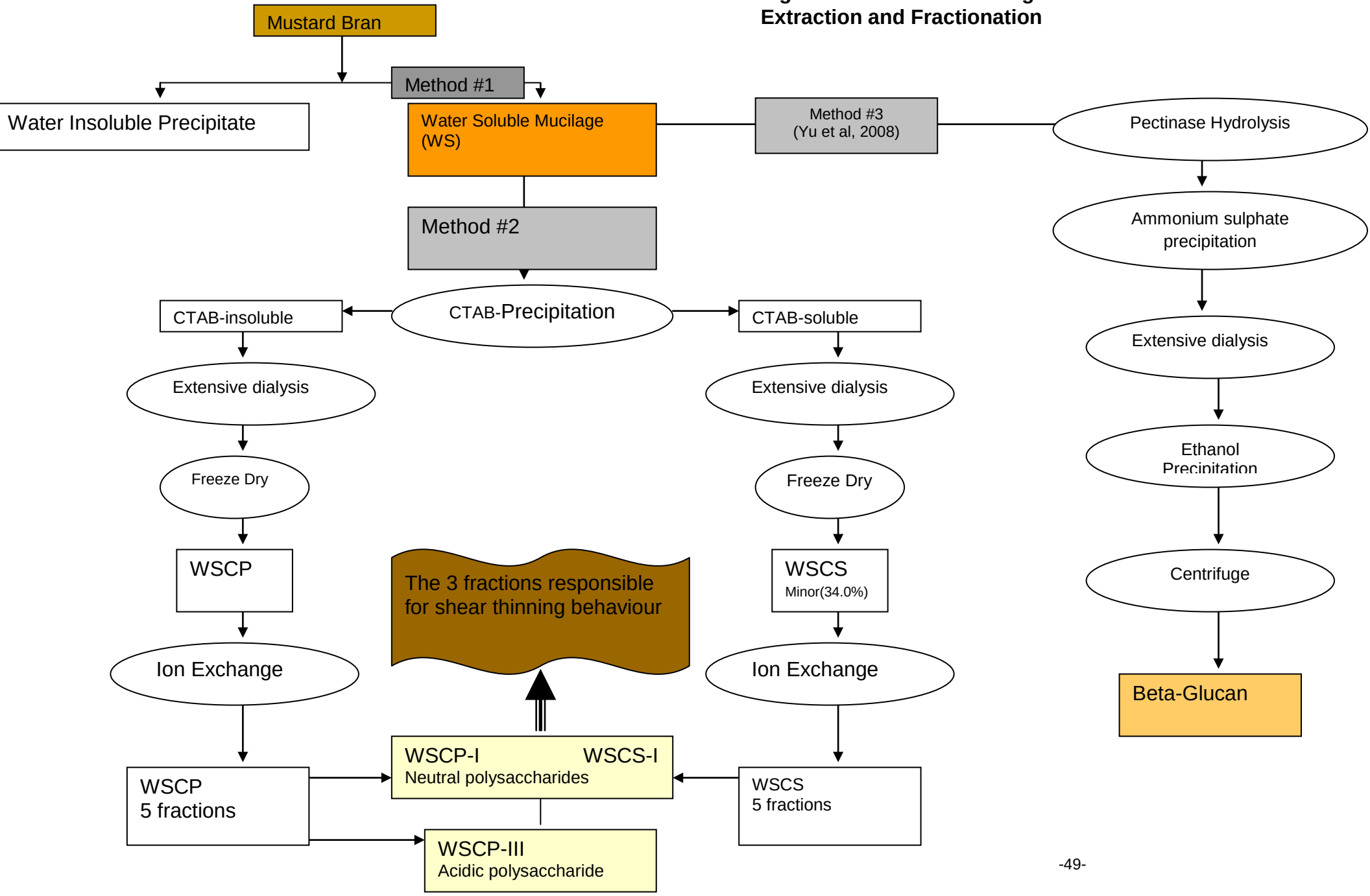


Figure 12 outlines extraction and fractionation processes. Method #1 is the patented aqueous extraction method from yellow mustard bran that yielded a water-soluble yellow mustard mucilage (WS-YMM) fraction (55.6%) and a water insoluble fraction (WI-YMM) (38.8%). Approximately 30-40% of the bran can be extracted as gum although rheological properties vary with extraction conditions. The resulting water soluble YMM interacts with galactomannans improving the viscosity and gel structure for use in products applicable to cosmetic and skin formulations.

Yellow mustard mucilage offers some unique rheological properties in aqueous systems and excellent emulsion capacity and stability in oil/water systems making it commercially viable as a hydrocolloid gum for food and non-food applications. The basic hydrocolloid functionalities of yellow mustard mucilage have been identified as:

- Emulsifying capacity and stability: Water soluble yellow mustard mucilage ranked third, after fenugreek gum and methylcellulose, for its emulsion capacity and stability. Its capacity surpassed gum arabic, pectin, microcrystalline cellulose, xanthan, oat, guar, locust bean gum, carrageenan and gellan (Cui et al., 2006)
- WS mucilage and WI mucilage exhibit greater emulsion capacity, but lesser foam stability than xanthan, guar, and arabic gums.
- Reduces surface tension
- Shear thinning behaviour exhibited by yellow mustard mucilage and its fractions resemble those of xanthan gum dispersions at all concentrations, but not guar gum (Cui et al, 1993)
- Synergistically interacts with galactomannans (e.g. locust bean gum, guar gum)

Figure 12 Mustard Mucilage R&D: Extraction and Fractionation



Further fractionation of the water soluble fraction by cationic detergent precipitation (CTAB) (Figure 12, Method #2) resulted in a precipitate consisting mainly of a CTAB insoluble precipitate (WSCP-52%) consisting of pectic polysaccharides (galacturonic acid, galactose, rhamnose) and a fraction predominantly composed of 1,4-linked B-glucose polymers (Cui et al, 1993b).The CTAP soluble fraction (WSCS-34%) was also comprised of 2 polysaccharide fractions consisting primarily of the neutral polysaccharide (1,4-linked β-D-glucose) and 12.5% uronic acid as glucuronic acid. It did not contain any rhamnose nor pectic-like substances.

The β-glucan backbone is the major component responsible for the shear thinning behaviour of yellow mustard polysaccharides. The 1,4-linked β-glucan backbone shares a similar backbone to xanthan gum (Cui et al, 2006).

Functionally, WSCP appeared to be more elastic that WSCS but both fractions contributed to viscoelastic character of WS mucilage. The viscoelasticity was typical of a weak gel (complex viscosity higher than apparent viscosity).

However, differences were noted between the two fractions with respect to the effect of temperature, pH, sucrose and salt concentration on apparent viscosity. This was attributed to differences in molecular size distribution, chemical composition and linkage positions.

Ion exchange chromatography was used to further fractionate the WSCP and WSCS (continuation of Method 3). Three of the resulting ten fractions (WSCP-I, WSCP-III and WSCS-I) were found to be responsible for the shear thinning behaviour of YMM. Shear thinning ability was more pronounced in the WSCP-I fraction, than in the other two fractions. The WSCS-1 fraction was comprised primarily of 1,4-linked β-D-glucose.

Cui’s research group involving Agriculture and Agri-Food Canada (Guelph), University of Guelph and University of Manitoba evaluated the use of the enzyme pectinase followed by ammonium sulphate precipitation (Figure 12, Method #3) as a more economical way to remove the pectic-like polysaccharides and produce a β-glucan backbone chain rich water-soluble fraction (Wu et al, 2009). This non-pectin polysaccharide (NPP) exhibited strong shear thinning flow behaviour and a weak gel structure which became stronger under acidic conditions. It also showed thermal stability over the temperature range of 5 to 90°C.

4.3.3 YELLOW MUSTARD MUCILAGE INTERACTIONS

Hydrocolloids are only one very small part of a food matrix and yet can have pronounced effect on other ingredients resulting in changes to functional characteristics and quality attributes of a food. Thus it is critical to determine the interactions of yellow mustard mucilage with other hydrocolloids, including starch.

Research shows that the synergistic interactions between YMM and galactomannans (locust bean gum (LBG), guar gum (GG) and fenugreek gum (FG) are influenced by the mannose/galactose (M:G) ratio in galactomannans.

Greater interactions were seen for LBG/YMM than guar/YMM which were greater than fenugreek/YMM. These galactomannans have a 4:1, 2:1 and 1:1 ratio of mannose to galactose to respectively, indicating that the M:G ratios of the galactomannans contribute to the increased synergism with YMM. These characteristics are similar to the interactions between galactomannans and xanthan. The (1,4)-linked β-glucosidic backbone chain is the active component of YMM responsible for the synergistic interaction with galactomannans.

Interactions have been investigated for buckwheat starch and pea starch (Liu et al, 2006), native and acylated pea starch (Liu & Eskin, 1998), and wheat and rice starches (Liu et al, 2003). YMM extracted from yellow mustard bran via the patented method of Cui *et al.* (2003) affected the functional and rheological properties of the studied starches in the following manner:

- Interactions between YMM/LBG and native and acylated pea starches demonstrated increased starch paste viscosity and altered degrees of pseudoplasticity of the starch pastes.
- Addition of YMM and YMM/LBG mixtures to wheat and rice starches increased the viscosity
- Addition of YMM to wheat, rice and buckwheat starches resulted in gels with increased hardness, adhesiveness, and chewiness. The YMM/LBG mixture decreased gel hardness.
- Presence of YMM decreases the swelling power of wheat, rice, buckwheat and peas, but also decreased the degree of syneresis in resulting gels.

These types of studies provide indications of how YMM may react in food matrices but more research is needed to determine how a food matrix is affected.

Food products prepared with a 9:1 ratio of LBG/YMM mixture at very low total gum concentrations showed excellent stability and rheological properties compared with commercial products containing xanthan gum with or without alginates. Building upon the interactions between LBG and YMM, Cui and his

colleagues have found that the addition of a small amount of LBG to commercial yellow mustard powders or yellow mustard flours significantly enhanced the gel strength of prepared meat emulsions after processing (Cui et al., 2006).

End of Mustard 21 Report Extract

New information added

Commercialization of YMM

Yellow mustard mucilage is produced from mustard bran, a by-product of mustard flour production. In the early 2000s, Natunola Health had commercialized a patented process (Cui et al, 2003, Patent Canada 2270750) to produce a mucilage extract. The glucan based preparation, however, did not have regulatory approval as a food ingredient and could only be used for non-food applications.

The mustard mucilage extract was sold into the cosmetic industry for use as emollient, film former, skin conditioner and viscosity control agents for aqueous systems. Products applications included anti-aging formulations, creams, lotions, liquid soap, self-tanning solutions, and sunscreen formulations.

However, the company is no longer producing YMM due to a limited market and competition from oat mucilage (Natunola Health, personal communication, 2016).

Continuation of Research into Synergistic Interactions

Investigations by Cui and colleagues on characterizing the synergistic interactions between the non-pectic polysaccharide (NPP) fraction from water soluble yellow mustard mucilage have continued since 2006. Using the pectinase/ammonium sulphate extraction method, the isolated NPP fraction was blended with four types of galactomannans (fenugreek gum, guar gum, tara gum and locust bean gum) to study how the blending ratio, mannose/galactose ratio, total polysaccharide concentration and pH affected synergistic interactions to determine mechanisms of action. The strongest synergies occurred at a total polysaccharide concentration of 0.5% and a GM/NPP blending ratio of 3/7, at neutral pH (6.5) (Wu et al., 2009b).

Detailed structural information on this NPP was obtained through nuclear magnetic resonance (NMR). NPP is comprised of a β-1,4 linked glucose backbone, with β-mannose, 1,6 linked to the backbone chain and the β-galactose 1,2 linked to the glycosidic backbone chain. The methyl groups were substituted

to the 2,3,6 positions of the glucose residues and the 2,3, positions of mannose and galactose residues (Wu et al., 2011a). Further structure studies using stress relaxation tests showed how the different galactomannans interacted with the yellow mucilage NPP (Wu et al, 2011b)

The previous studies have all examined the chemical, structural and rheological properties of yellow mustard mucilage and its various fractions, but not its emulsifying properties. The emulsifying properties (stability, oil distribution, zeta-potential and freeze thaw stability) of the water soluble mucilage (WSM) in oil-in-water emulsions was compared to gum Arabic and citrus pectin, two commercial gums used by the food industry. Providing technical information about these properties to the food industry may support the potential of WSM as a novel ingredient for food emulsions. Of the three gums tested, WSM exhibited the best emulsion stability and the highest surface activity under the conditions tested. WSM showed the best freeze-thaw stability indicating it would be a good ingredient for frozen food products.

Further research is needed to study interactions in specific food products and to better understand the synergistic interactions with other food components (proteins, polysaccharides) on its emulsifying properties (Wu et al, 2015)

To further strengthen YMM as a food ingredient, the antioxidant property of water soluble yellow mucilage was explored and compared to the antioxidant properties of pectin and xanthan gum. The researchers found that while the overall antioxidant activity of the 3 polysaccharides was lower than for ascorbic acid and BHA, WSM exhibited the strongest antioxidant properties followed by citrus pectin and xanthan gum. Further studies would be needed to investigate the possible applications of WSM in food for food quality, product shelf life and replacement of synthetic antioxidants in food products (Wu et al., 2016).

In the Mustard 21 Report, a summary of Xanthan gum was provided as YMM has functional properties very similar to this microbial hydrocolloid. Information from that report has been attached as Appendix 2.

4.3.4 RESEARCH GAPS: YELLOW MUSTARD MUCILAGE

Similar to the investigation into mustard proteins, research is still needed to ensure yellow mustard mucilage can meet both industry, regulatory and consumer needs. However, one factor is critical to its success. YMM is one small component of mustard bran and its extraction, concentration and purification, while a value added product, will require commercial markets for the other mustard co-products.

The following list provides some direction for future research.

Functional Characteristics

- A better understanding of the mucilage molecular properties, modes of action and relationship to food functionality
- The water soluble mustard mucilage has seen extensive research. Are there opportunities for the water insoluble fractions?
- Effects of chemical or enzymatic modification of YMM and its impact on other ingredients in food systems
- Determining the interactions between YMM and other food ingredients components (other proteins, carbohydrates, fibre, minerals, vitamins etc)
- More comprehensive studies comparing properties of xanthan in food systems (or non-food systems) to YMM
- Use of YMM in specific food applications
- Detailed specifications for all types of YMM products developed
- Acceptable sensory characteristics of developed food products

Nutritional Characteristics and Health Benefits

- Determining opportunities for mustard bran or YMM to be used as dietary fibre including usage levels for functionality versus physiological benefits
- Establishing a physiological effect for its use as a dietary fibre, as defined by Health Canada and US FDA.
- Investigating the bioactive properties of yellow mustard mucilage

Processing

- Development and optimization of extraction, isolation and purification procedures for large scale production of mustard mucilage, both native and modified
- Optimization of processes that could enhance the functionality of proteins
- Development of food safety and quality programs

Safety Considerations

- Microbiological safety
- Presence of organic and non-organic chemical contaminants (i.e. heavy metals, mycotoxins etc)

- Dietary exposure evaluations
- Allergenicity: ensure the YMM does not contain the allergenic protein

4.4 Bioactive Compounds in Mustard

Mustard contains two phytochemicals of great interest to the food, non-food, and pharmaceutical industries: glucosinolates and phenolics.

4.4.1 Glucosinolates

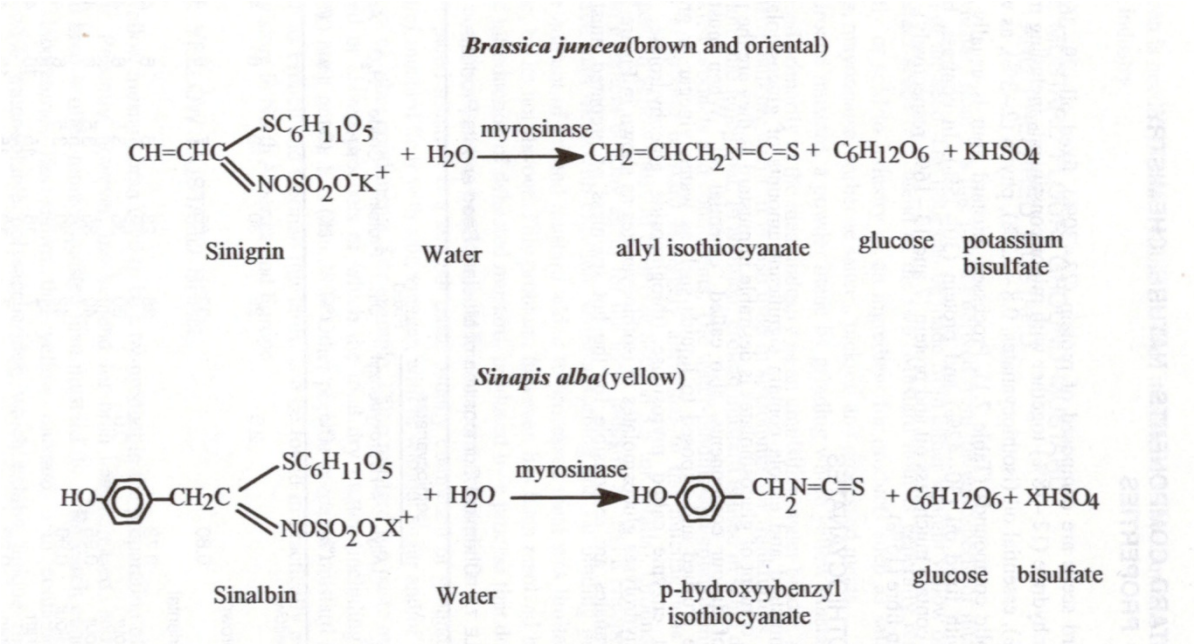
Glucosinolates (GLS) are a family of about 120 sulphur-containing secondary plant metabolites. Glucosinolates of most importance to animal and human health are produced mainly by plants in the family *Brassicaceae* and include rapeseed, cabbage, cauliflower, Brussels sprouts, turnip, radishes, mustard seed and horse radish (Fahey et al., 2001). Upon disruption of plant tissues the enzyme myrosinase (thioglucoside glucohydrolase 3.2.3.1) is released and catalyzes the hydrolysis of glucosinolates to a variety of bioactive compounds including isothiocyanates (ITC), thiocyanates, nitriles, oxazolidine-2-thiones and indole products. While GLS themselves are not toxic; their hydrolytic products are.

Increased consumption of cruciferous vegetables has been associated with a reduced risk of lung, stomach, colorectal, breast, bladder and prostate cancer as well as a reduced risk of myocardial infarction (Traka et al., 2009; Walley and Buchanan-Wallaston, 2011). It was thought the main health concern due to the presence of these compounds in foods was the potential for increase in goitre (enlargement of the thyroid gland) as the thiocyanate ion products of ITCs and oxazolidine-2-thiones degradation have been shown to have effects on the thyroid gland in animals and humans. However, there does not appear to be any evidence to support a causative role for dietary glucosinolates in human goitre (Heaney and Fenwick, 1995).

The main glucosinolate in *Brassica juncea* (brown/oriental mustard) is **sinigrin** which when hydrolyzed by myrosinase yields allyl isothiocyanate (AITC), which is important for flavour and pungency. Hydrolysis of the glucosinolate **sinalbin** in yellow/white mustard (*Sinapis alba*) yields para–hydroxybenzyl isothiocyanate (p-HBITC) (Figure 13).

Glucosinolates and isothiocyanates have been linked to anticancer, antibacterial and antifungal activity. Apart from isothiocyantes, mustard seeds produce other metabolites with antimicrobial and antioxidant properties such as phenolic acids and phytin (Cui and Eskin, 1998).

Figure 13 Mechanism of enzymatic hydrolysis of mustard glucosinolates (Cui & Eskin, 1998)



Both solvent extraction from natural plant sources and chemical synthetic procedures are used in the commercial production of allyl isothiocyanate (AITC). Historically, AITC has been extracted from the dried seeds of *Brassica nigra* (black mustard) for various industrial and therapeutic applications (Merck, 2006). Before being extracted, AITC is liberated from the glucosinolate sinigrin through reaction with myrosinase (Romanowski, 2000). Chemical synthetic methods for AITC production from allyl iodide and potassium thiocyanate were published in the 1920s and variants of this process currently remain in use (Fan, 2012). However, the use of pure AITC has limitations because of the strong pungent smell associated with volatile AITC which causes eye and nasal irritation and a burning sensation of the skin and tongue.

Cost of glucosinolates

The SMDC board requested information on the cost of pure glucosinolates and isothiocyanates. Examples of two chemical supply companies—Sigma-Aldrich (Canadian pricing) and C2-BioEngineering (Euro pricing) -- providing pure sources of these compounds are provided in Table 15.

Table 15 Cost comparisons of purified glucosinolates and respective isothiocyanates from chemical supply companies.

Glucosinolate	Isothiocyanate	Source	Amount	Cost
Sinigrin (hydrate)-from horse radish		Sigma-Aldrich	500 mg	$CAD 629.00
Sinigrin (analytical standard)		Sigma-Aldrich	10 mg	$CAD 127.50
Sinigrin (standard-natural source, not identified)		C2 Bioengineering	500 mg	€268 ($CAD 390.00)
	Allyl isothiocyanate (95% purity)-synthetic	Sigma -Aldrich	500 g	$CAD 152.00
	Allyl isothiocyanate (analytical standard-synthetic)	Sigma-Aldrich	1 g	$CAD 65.60
Sinalbin (standard-natural source, not identified)		C2-Bioengineering	500 mg	€711 ($CAD1035.00)
	Benzyl isothiocyanate (98% purity) (benzyl mustard oil)*	Sigma-Aldrich	25g	$CAD 132.50
Rapeseed (total glucosinolates-high level standard)		Sigma-Aldrich	20 g	$CAN 317.00
Rapeseed (total glucosinolates-mixture from *Brassica napus*)		C2-Bioengineering	500 mg	€1017 ($CAD1481.00)

*Note: the hydroxybenzyl isothiocyanate that is the glucosinolate breakdown product is not available commercially. This is a methylbenzyl isothiocyanate.

4.4.1.1 Antimicrobial Activity of Glucosinolates and ITC

There is growing interest in the use of natural plant products as antimicrobials to reduce the use of conventional antimicrobial preservative compounds such as sorbates, benzoates, organic acids or combinations in food preservation and possibly to overcome the emergence of antibiotic resistance in bacterial pathogens. Increasing negative consumer perception of synthetic food additives and growing interest in finding natural alternatives to the synthetic ones as well as the movement toward “clean labels” whereby all ingredients are natural and pronounceable is also driving the research into natural antimicrobial agents.

A number of factors must be taken into considerations when developing natural antimicrobials for use in foods (Davidson et al, 2013):

a) Keep the form of the antimicrobial as close to the natural source as possible (i.e. mustard meal (or extract) versus a purified form allyl isothiocyanate). The more purified an antimicrobial, the higher the likelihood it would be considered a food additive.
b) Regulatory authorities have oversight of antimicrobials added to foods and the safety of any antimicrobial must be considered.
c) Acquiring toxicology data is expensive and is the reason there is a lack of data on purified natural antimicrobials.
d) Development of activity validation methods as currently there are no approved methods for determining food antimicrobial activity.
e) Determination of the potential for resistance development, influence of biofilms on activity and effect of antimicrobials on natural microflora.
f) Effect of the antimicrobial on sensory characteristics of the food
g) Effect of the natural antimicrobial in a food matrix as the food components (protein, fat, fiber, cations etc), pH and form (liquid or solid) may inhibit its activity.
h) Evaluate stability of antimicrobials during processing and storage
i) Developing methods to enhance antimicrobial efficacy (liquid, vapour, encapsulation, incorporation into packaging, using multiple food safety hurdles etc)

Isothiocyanates, and particularly allyl isothiocyanate (AITC), have been widely studied as antimicrobial agents and their antimicrobial spectrum is wide inhibiting both Gram-positive (*Listeria monocytogenes* and *Staphylococcus aureus*) and Gram-negative bacteria (*Cytophaga*, *Escherichia coli* O157:H7, *Pseudomonas corrugata*, *Salmonella* Montevide, *Salmonella* typhimurium, and *Serratia grimesii*), yeast (*Endomyces fibuliger*), and fungi (*A. flavus*, *Aspergillus niger*, *B. cinerea*, *Cladosporium cladosporioides*, *Penicillium citrinum*, *Penicillium commune*, *Penicillium expansum*, and *Penicillium roquefort*) at concentrations in the nanograms per liter range in vapor phase and in liquid media (Davidson et al., 2013). Although the effectiveness of allyl isothiocyanates varies among bacteria, Gram-positive bacteria are more resistant than Gram-negative bacteria (Obaidat & Frank, 2009; Schirmer & Langsrud, 2010). AITC is antimicrobial in both liquid and gas forms, but gaseous AITC has greater antimicrobial potency (Delaquis and Sholberg, 1997).

In Canada, Dr. Rick Holley (University of Manitoba) has been working on the antimicrobial effects and mechanisms of action of glucosinolates (allyl isothiocyanate and ρ-hydroxybenzyl isothiocyanate) and mustard components on food spoilage and pathogenic microorganisms for more than fifteen years. Graduate students in his laboratory have extensively studied the effects of pure mustard components and mustard flours on the survival of a significant food pathogen, *Escherichia coli* 0157:H7 inoculated in ground beef, dry hams and in dry, fermented sausages as well as other

food pathogens (*Listeria monocytogenes* and *Campylobacter jejuni*) in comminuted meat products and in chicken. Various themes have been explored including determining effective doses, exposure times, mechanisms of delivery (microencapsulation, edible films, antimicrobial films), endogenous formation of the ITC from the glucosinolate by microorganisms, and mechanisms of action of the isothiocyanate. Both AITC and the yellow mustard isothiocyanate, p-HBITC have been investigated.

Why the emphasis on *Escherichia coli* 0157:H7? The incidence of this pathogenic organism has increased significantly in recent years and its presence in finished food products is considered a Health Risk Category 1 concern by Health Canada. It causes life threatening complications such as hemorrhagic colitis in children, the elderly and the immune-compromised and only a few cells are required for infection. Food-borne outbreaks of *E.coli* 0157:H7 have been attributed to contamination of improperly cooked ground beef and dry fermented sausages. Canada and the United States require a validated 5 log reduction in the number of *E.coli* 0157:H7 in dry and semi-dry sausages as this organism can survive traditional fermentation conditions (CFIA, 2016). While the regulatory authorities have provided processing options to achieve this target (e.g. heat) food processors are looking at alternative manufacturing processes to preserve the physical and sensory characteristics of this fermented product which is traditionally consumed uncooked.

A 5-log reduction means lowering the number of microorganisms by 100,000 fold. Thus if there are 100,000 pathogenic bacteria present in a food, a 5-log reduction would reduce the number of microorganisms to one.
In terms of numbers: 1 log =10 organisms or 10^1; 2 log=100 organisms or 10^2 organisms and 6-log CFU/g is equal to 1,000,000 (1 million) or 10^6 microorganisms
A colony forming unit per gram food (CFU/g) represents a mass of bacteria or yeast growing together.

The overall focus of Holley's research program has been to investigate non-thermal processes to eliminate *E.coli* 0157:H7 and other food-borne pathogenic microorganisms using natural antimicrobial agents; thus the addition of the antimicrobial agent allyl isothiocyanate (AITC) or mustard preparations containing the ITCs to meat and poultry products has been studied. Summaries of some of this research are provided in Table 16. The testing has been done on a cocktail of 5 non-virulent strains of *Escherichia coli* 0157:H7.

Table 16 Effect of AITC and deheated mustard flours on *E.coli* 0157:H7 in meat systems

Purpose	Results	Reference
Studied effect of a) volatile AITC on filter paper insert in packaging and b) deheated (cold) mustard flour and non-deheated (hot) mustard flour added at 10% and 20% to refrigerated ground beef inoculated with a five strain cocktail of *E.coli* 0157:H7 (at 3 log CFU/g) and compared with the effects of pure AITC at 1300 ppm.	In ground beef: • AITC at 1300 ppm eliminated 3 log cfu/g *E.coli* 0157:H7 by day 15; reduced >4.5 log *E.coli* 0157:H7 at 25d storage at 3°C at the 6-log microbial concentration. • Deheated mustard at 10% or 20% did not eliminate *E.coli* 0157:H7, a 3 log cfu/g reduction in 18d at 4°C. • Non-deheated mustard at 20% completely eliminated pathogen at D3 whereas 10% content and the pure AITC took 18d and 15d, respectively	Muthukumarasamy et al, 2003, 2004
To test the antimicrobial potential of AITC on a filter paper disk packaged with ground beef on viability of five strain cocktail of *Escherichia coli* 0157:H7.	• AITC impregnated on filter disc eliminated *E.coli* 0157:H7 at 3 log inoculum level after 18d at 4°C or 10d at -18°C • Samples with 6 log inoculums content: a >3 log reduction noted after 21d at 4°C, 1 log reduction after 8 d at 10°C and 35d at -18°C	Nadarajah et al, 2005a.
To determine effect of addition of 5, 10 and 20% non-deheated (hot) mustard flour to ground beef patties stored at 4°C under nitrogen for 21 days on viability of *E.coli* 0157:H7 and to evaluate sensory acceptance of cooked ground beef patties	• By day 21 of storage, reduction of 0.5, 3 and 5.4 $\log_{10}$ reduction in number of *E.coli* 0157:H7 from initial levels of 6 log cfu/g in meat containing, 5%, 10% and 20% mustard flour, respectively. • *E.coli* 0157:H7 at a 3 log inoculums level was eliminated by day 18, 12 and 3 days with 5%, 10% and 20% mustard flour respectively • Studies show that AITC use at 20% levels completely eliminated *E.coli* 0157:H7 from ground beef but generated a strong odour not acceptable to consumers. Possible to use 5%-10%; AITC noticed but still consumer acceptance.	Nadarajah et al., 2005b
Dry cured hams (Wesphalian ham) inoculated with 7.5 log cfu/g *E.coli* 0157:H7 5 strain cocktail and surface powdered with 4 or 6% (w/w) deodorized yellow mustard powder for 80d.	• At 21d, pathogen reduced by 3 log cfu/g with YM application compared to 1 log reduction in control. At 45d, > 5 log cfu/g reduction noted, whereas it took 80d for control to achieve this reduction. • Addition of ym powder accelerated reduction of the pathogen, compared to control.	Nilson & Holley, 2012

4.4.1.2 Evaluating Delivery Mechanisms

As the use of AITC in the meat industry has been limited due to its volatility and sensory profile, Holley's group studied whether the microencapsulation of AITC and subsequent incorporation into dry fermented sausage and refrigerated chopped beef could be effective and reduce the sensory impact. Pure AITC was microencapsulated into gum acacia and tested at concentrations of 500, 750 and 1000 ppm AITC against a cocktail of 5 non-virulent strains of *E.coli* 0157:H7 in fermented dry sausage. After 28 days of

drying, the pathogenic organism had been reduced by 5.2 to 6 logs CFU/g, meeting the regulatory requirements for a 5 log reduction. However, from a sensory perspective incorporation of 1000 ppm AITC resulted in a strong bitter sausage. Lower concentrations significantly affected flavour, appearance and texture, but could be acceptable (Chacon et al, 2006a). When microencapsulated AITC at concentrations ranging from 174 to 4980 ppm was added to packaged chopped refrigerated beef containing the *E.coli* cocktail and then stored under nitrogen for 18 days at 4°C. Reductions in *E.coli* were only observed at concentrations >1481 ppm AITC with complete elimination at 4980 ppm AITC. Faint to strong AITC odours were noted upon opening packages with the higher AITC treatments causing eye watering (Chacon et al, 2006b).

Holley's team tested AITC and oriental mustard extracts incorporated into edible antimicrobial coatings against other food pathogens-*Campylobacter jejuni* (gram negative), *Salmonella* (gram negative) on fresh, refrigerated vacuum-packed chicken breasts and *Listeria monocytogenes* (gram positive) on refrigerated, cured roast chicken. The antimicrobial agents were used in combination with malic acid or ethylenediamine tetraacetic acid (EDTA) to enhance antimicrobial activity. Factors influencing the stability and antimicrobial activity of allyl isothiocyanate as well as factors enhancing sinigrin hydrolysis by these 3 pathogens were evaluated. Highlights of the results of these studies are outlined below:

- Determined that minimum inhibitory concentrations of AITC against strains of *Salmonella* , *Listeria* and *Campylobacter* decreased as temperature decreased confirming that AITC is more stable at lower temperatures (4°C to 21°C). AITC was more effective at acidic pH against *Salmonella*, and at neutral pH against *Listeria monocytogenes* (Olaimat & Holley, 2013)
- *C. jejuni*, *Salmonella* and *L. monocytogenes* strains and mixtures had the ability to degrade sinigrin to form inhibitory concentrations of AITC, and sinigrin hydrolysis was significantly enhanced by higher incubation temperatures, the presence of 10mM ferric or ferrous irons and the presence of <0.25% glucose (Olaimat et al, 2014a)
- Effect of combinations of oriental mustard, malic acid and EDTA: the addition of malic acid improved the antimicrobial activity of oriental mustard extract against *L. monocytogenes*, while EDTA improved its activity against *Salmonella*, most likely due to differences in the cell wall structure between gram positive and gram negative organisms (Olaimat & Holley, 2014)
- Determined that *k*-carrageenan/chitosan coatings containing either AITC, mustard extract alone or combined with EDTA, malic or acetic acid significantly reduced *C. jejuni* and *Salmonella* on fresh, refrigerated, vacuum packed chicken

breasts and *L. monocytogenes* on refrigerated, cured roast chicken (Olaimat et al, 2014b; Olaimat & Holley,2015, 2016).

Lara-Lledo et al (2012) found that a polymeric film containing oriental mustard extract with 5% (w/w) sinigrin was more inhibitory than film containing 6% (w/w) sinalbin in a yellow mustard extract against *L. monocytogenes* on vacuum-packed bologna at 4°C. When both yellow and oriental mustard powders were incorporated at the same mustard concentration in dry sausage, yellow mustard was more active against *E.coli* 0157:H7. This was attributed to the formation of greater amounts of ρ-HBITC than AITC as there is ~2.3% ρ-HBITC in yellow mustard compared to 0.8% AITC in oriental mustard (Cordeiro et al, 2014b). Oriental and yellow mustards also have high phenolic contents which may be responsible in part for their antimicrobial activity as some work has shown that the total phenolic content of yellow mustard is two-fold greater than in oriental mustard (Wu, 2013).

Holley's team also found that incorporation of sinigrin into nanoparticulate carboxymethyl cellulose films and conventional carboxymethyl cellulose films containing sinigrin were effective in reducing *E.coli* by more than 4 logs after 5 days and > 5 log at 18d storage at 8°C (Herzallah and Holley, 2015).

Since mustard flour can be added as a spice (active myrosinase) and/or binder (inactive myrosinase) in dry-cured sausages, Holley's team continued to evaluate the antimicrobial effect of non-deheated (active myrosinase) and deheated (inactive myrosinase) yellow mustards in sausages. Non-deheated yellow mustard flour at 2%, 4% and 6% (wt/wt) or 6% (wt/wt) deheated yellow mustard flour was added to dry sausage batter inoculated with about 7 log CFU/g *E.coli* 0157:H7. It was found that all levels of non-heated mustard powder resulted in significant reductions of *E.coli* 0157:H7 during 30 days of drying. However, it was the addition of 6% deheated yellow mustard (myrosinase enzyme destroyed by heating) in dry sausage that achieved the 5-log reduction required by food regulatory agencies of the pathogen *E.coli* 0157:H7 in only 6 days (Grauman and Holley, 2008). Further investigations showed that neither the meat enzymes nor spice mix affected the sinalbin concentrations. However, all bacteria tested were able to degrade sinalbin and form *p*-hydroxybenzyl isothiocyanate. This occurred intracellularly, and depending upon the microorganism, at different rates of glucosinolate breakdown [*E.coli* 0157:H7 > *Staphyococcus carnosus* > *Pediococcus pentosaceus* (Luciano et al, 2010)].

Similar results were found when ground "cold" black or brown/oriental mustard flour, containing the allyl glucosinolate (sinigrin) was incorporated into the dry sausage formulations. In this case, *E.coli* 0157:H7 (and the two fermentation starter organisms) was able to convert the sinigrin into allyl isothiocyanate. In these experiments, AITC had stronger antibacterial activity against *E.coli* 0157:H7 than did *ρ*-HBITC. It appears that

deodorized ground mustard when used in dry fermented sausages can be converted to an effective *E.coli* 0157:H7 control agent where the pathogen itself activates the glucosinolate to form the antimicrobial agents, p–HBIT or AITC. Holley's team has found endogenous myrosinase-like activity in other bacterial species as well including lactic acid bacteria used as starter cultures in meats and cheese such as *Pediococcus pentoseceus* and *Staphylococcus carnosus* (Graumann and Holley, 2008), *Lactobacillus curvatus* and *L. plantarum* (Luciano et al., 2011) and pathogenic organisms such as *Salmonella typhyimurium*, *Enterococcus facecalis* and *Listeria monocytogenes* (Herzallah et al, 2011).

The ability of *E.coli* 0157:H7 to degrade sinigrin endogenously was further investigated. Research indicated there was sequence homology between plant myrosinase and enzymes encoded by genes from β-glucosidase families in *E.coli* 0157:H7. Using single and double mutants of the organism that eliminated glucosidase activity, the results suggested that two *E.coli* genes, *bglA* and *ascB*, encoding 6-phospho-β-glucosidase could play a role in the degradation of sinigrin by E.coli 0157:H7 (strain 02-0304)(Cordeiro et al., 2015).

Autoclaving yellow mustard powder also appears to cause more rapid pathogen reduction as well, possibly due to the release of other antimicrobial metabolites during the thermal treatment (Luciano and Holley, 2010, 2011). Cordeiro and colleagues (2014a) found that yellow mustard derivatives were more potently antimicrobial than Oriental mustard.

4.4.1.3 Mechanism of Action

A key aspect to the development of antimicrobial agents is determining the mechanism of action and optimizing conditions of use. Luciano and Holley (2009) found that AITC is a more effective antimicrobial at low pH values and its degradation reduces this activity, suggesting that AITC may be more stable at low pH and would work better in more acid foods. In the presence of water, AITC degrades to form 3 decomposition products: diallylthiurea (80%), diallyurea and diallyl disulfide, which do not have any antimicrobial activity. In addition, AITC appears to have a multi-targeted mechanism of action, perhaps inhibiting several metabolic pathways and damaging cellular structure (inhibited the catalysis of thioredoxin reductase involved in DNA synthesis and acetate kinase, involved in energy generation. [Note: the mechanism of action of ITCs in general is being more thoroughly investigated by the health community and will be discussed later in this report.]

Further investigations by Cordeiro and coworkers (2014b) showed that the ability of *E.coli* 0157:H7 to sometimes resist the antimicrobial effect of AITC was due to an inherent defense mechanism system (BaeSR).

From a sensory perspective, the use of ground deodorized mustard in dry, fermented sausage at concentrations required to meet regulatory control of *Escherichia coli* 0157:H7 may have a negative effect on consumer acceptance. The overall sensory acceptance of dry fermented sausages containing 3% and 4% mustard was reduced compared to the control sausages or those containing 1% mustard (Li et al., 2013)

Beyond meat applications, AITC is being tested in different food systems such as bakery products, cheese and packaged nuts.

The addition of antimicrobials that can act during the storage of bakery products to control the presence of the mycotoxigenic fungi is of great interest to the bakery industry. Most bakery goods contain propionate, sorbate or benzoate salts to prevent mold growth, but consumers want less synthetic preservatives in food. It was found that allyl isothiocyanate in mustard essential oils can inhibit fungal growth in bakery products (Nielson and Rios, 2000; Suhr and Nielsen, 2003 and Azaiez et al, 2013) and fresh pizza crust (Quiles et al, 2015a) and wheat tortillas (Quiles et al., 2015b) where it was found that gaseous allyl isothiocyanate from oriental mustard extracts was more effective than p-hydroxybenzyl isothiocyanate from yellow mustard extracts in inhibiting production of aflatoxins.

The shelf life in cheese was extended from 4.5 to 13 weeks and to 28 weeks with 1 and 2 AITC labels respectively, (Wasauro interior labels). However, cheese stored up to 12 weeks with an AITC label had an unacceptable mustard flavor which decreased to an acceptable level between weeks 12 and 28. The authors proposed AITC could be an alternative to modified atmosphere packaging in cheese, extending shelf life and protecting against problems such as pinholes and leaking seals in cheese packaging (Winther and Nielsen, 2006).

Maynes and colleagues (2015) found that AITC when applied to bioactive packaging for specific foods (almonds, peanuts, nuts, hazelnuts, pistachios and apples, peaches, grapes and strawberries) inhibited the growth of mycotoxigenic fungi.

Researchers are also investigating combinations of essential oils. Clemente and co-workers (2016) found that AITC induced cell arrest and was 10 times more bactericidal/bacteriostatic than cinnamon essential oil. There was an additive effect when both were combined. It was suggested these combinations of essential oils could be used to design active packaging.

4.4.1.4 Antimicrobial Agents from *Sinapis alba* Oils

White mustard essential oil (WMEO) also has the potential to be commercialized as an antimicrobial agent. WMEO is an essential oil with very low volatility. WMEO contains *para*-hydroxybenzyl isothiocyanate (p-HBITC), derived from the glucosinolate sinalbin in white/yellow mustard upon its hydrolysis by the endogenous enzyme myrosinase. Due

to its instability in aqueous environments WMEO must be extracted using solvents or supercritical carbon dioxide (Ekanayake et al. 2016).

According to Proctor and Gamble US Patent 8,697,150 (Ekanayake et al, 2014) and a GRAS notification submitted on behalf of ConAgra (GRAS Notice (GRN) No.442) preparation of ρ-HPITC involves cold pressing mustard seeds to remove most of the fixed oil and grinding the press cake or seed to a fine powder. The partially defatted mustard press cake is moistened in the presence of ethyl acetate and ascorbic acid (acts as an enzyme activator) activating the myrosinase enzyme. The addition of ascorbic acid hastens the activation of myrosinase reducing the time for extraction and makes the process more efficient. After the short reaction period, the ethyl acetate extract containing ρ-HPITC and the fixed oil in the partially defatted mustard cake is removed by centrifugation. Low temperature evaporation of ethyl acetate under reduced pressure yields white mustard essential oil (WMEO) containing ρ-HPITC.

To stabilize the ρ-HBITC in the WMEO and to provide a means of easy dispensing, WMEO is mixed with maltodextrin. WMEO was to be used as an antimicrobial system for the control and prevention of sporadic recontamination, complementary to and not a substitution for existing process controls that ensure microbial destruction and product stability.

In the GRAS petition, it was proposed WMEO could be added to non-carbonated beverages, ketchup, sauces/gravies in frozen meals and egg substitute products at the following levels:

- Non carbonated beverages: 25pp (ρ-HPITC): energy and sport drinks, fruit-flavoured drinks, fruit juice and juice drinks
- Ketchup: 150 ppm ($-HBITC)
- Sauces/Gravies in frozen meals: 500 ppm (ρ-HPITC)
- Egg substitutes: 250 ppm (4HBITC)

Based upon this technology and application, ConAgra submitted a GRAS (Generally Recognized As Safe) petition to the US Food and Drug Administration in April 2012 for the agency review. However the petition was withdrawn from the GRAS process in 2014.

Discussions with Dr. Jairus David (Personal communication, 2016) of ConAgra Foods revealed that Proctor & Gamble (P&G) retained ownership of the WMEO technology and continued limited investigations evaluating WMEO in foods. David and colleagues from P&G found that a 12% preparation of WMEO was most effective when combined with commercial natural antimicrobial agents (citrus flavonoid and acid blends, olive extract, nisin and lauric arginate) against food borne and spoilage microorganisms

(Techathuvanan et al, 2013). The white mustard essential oil (14% solution) was also used as an antimicrobial agent in processing sauces containing particulates and effectively reduced inoculated *Salmonella* counts by 0.8 to 2.7 log (cfu/g) in frozen sauce with particulates in dose-dependent manner and from 0.7 to 2.4 log (cfu/g) in a simulated unintended abuse condition for a frozen food (David et al., 2013).

WMEO is more effective against pathogenic and spoilage gram-negative bacteria (*Escherichia coli*, *Salmonella enterica,* E*nterobacter aerogenes*) than gram-positive bacteria (*Listeria monocytogenes* , *Bacillus cereus* and *Lactobacillus fermentum*) with it activity being strain specific. It was found the food composition does affect its effectiveness with foods containing high fat content rendering the WMEO inactive due to the affinity for the essential oil to the lipid phase of the food (Monu et al., 2014).

Further to this information, conversations with Dave Grex, R&D Director at Newly Weds Foods at the IFT 2016 Food Expo (personal communication, July 2016) revealed that Newly Weds Foods had purchased the WMEO patents from P&G and contracted a researcher at the University of Arkansas to study the effect of WMEO against pathogenic organisms in red meat systems. After more than 2 years study, it was found that while WMEO was an effective antimicrobial agent, the sensory profile in the raw red meat systems was not acceptable. Consequently, Newly Weds Foods stopped work on this compound, instead focusing their efforts on other antimicrobials with a better flavour profile (e.g. dried vinegar). The antimicrobial research was never published. However, P&G retained the rights to use WMEO in beverage applications.

4.4.1.5 Other Avenues of Antimicrobial Research

Capitalizing on the ability of many microorganisms (bacteria and fungi) to degrade glucosinolates, research is focused on the characterization of degradation by intact cells as well as by select microorganisms with high myrosinase activity. These microorganisms could become potential agents to degrade glucosinolate-rich wastes from the agricultural industry and produce valuable glucosinate-derived products, assist with feed detoxification and produce mustard flavours (allyl isothiocyanate production) (Rakariyathan et al, 2005).

Due to the interest in the link between isothiocyanates and cancers, there has been significant research into mechanisms of action of ITC, particularly in *in vitro* models and in cell models. However, the antimicrobial mechanisms of ITC in bacteria and fungi are not well understood. A recent review paper (Dufour et al, 2015) outlined some putative mechanisms but stressed work in this area was in its infancy. ITCs affect basic physiological and metabolic processes in bacteria by causing an abrupt inhibition of stable RNA synthesis with DNA synthesis being affected after longer exposures. AITC has the strongest effect. Others have found that the induction of the stringent response during ITC treatment was responsible for inhibition of bacterial growth and inhibition of

the prophage induction in Shiga toxin-harbouring bacteria (*E.coli* 0157:H7). Further studies showed the possibility of ITCs binding with specific amino acids to prevent their transport into the bacterial cell or affecting activity of RNA transferases. If ITC are to play a role in health care, further work to investigate synergistic actions between ITC and other antibiotics are

4.4.1.6 Future Research Opportunities

As outlined in the numerous research papers, considerable work is needed to fully understand the use of mustard and its isothiocyanate products as antimicrobial agents for foods or as potential antibiotics:

- Gain a better understanding of the antimicrobial mechanisms of ITCs
- Better understanding of the mechanisms involved in bacterial glucosinolate-isothiocyanate conversions to optimize the use of cold/autoclaved mustard powder for the control of *E coli* 0157: H7 and perhaps other food pathogens in dry fermented sausage.
- Further studies using one or more shiga-toxin producing *E.coli* 0157:H7 strains that cause human illness from contaminated dry sausage are necessary for validation before mustard powder can be used industrially for this purpose (Health Canada, 2000).
- Optimization of the thermal heating process to enhance antimicrobial properties in yellow mustard powders and understand the mechanism of interaction between phenolic compounds and isothiocyanates.
- Enhance the effectiveness of AITC by combining with other natural antimicrobials.
- Determine effects of the addition of mustard extract on the organoleptic qualities and consumer acceptance of foods.
- Further research is needed on the encapsulation of natural antimicrobials and interaction of these antimicrobials with physical processes to improve the applicability of antimicrobial delivery systems to target organisms.
- Pathogenic bacteria showed myrosinase-like activity. Isolate and purify the responsible enzymes and study factors to enhance activity.

4.4.2 PHENOLICS AND ANTIOXIDANT ACTIVITY

Antioxidants are the principle ingredients which protect the quality of foods by slowing oxidative breakdown of their lipid (oil) components. Commercial antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tert-butyl hydroquinone (TBHQ) are synthetic compounds and there is a growing interest in replacing them with natural ingredients such as lutein or Vitamin C (ascorbic acid) or vitamin E (tocopherol). Natural antioxidants can be enzymatic or non-enzymatic and cover a wide range of compounds (Figure 14) including phenolic compounds, tocopherols, phospholipids, or amino acids. Under the Canadian Food and Drug Act

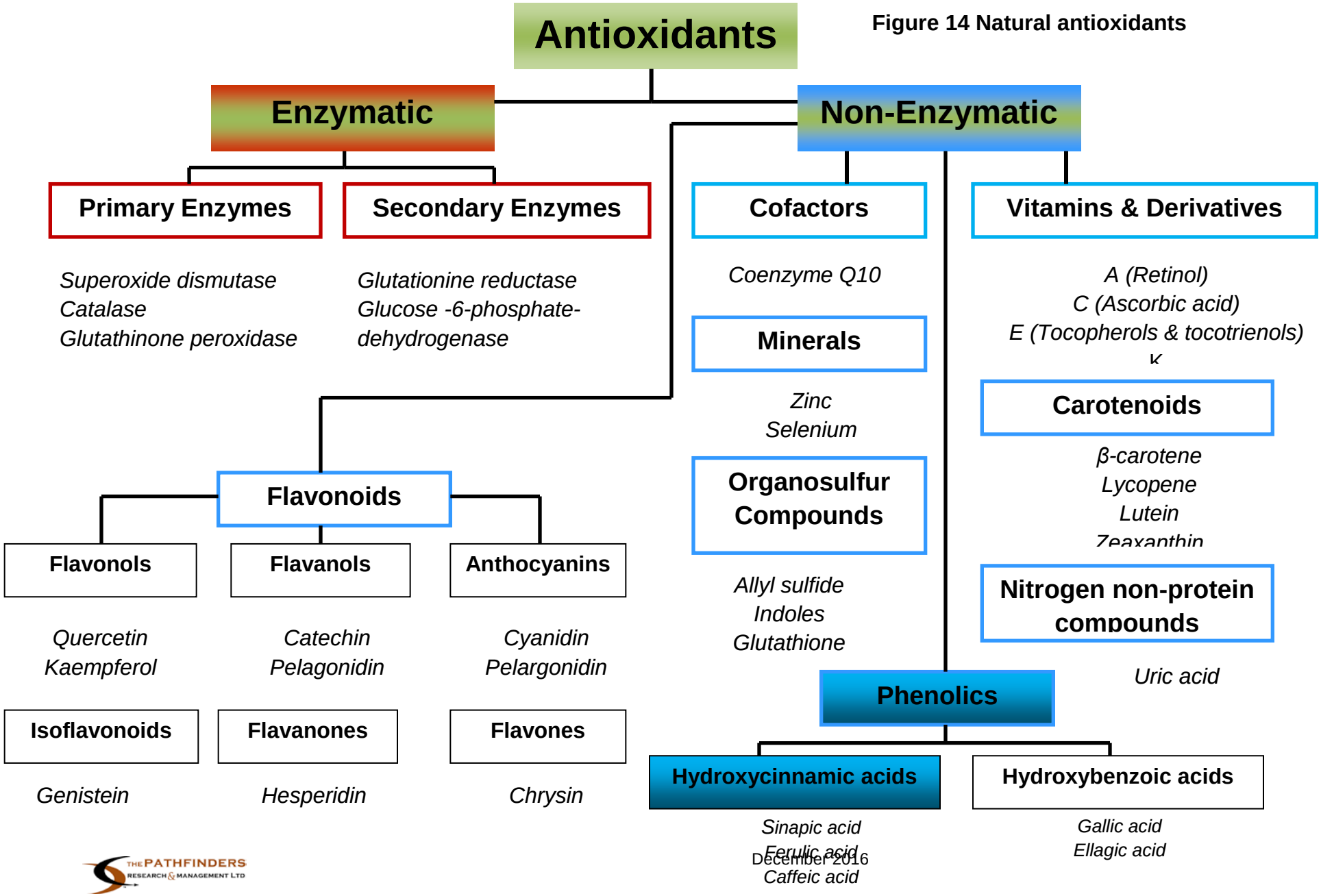
and Regulations, antioxidants added to food products are considered food additives and are regulated under Division 16 along with other food additives.

Consumption of antioxidants has become a major health topic and several foods are consumed primarily because they are rich in antioxidants and offer numerous health benefits (e.g. blueberries). Considerable research is ongoing to investigate and confirm the links between reactive oxygen and nitrogen species and the effect of antioxidants on chronic diseases such as cancer, diabetes, autoimmune conditions and eye diseases.

The most common water-soluble antioxidant compounds in plants and foods are the phenolic compounds. Phenolics are one of the most ubiquitous groups of secondary metabolites found in the plant kingdom primarily formed by the plant as a defense mechanism in response to biotic and abiotic stress (Bhattacharya et al., 2010). In foods, phenolics may contribute to the bitterness, astringency, colour, flavour, odour and oxidative stability of products. Phenolic compounds are primary antioxidants that act as free radical acceptors (de Beer et al., 2002).

In the Brassicaceae species, sinapic acid and its derivatives are considered to be the most important phenolic compounds. Sinapic acid is one of the four most common hydroxycinnamic acids (others include ferulic, caffeic and coumaric acids) (Figure 14) and is present in fruits, vegetables, oilseeds and cereal grains. It can be found in its free form but like other hydroxycinnamic acids, it is also found in the form of esters. Sinapic acid has been proposed as a potent antioxidant and shows antimicrobial, anti-inflammatory, anticancer and anti-anxiety activities (Niciforovic and Abramovic, 2014).

Figure 14 Natural antioxidants



Note: It is difficult to compare results for phenolics in the scientific literature due to differences in the methods of sample preparation, extraction procedures (e.g. solvent, time, temperature), methods of purification and fractionation (e.g liquid-liquid or liquid-solid), and analysis and quantification (e.g. spectrophotometric or chromatographic) (Naczk et al., 2004).

In general, there appears to be limited information on the total phenolic content and phenolic profile in the various mustard varieties, flours, meals and mustard commercial products.

Early investigations into the antioxidants from low pungency mustard flour identified three fractions with variable antioxidant activity as measured in a β-carotene-linoleate model system. Fraction 1 showed very high antioxidant activity and it was attributed to the presence of the phenolic compounds (Shahidi et al, 1994). It was then shown that adding 1-2% of an 85% methanolic extract of low pungency ground mustard seed to comminuted pork was as effective as adding 200ppm of the synthetic antioxidants BHT and 30 ppm of TBHQ on the oxidative stability of the meat product. In addition, the mustard ingredient addition enhanced cooking yield without affecting product colour (Saleemi et al, 1993).

In mustard, sinapine (the phenolic ester of sinapic acid) is the major phenolic acid constituting over 90% of the 70% methanol extract of mustard press cake (Thiyam et al, 2006). In addition to sinapic acid and sinapine, other phenolics including kaempferol-sinapoyl-trihexoside, sinapoyl-hexoside, disinapoyldihexoside, and disinapoyl-hexoside have been found in defatted oriental mustard (Engels et al, 2012).

Research conducted at the University of Manitoba (as part of a Mustard 21 project report entitled “Biorefining of Mustard”) showed the variation in total phenolic content (TPC) and individual phenolics in 7 mustard seed samples, deheated mustard flour and ground yellow and brown mustard flours obtained from GS Dunn (Table 17). There was significant variation in TPC among samples ranging from 10.84 mg sinapic acid equivalents (SAE)/ g to 42.31 mg SAE/g. Similar variation was observed for the individual phenolics, sinapine and sinapic acid (Thiyam-Hollander etal., 2014). For comparison, the amount of sinapic acid derivatives in rapeseed meal varies from 6.39-18.37 mg/g depending on the variety and the oil pressing method (Khattab et al, 2010).

Table 17 Characterization of sinapine, sinapic acid and total phenolic compounds quantified from mustard flour and seed extracts (extracted from Thiyam-Hollander et al., 2014)

Flour/Seed Samples	Sinapine (mg/g)	Sinapic Acid (mg/g)	Total Phenolics (mgSAE/g)
Mustard Flour 1	18.7	0.44	35.39
Mustard Flour 2	17.54	0.48	42.31
Mustard Flour 3	18.71	0.36	30.52
Mustard Flour 4	17.78	0.44	38.27
Mustard Flour 5	26.29	0.21	15.22
Mustard Flour 6	15.91	0.47	37.54
Mustard Flour 7	13.04	0.16	11.46
Deheated mustard flour	15.31	0.48	36.21
Ground yellow mustard	7.81	0.15	15.08
Ground brown mustard	21.34	0.16	10.84
High erucic acid rapeseed[a]	15.1	0.28	
Canola seed[a]	11.89	0.20	
Canola seed[a]	11.39	0.29	

SAE: sinapic acid equivalent [a]Reported values obtained from another researcher

Further work by this same group determined the phenolic profiles and antioxidant capacity in the hulls and cotyledons of canola and mustard (Mayengbam et al, 2014). Sinapic acid, sinapine, sinapoyl glucose and canolol are the potent antioxidants in both canola and mustard seeds and extracts where canola cotyledons had higher total phenolics than mustard cotyledons. Sinapine was the major phenolic in hulls and cotyledons with the cotyledons containing more phenolics than hulls (Table 18). The table also illustrates the differences in values when phenolics are quantified using different methods (high performance liquid chromatography (HPCL) versus the traditional chemical Folin-Ciocalteu method).

Table 18 Profile of sinapic acid and its derivatives in different fractions of mustard and canola (extracted from Mayengbam et al, 2014)

Sample	Sinapoyl glucose (mg/g)	Sinapine (mg/g)	Sinapic Acid (mg/g)	Total Phenolics (HPLC) (mg/g)	Total phenolics (Folin-Ciocalteu) (mg/g)
Canola cotyledon	8.71	12.20	0.22	20.20	16.89
Canola seeds	5.45	8.35	0.15	14.06	10.69
Canola hulls	1.35	1.15	0.04	3.57	4.50
Mustard cotyledon	0.67	10.62	0.18	11.45	10.60
Mustard seed	0.66	10.17	0.19	11.12	13.31
Mustard hulls	0.41	4.74	0.90	5.67	6.24

Canola : source: Nexera variety (Dow AgroSciences)
Mustard: source: GS Dunn Ltd

In order to identify the different antioxidant mechanisms, antioxidant activity of compounds is usually measured in a number of different ways including the following:

- Reducing power assay-indicator of electron donating activity
- DPPH-radical scavenging assay-DPPH radical accepts an electron or hydrogen from an antioxidant stabilizing the radical
- Ferrous-ion chelating ability-metal ions can accelerate lipid oxidation. Antioxidants can form bonds with the metals and stabilize the oxidized form of the metal.
- Alkylperoxyl radical scavenging capacity (using β-carotene-linoleic acid emulsion system)-the presence of antioxidants prevents the bleaching (loss of yellow colour) of β-carotene.

In many cases, the type of extraction solvent (e.g. water, ethanol, methanol, acetone) influences the amount of total phenolic acids compounds extracted and affects the antioxidant activity. A high total phenolics content (TPC) value does not always correspond to high antioxidant activity. As an example, Slovenian researchers compared the TPC values in five oilseed meals, including white mustard, and found that while white mustard meal had the highest TPC value, it exhibited the weakest antioxidant activity. However the mustard extracts prevented the oxidative deterioration of safflower oil when the oil was incubated with the meal extracts. This indicates that mustard meal prevents the formation of primary and secondary oxidation products and could have potential as a natural antioxidant in lipid based foods (Terpinc et al., 2012)

The antioxidant activity of sinapic acid and its derivatives in canola meal and in Indian mustard meals was evaluated byThiyam's group (2006). Mustard meal contained about 4-10 mg sinapic acid derivatives per gram oil-free meal with 90% being identified as sinapine. Mustard meal showed 2-3 times lower free radical scavenging activity compared to extracts of cold-pressed rapeseed cake. Sinapine contributed about 40% to this activity while the sinapic acid made a negligible contribution. Generally it was found that sinapine had a significant but lower free radical scavenging activity compared to sinapic acid. The ability of sinapic acid and sinapine to act as antioxidants and prevent the formation of lipid oxidation products in purified rapeseed soils was compared to the various tocopherolos. In the study, the sinapine-rich fraction did not inhibit the formation of hydroperoxides whereas sinapic acid rich fractions could. In addition, it appeared that the Indian mustard meals had lower activities than the cold pressed or extracted canola meal.

Recently, it has been shown that roasting oil seed oils such as sesame and rapeseed resulted in the formation of a potent radical scavenging compound, canolol (2,6-dimethoxy-4-vinlyphenol) due to the decarboxylation of sinapic acid. Canolol has been

shown to improve the oxidative stability of roasted rapeseed (Wakamatsu et al., 2005; Kumazawa et al., 2003)

Researchers in India also found cananol in high erucic acid mustard (HEM) varieties of *Brassica juncea, B. juncea* var. oriental, *B. nigra* and *S. alba* with the highest values found in the rapeseed. They determined that roasting the HEM varieties gave higher canolol values and improved the oxidative stability of oil (Shrestha et al, 2012, 2013, 2014)

Thiyam-Hollander and co-workers (2014) identified and quantified canolol and related sinapate precursors in commercially produced Indian mustard oils and found it in 2 of the 4 Indian mustard oils tested (Table 19); however, no canolol was found in commercial Canadian mustard seeds or flours.

Table 19 Presence of canolol in commercially produced Indian mustard oils (extracted from Thiyam-Hollander et al, 2014).

Oil Samples	Canolol (mgSAE/g		Total Phenolics (mg SAE/g)
Dabar mustard oil	0.157		0.241
KTC mustard oil	Not detected		0.203
Gagan mustard oil	0.145		0.145
Good Choice mustard Oil	Not detected		0.317

SAE: sinapic acid equivalents

Recognizing the potential value of co-products from mustard (e.g. protein for food or the high erucic acid oil as a biofuel), alternative methods of extraction to retrieve valuable bioactive compounds such as the phenolics and glucosinolates are being investigated.

Diosady's laboratory at the University of Toronto developed an ultrafiltration-diafiltration based technique to simultaneously prepare a mustard protein isolate and recover the phenolic-rich fraction from seed meal using a high shear rotating disc membrane module with emphasis on reduced membrane fouling. Although the yield of the membrane process was less (31.4%) compared to the protein precipitation method (42%) a protein isolate of high purity (96%) with improved functional properties and low phenolic content was obtained. The recovered phenolic-rich fraction contained about 16% sinapic acid and 62% sinapine, and retained about 87% of the original radical scavenging function (Das et al, 2009).

Continuing in this vein, Diosady's group used a combination of membrane processing followed by ion-exchange chromatography to recover and purify sinapic acid from the waste water generated from mustard protein processing. Sinapic acid was recovered by

concentrating the starting waste permeate by nanofiltration followed by alkaline hydrolysis which recovered about 95% of the sinapic acid originally in the waste permeate. It was further purified by ion exchange chromatography. Recovering the waste stream improved the economic viability of protein isolate production and suggested the possibility of water reuse (Prapakornwiriya and Diosady, 2008)

Work progresses on developing methods to rapidly extract phenolics with higher yield in order to quantify, isolate and identify the phenolics present in white mustard. The University of Idaho is investigating novel extraction methodologies to recover bioactives from mustard meals where the oil is being used for the biofuel industry. An ultrasound-assisted extraction (UAE) procedure for recovery of phenolics from *B. juncea* was optimized and resulted in increased phenolic yield and antioxidant activity compared to the conventional extraction method. They found that inclusion of ultrasonics meant lower extraction temperatures could be used (75°C for UAE versus 80°C for conventional) and treatment duration could be significantly reduced (30 minutes for UAE compared to 24h or 7 days with conventional). While, the antioxidant activity was retained, it appeared that sinapic acid was more susceptible to sonochemical degradation than sinapine (Dubie et al, 2013).

UAE was also used to enhance recovery of sinapic acid and its derivatives from three white mustard seed varieties developed in Poland ((Szydlowska-Czerniak et al., 2015

4.4.2.1 Antimicrobial Activity of Sinapic acid

Sinapic acid does have antimicrobial activity and its effect has been studied on both plant and human pathogens, particularly in the last fifteen years (Niciforovic and Abramovic, 2014). It appears that sinapine (the ester of sinapic acid, derived from ethanolic extracts of rapeseed) does not have antibacterial activity and it requires alkaline hydrolysis to release the sinapic acid (Nowak et al., 1992).

Sinapic acid was extracted from defatted yellow mustard meal and found to have antibacterial activity against *E.coli, S. enteritidis*, and *S. aureus* (Tesaki et al, 1998). Engels and co-workers (2012) at the University of Alberta extracted phenolic compounds from defatted Oriental mustard (*Brassica juncea* L) seed meal and characterized the phenolic compounds using ultra-high performance liquid chromatography. The crude extract and a purified phenolic fraction exhibited selective antibacterial effects against Gram negative and Gram positive bacteria including *Staphylococcus aureus* and *Listeria monocytogenes* whereas the lactic acid bacteria *Lactobacillus plantarum* was resistant. Alkaline hydrolysis of the crude phenolic extract enhanced the antimicrobial activity of the preparation suggesting that phenolics are the major inhibitory compounds. [Note: for this study, the defatted mustard meal was obtained from Biofume Technologies (Saskatoon); however, this company no longer exists].

4.4.2.2 Opportunities for Mustard Phenolics Research

From the literature review, a number of research initiatives have been identified:

- Better understanding of residual phenolics in these oilseeds and how structural changes or enhancement techniques during roasting and processing could lead to better practices, improved functionality and oxidative stability within the finished product.
- Determine the levels of antioxidants in mustard as impacted by cultivation techniques, cultivar, growing conditions, ripening conditions and stress conditions (UV radiation, pest infections, air pollution, extreme temperatures).
- Need to quantify phenolic constituents, namely sinapic acid derivatives, to relate individual antioxidative potential.
- Study how naturally derived phenolic extracts could be used as an antioxidant in lipid and lipid-containing systems, for food as well as non-food applications.
- Optimize phenolic extraction procedures from the meal and press cakes resulting from rapeseed and mustard oil processing.

4.5 Antimicrobial Packaging

Consumer demand for natural food ingredients is spurring research into the use of natural antimicrobials agents as replacements for chemical additives in foods and food packaging. As AITC is considered to be a natural, potent antimicrobial agent, research into potential active packaging applications is on the rise.

Active packaging is one of the innovative packaging technologies used to prolong the shelf life, enhance the quality and safety of food and protect the environment. Traditional “passive” packaging systems protect the food product only from external environmental hazards. New technologies are use to modify and monitor the internal and external environment and provide multiple barriers or hurdles to protect food.

Active packaging can be classified into three systems (Yezza, 2008):

- active scavenging systems (absorbers);
- active releasing systems (emitters); and
- controlled release packaging.

These systems cover a wide number of mechanisms including oxygen scavenging, moisture absorption and control, carbon dioxide and ethanol generation and antimicrobial migrating and non-migrating systems (Suppakul et al., 2003). Controlled release packaging describes a new generation of packaging materials that can release active compounds of any type (antimicrobials, antioxidants etc.) at rates suitable for a

wide range of food applications (Yam et al., 2006). Active packaging can be considered an alternative to the use of preservatives or modified atmosphere packaging (MAP).

Controlled release packaging uses packaging as a delivery system to release the antimicrobial agent into food in an effective manner, thereby extending product quality, safety and shelf life (Balasubramanian et al., 2009). The concept and application of controlled release of substances has been used for prescription medications such as drugs and antimicrobials and other compounds such as vitamins and antioxidants, as well as for the controlled release of flavours, enzymes, sweeteners and other food preservatives in foods. This technology has been extended to the development of packaging films containing antimicrobial agents.

Antimicrobial packaging can take many forms (Appendini and Hotchkiss, 2002):

- Addition of sachets or pads containing volatile antimicrobial agents
- Incorporation of volatile or non-volatile antimicrobial agents directly into polymers
- Coating or adsorbing antimicrobials onto polymer surfaces
- Immobilization of antimicrobials to polymers by ion or covalent linkages
- Use of polymers that are inherently antimicrobial.

With this technology, packets, packaging films or coatings contain an antimicrobial agent which is either stationary or is slowly released into the food environment to control the growth of microorganisms, and extend the shelf-life of food products (Nerin, 2012). Table 20 provides examples of antimicrobial agents which can be incorporated directly into polymers used for food packaging.

Table 20 Potential antimicrobial agents for incorporation directly into polymers used for food packaging (Appendini and Hotchkiss, 2002; Suppakul et al, 2003; Steven and Hotchkiss, 2003)

Antimicrobial Agent	Polymer/Carrier	Main Target Organisms
Organic Acids/Anhydrides (Propionic, benzoic, sorbic, acetic, malic, lactic acids)	Edible films, EVA, LLDPE	Moulds
Inorganic Gases (Sulphur dioxide, chlorine dioxide)	Various polyolefins	Moulds, bacteria, yeast
Metals (silver, copper)	Various polyolefins	Bacteria
Fungicide (benomyl, imazalil)	LDPE	Moulds
Bacteriocins (nisin, pediocins, lacticin)	Edible films, cellulose, LDPE	Gram positive bacteria
Enzymes (lysozyme, glucose oxidase, chitinase, lactoperoxidase)	Cellulose acetate, PS	Gram positive bacteria
Spices (cinnamic, caffeic, p-coumaic acids, horseradish (allylisothiocyanate))	Nylon/PE, cellulose	Moulds, yeast, bacteria
Essential Oils (plant extracts) (grapefruit seed extract, hinokioiol, bamboo powder, Rheum palmatum, Coptis chinesis extracts, mustard extracts)	LDPE, cellulose	Moulds, yeast bacteria

No single antimicrobial agent can cover all the requirements for food preservation. Researchers and manufacturers must consider the target organism to be eliminated, food type and microbial surface population, the effectiveness of the antimicrobial agent against the targeted organism, the types of films used and how it is to be attached to the packaging surface.

4.5.1 Research on AITC antimicrobial activity in packaging

As described previously, most of the research conducted on allyl isothiocyanate uses a pure (>94%) form of AITC sold by chemical companies (Sigma, Aldrich etc) which is then blended with another ingredient such as vegetable oil to depress its strong odour and lachrymatory (eye tearing) properties. Some of the research uses natural mustard extracts or mustard essential oils.

AITC vapor is a more effective antimicrobial agent than liquid AITC (Sekiyama et al, 1996). In the vapour phase, minimum inhibitory concentration of AITC against bacteria, yeasts and molds have been estimated at 34-100, 13-37 and 16-62 ng/mL respectively (Isshiki, et al, 1992). Suhr and Nielson (2003) investigated the antimicrobial effect of

several essential oils including AITC against rye bread spoilage fungi and found that smaller compounds such as AITC and citral were most effective when added as volatiles. AITC vapour was 500 to 1000 times more effective than the same amount of liquid in agar. The minimum inhibitory concentration to control fungal growth was 250 times lower for AITC in the gas phase than in the liquid phase.

While AITC is considered to be an effective antimicrobial agent, its use in food systems is limited because of its strong odour which can affect the taste of food (Delaquis and Mazza, 1996, Chacon et al., 2006).

Thus researchers are investigating ways to incorporate AITC into packaging such than the continuous release of minimal amounts will prolong the effective treatment time, enhance efficacy of AITC and reduce the impact of its odour. This includes combining AIT with other methods of packaging (e.g. using modified atmosphere) or processing (high pressure processing) to enhance effectiveness of AITC and to reduce the amount needed. Due to volatility and strong odour of AITC, controlled release is needed to prevent its excessive accumulation that may impact the sensory attributes of the food products.

The following summaries are examples of how AITC is being incorporated into different polymer carriers and investigated for its release behavior:

- Winther and Nielson (2006) investigated the antimicrobial effect of AITC labels against cheese-related fungi such as *Penicillium commune*, *P. nalgiovense*, *P. roqueforti*, *Aspergillus flavus*, *Geotrichum condidum* and *Debaromyces hansenii*. AITC labels were placed in packaging prior to sealing under modified atmosphere. All inoculated microorganisms were inhibited on the cheese surface and the shelf life of cheese extended. However, there were issues with the cheese having an unacceptable mustard flavour up to 12 weeks exposure. Between weeks 12 and 28, the off-flavour decreased to an acceptable level which was attributed to reactions with nucleophiles (-SH and –OH groups).

- Nadarajah et al (2005a) investigated the antimicrobial activity of AITC which was placed on ground meat patty packaged in nylon/EVOH/PE (EVOH-ethylenevinlyalcohol; PE-polyethylene) under 100% nitrogen against mesophilic bacteria and *E.coli*. Results showed that AITC can substantially reduce the numbers of *E.coli* 0157:H7 in fresh ground beef during refrigerated and frozen storage, but it was not effective against mesophilic bacteria.

- Nielson and Rios (2000) investigated antimicrobial effect of AITC against moulds and yeasts in bread. AITC was added in PA/EVOH/PA bags of bread packaged

under modified atmosphere. It was found that AITC could be fungicidal and fungistatic depending on the concentration of AITC and the number of fungus spores.

- Shin and coworkers (2010) incorporated AITC in high density polyethylene (HDPE) film with modified atmosphere packaging (MAP) and showed this combination could be used to inhibit the growth of fresh poultry-related pathogens such as *Salmonella typhimurium* and *Listeria monocytogenes* in fresh chicken samples.

AITC incorporation is also being investigated for use in edible films (Table 21). Unlike synthetic films, edible films and coatings are biodegradable and sourced from renewable materials. Edible films are thin layers of edible, food-grade suspensions molded as solid sheets onto inert surfaces. They are dried and put into contact with food as wrappings, pouches, capsules, bags or casings for further processing. Films can be made from starches, cellulose derivatives, chitosan/chitin, gums, animal or plant based proteins and lipids. Not only do edible films act as barriers, they can also act as carriers for antioxidant or antimicrobial additives to extend a food shelf life while maintaining mechanical integrity and handling characteristics (Mellinas et al, 2015).

Various technologies are being investigated for incorporation of AITC or mustard extracts into packaging films. Conventional packaging films are produced by mechanically extruding melted plastic materials to form thin films. Instead of mechanical force, electrospinning uses electrostatic force to produce ultrafine layers of films from a variety of polymers, fibers and particles films that contain fibers with diameters in the nanometer range. By manipulating the polymer characteristics, the controlled release of active compounds (antioxidants, antimicrobials) can be achieved. AITC is being incorporated into different composition of electrospun fibers to enhance is effectiveness at lower use concentrations.

- Encapsulated AITC in gum Arabic and chitosan microparticles to significantly reduce the odor of AITC in kimchi, a fermented cabbage product, while reducing microbial growth. Loading of AITC-containing microparticles was limited to less than 01% (w/w) to prevent AITC off-flavour product (Ko et al., 2012)
- Encapsulated AITC in electrospun soy protein isolate (SPI) and poly(lactic acid) (PLA) fibers to control release of AITC (Vega-Lugo and Lim, 2009)
- Mix with a compatible diluent. Canola oil has been shown to be effective in depressing the vapor pressure of AITC (Lim and Tung, 1997)
- Formation of an AITC-canola oil blend sealed in high-density polyethylene film to inhibit the grown of *Listeria monocytogenes* and *Salmonella typhimurium* in raw chicken packaged in modified atmosphere packaging (Shin et al, 2010)

- Systematic study of the effects of relative humidity, temperature, particle size and lipid content on the release kinetics of AITC from mustard seed meal powder.
- Potential use as a source of AITC in active packaging (Dai and Lim, 2014). Need to further study the residual AITC during storage in actual food packaging systems.

4.5.2 COMMERCIAL APPLICATION: AITC ANTIMICROBIAL PACKAGING

Mitsubishi-Kagaku Foods Corporation

Mitsubishi-Kagaku Foods Corporation (Japan) distributes a number of forms of antimicrobial packaging based upon allyl mustard oil derived from wasabi (Japanese horseradish) or mustard under the trade name, Wasaouru (http://www.mfc.co.jp/wasaouro/e/index.html).

In 2004, Mitsubishi-Kagaku Foods Corp, a wholly-owned unit of Mitsubishi Chemical Corp, agreed to acquire the Wasaouro business of CAREX Inc (Japan) from Mitsubishi Chemical Holdings Corporation. Carex Inc was a manufacturer of antibacterial and freshness products.

The active ingredient (AIT) is available in a variety of formats including sheets, labels, films and water soluble for use in a number of Ready-to-Eat (RTE) Japanese foods (Table 22). In Japan, the use of AIT in food packaging is allowed only when this compound is extracted from a natural source (Perez-Perez et al., 2006).

In this system, AITC is temporarily impregnated in the adhesive layer of the label. To prevent premature release of the antimicrobial volatile, the adhesive layer is protected by a high barrier film, such as metalized polyester. To activate the label, the protective film is removed and the label is affixed to either the exterior or interior of a food package, depending on the AITC permeability of the package structure.

Allyl isothiocyanate (or Volatile Oil of Mustard-VOM) received FDA approval as a GRAS substance for food shelf life extension applications in 2004 with extended uses approval in 2006.

Table 21 Biopolymer research for controlled release of AITC

Biopolymer	Matrix	Results	Reference
α- and β-cyclodextrins encapsulated pure AITC in l-polylactide -co-polycaprolactone films as slow release additives	Packaged cheese Fungi	5% α-cyclodextrin –AITC films more effective at controlling fungal growth than the β-encapsulated films	Plackett et al, 2008
AITC from finely ground mustard seed powder encapsulated within electrospun fibers	No food, no microorganisms; Studied the release kinetics of AITC from the biopolymer	Electrospun fibers with different PLA:PEO ratios could be used as carriers for controlled release of AITC	Dai & Lim, 2014
Corn oil/gum Acacia microencapsulation Pure AITC	5% and 10% AITC microcapsules in finely chopped beef packed under nitrogen, stored at 4C for 18 days *Escherichia coli* 0157:H7	AITC-gum Acacia microcapsules producing > 1000ppm AITC effectively reduced growth of *E.coli*	Chacon et al, 2006b
Pure AITC (>95%) Gum Arabic aqueous solution Chitosan Tween 80 Spray dried mixture to form microparticles	AITC microparticles added to Kimchi (fermented vegetables) Purpose was to control the growth of the fermentation bacteria to prevent off-flavours and enhance shelf life	Gum Arabic-AITC microparticles at loadings <0.1% (w/w kimchi) effective in eliminating excess bacterial growth without reducing product sensory quality after 15 days storage at 4C and 10C	Ko et al., 2012
AITC - -cyclodextrin complex followed by encapsulation in electrospun poly(lactic acid) and soy protein isolated ultrafine fibers.	Studied release kinetics under different conditions of relative humidity and moisture.	Suggested moisture could be used to activate release of AITC from PLA/SPI fiber carriers.	Vega-Lugo and Lim (2009)
Edible film comprised of chitosan, AITC, barley straw arabinoxylan	Microemulsions obtained by high pressure processing (HPP) used to form the composite films Thin films added to non-food system and to surface of RTE turkey slices inoculated with *Listeria monocytogenes*	HPP enhanced antimicrobial activity of AIT. HPP –AIT films could reduce L. monocytogenes by 4 logs in non-food system Bacterial populations reduced slowly by about 3 log cycles over 35 d storage; food composition affects efficacy of AIT	Guo et al, 2015
Poly(lactic acid) fiber of submicron	Grapes and ready to eat (RTE)	AITC released in time-dependent manner	Kara et al, 2015.

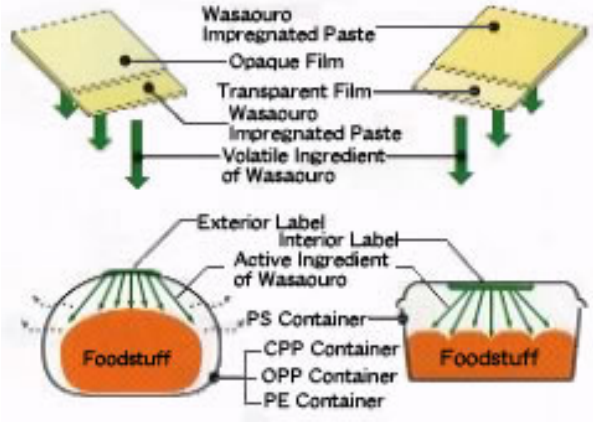
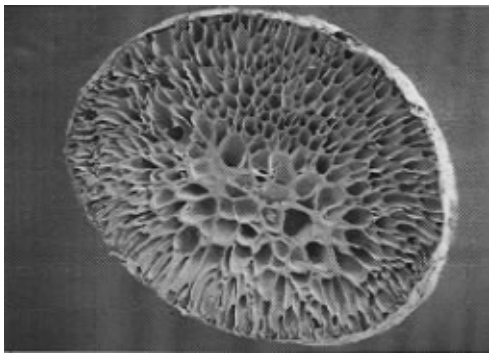
size encapsulated AITC and electrospun grafted onto PLA films	deli turkey meat Inoculated with E.coli K12 or Listeria innocua.	over 3 weeks. Released AITC suppressed growth of both E.coli and L. innocua by at least 2 logs over a 40h test period at 22C	
Biodegradable film comprised of AITC and polylactic acid (PLA) and sugar beet pulp	Studied storage and handling of films to evaluate stability over time Liquid test tube method used to test AITC activity against *Salmonella* Stanley H0558	Regardless of storage conditions and handling all films inhibited *Salmonella* growth during 24h at 22C. Difference between maximal and minimal microbial inactivation was less than 0.5 log cycles	Li et al, 2012
AITC (pure) or deodorized oriental mustard extract in edible k-carrageenan/chitosan based coatings	4 strain *Campylobacter jejuni* cocktail or *Salmonella* cocktail Vacuum-packaged fresh chicken breasts dipped in AITC-edible coating and stored at 4C.	k-carrageenan/chitosan based coatings containing > 50 ul/g AITC eliminated C. jejuni after 5 d at 4C and > 300mg/g oriental mustard reduced C. jejuni up to 3.75 log. For *Salmonella* , coatings containing 250mg oriental mustard extract/g or 50ul AITC/g reduced *Salmonella* on chicken breasts by 2.3 logs at 21 d at 4C. When EDTA added to coating, *Salmonella* reduced by 2.3 log at 5 d and 3 log at 31d.	Olaimat et al., 2014, 2015.

Two GRAS submissions have been made to the US FDA for notice of a GRAS Exemption Claim for “Volatile oil of mustard (VOM) as a shelf life extension and anti-spoilage agent for certain foods”. This first submission by Carex Inc (Japan) made on June 27, 2003 was reviewed and a “Letter of No Objection” issued by the FDA on January 4, 2004. The submission was “for use as a shelf-life extension agent or anti-spoilage agent in meat, fish, shellfish, and poultry products and in baked pies at levels that would typically be 150 micrograms per liter of air in a food container system.” A second GRAS submission to the US FDA was made by Mitsubishi-Kagaku Foods Corp. in 2005 for “Allyl Isothiocyanate in a food shelf life extension and anti-spoilage system” for similar methods of delivery as the 2003 submission but extended to additional uses including a) foods in food storage packages for use in home b) in food service establishments in restaurants, cafeterias and grocery stores and c) in farm fields to pack raw agricultural commodities.

Two delivery technologies are described in the GRAS notifications, each delivering similar levels of VOM (122-203 µg/L) into the air within the atmosphere of the food container. In the first method, a patch, made of polyethylene (or other conventional polymer) and containing VOM at a specified level (up to 5.4 mg VOM), was affixed to a flexible polymer by means of an adhesive or by heat-sealing. Mitsubishi states that the adhesive will comply with FDA's regulations concerning adhesive components (21 CFR 175.105). In the second method, the VOM was contained within layers of flexible film. In either method, the VOM will be maintained within microcapsules that dissolve when the humidity within the package reaches 70% or above, thereby releasing the VOM. Small amounts of VOM may deposit on the surface of the food or be absorbed into the food item. If the VOM is absorbed by the food or escapes through the packaging material, then the patch will release more VOM in order to maintain a steady state concentration of 122-203 µg/L in the packaging atmosphere. According to Mitsubishi, the proposed use of VOM is self-limiting and below that at which organoleptic changes occur.

Note, in the “Letter of no Objection” the FDA did not give approval for the use of VOM in meat and poultry products as this decision must be made by the Food Safety and Inspection Service of the United States Department of Agriculture (USDA). FDA did consult FSIS for both submissions and it was the conclusion the “proponents for GRN 000133 nor GRN 000180 did not provide sufficient data and information to enable FSIS to determine the efficacy and suitability of VOM for the intended use as a shelf-life extension agent or anti-spoilage agent in meat and poultry products”.

Table 22 Wasaouru Products Forms (http://www.mfc.co.jp/wasaouro/e/products/index.html)

Product Form	Applications	Photo
Sheets	TN and RN sheets for placing over RTE food boxes.	
	FN sheet: available in film-format or a Greenleaf Wasaouru-plastic disguised as salad garnish	
Labels	Exterior label attached to outside of gas-permeable wrapping material (cast polypropylene, oriented polypropylene and polyethylene) and used for cakes and breads Interior labels attached to underside of polystyrene and other containers.	
Beads	Cellulose beads for use in marine products and processed marine products, Releases mustard extract at a constant rate. Made of porous cellulose spheres from quality pulp and impregnated with Wasaouru, then covered in specially processed viscous paper to ensure gradual release.	

Powder and emulsion	For fish, shellfish, seaweed and other marine products. Delivers protective action in a water environment. Emulsion type can be used as additive or as spray with wrapped products or dissolved in water that food is place in.	
Home products	Greenleaf, sheets for lunch boxes and for specific Japanese foods (omochi-rice cakes)	

4.5.3 RESEARCH OPPORTUNITIES

Antimicrobial films will be targeted towards niche food sectors where shelf life and food borne illness are major concerns. Uptake will primarily be by big food industry manufacturers in the meat, poultry and fish sector and horticulture industries who can afford the technology and who have global markets and distribution systems.

Food packaging is highly regulated and packaging developers and manufacturers must ensure the antimicrobial agent and the packaging matrix for an intended food use is safe for consumers, regardless of the source of the antimicrobial agent (natural, chemical or synthetic)

Approved food-grade ingredients must be used. All countries have their own regulations for packaging components and permitted additives.

Netramai et al (2012) identified potential research opportunities and critical successf factors needed to support and encourage the widespread adoption of antimicrobials and AITC in active packaging applications including the following:

- A better understanding of how food components (protein, fat, carbohydrate, acidity etc) may enhance or hinder its antimicrobial activity

- Studies on how the stability of AITC/mustard extracts are affected by food processes, storage, handling and transportation of the food and/or the packaging material itself
- Investigations into the effect of other processing or preservation techniques on stability and efficacy of AITC
- Development of active delivery systems in the package to optimize the distribution and contact of the antimicrobial in the food

Critical Success Factors for Antimicrobial Films

- Regulatory approval in North America and Europe
- Sound scientific evidence of efficacy in targeted foods: for instance, a significant log reduction in number of food-borne pathogenic organisms or spoilage organisms or an extended shelf-life
- Buyer will have to see value
- Must increase efficiencies in some part of the supply chain
- Consumer acceptance: antimicrobial agent should have no effect on sensory characteristics of the food
- Must prove that any added cost is recoverable
- Used as part of an overall food safety hurdle strategy, not as a “magic bullet”

4.6 Mustard as a Pest Control Agent

There is renewed interest in investigating economically viable alternatives to chemical pesticides on food crops due to consumer pressure for less pesticide usage, the restriction or banning of traditional chemicals (e.g. methyl bromide as a soil fumigant which occurred in 2015), the decline in development of new actives and the drive for sustainable agricultural practices.

Biofumigation, a term coined over 2 decades ago, can be considered as a natural alternative to chemical fumigation. Plants in the *Brassica* genus have been used as cover crops and incorporated as green manures in vegetable and green house production systems to suppress common soil borne pathogens, nematodes and weeds (Haramoto and Gallandt, 2005; Norsworthy and Meehan, 2005). Commonly used biofumigant plants include brown mustards, white mustards and radishes as they are known to have high levels of glucosinolates. It is the isothiocyanates derived from glucosinolates in *Brassica* species that are considered the most active compounds although other compounds such as non-glucosinolate sulphur containing compounds, fatty acids, nitriles and ionic thiocyanates may also be effect pest control agents. Biofumigant crops can be used in a number of different ways for disease control: intercropping and rotations; incorporation of biofumigants crops into the soil; incorporation of Brassica seed meals (as liquid or granular/powders) into soil; and as green manures and trap crops (Clarkson et al., 2016).

Seed meals from *Brassica napus*, *B. juncea* and *Sinapis alba* have been shown to have biofumigation properties against weeds such as shepherd's purse, kochia and green foxtail, nematodes and soil borne pathogens (Melander et al, 2004).

There has been considerable research on the use of mustard greens, meals and allyl isothiocyanate (AITC) as a fumigant in the United States (e.g. CSNAR, 2016; University of Idaho, 2016), Europe and Australia. In Europe, the EIP-AGRI[15] initiative has one research program focused on how to control soil-borne diseases (EIP-AGRI, 2014). In Australia, biofumigants are being investigated as part of Horticulture Innovation Australia's Soil Wealth Program (www.soilwealth.com.au).

[15] EIP-AGRI: European Innovation Partnership "Agricultural Productivity and Sustainability" aims to find innovative solutions to challenges facing rural areas. http://ec.europa.eu/eip/agriculture/sites/agri-eip/files/fg13_soil_borne_diseases_starting_paper_2015_en.pdf

Commercial pest products made from mustard are available.

A Canadian example is the MustGrow product developed in Saskatchewan. MPT Mustard Products and Technologies (MPT) has commercialized a dry granular product “MustGrow” for use as a pre-plant soil treatment for the management of soil-borne nematodes and soil-borne diseases. MustGrow is manufactured from *Brassica juncea* (Oriental mustard) grown in Saskatchewan and is formulated to contain 3.5-4.0% AITC (MPT, 2013). Its main features include the following:
a) Its production from 100% natural de-oiled mustard seed oil,
b) For use in organic and conventional production
c) Biodegradable and environmentally safe and
d) Listed as an OMRI (Organic Materials Review Institute) fertilizer.

MPT has 3 products registered in the United States as fertilizer, turf product and pre-crop soil fumigant. In Canada, MPT has MustGrow products registered as a fertilizer and crop fumigant for management of red stele in strawberries, phytophthora root rot in raspberries and root lesion nematodes in strawberries and raspberries (MPT, 2013).

Discussions with Jeret Bode, General Manager, MPT (personal communication, July 2016), indicated the company is actively searching for partnerships/merger/sale to aid in commercialization and market expansion of products derived from MPT’s patented technologies. Currently, MPT has about 9000 tonnes of granular product distributed by Agrium and about 2 tonnes of a newly developed liquid product. The liquid product consists of a concentrated form of the glucosinolate plus the myrosinase enzyme suspended in glycol which when mixed with water can be delivered via a drip system to growing plants.

However biofumigants based upon pure AITC are also available and have been approved as “oil of mustard” products in the US since 1962. Isagro (USA) (Italian based parent company) gained U.S. EPA approval in 2013 for its “Dominus” biopesticide and is an emulsifiable concentrate containing 96.3% or 99.8% AITC (Isagro-usa, 2013) for use on strawberries, tomatoes, peppers, berries, cucurbits, melons and ornamentals.

Future Research Needs

From the various research groups involved in biofumigant research, the following topics generally reflect the research interests of this emerging industry:

- Developing standardized methods for measurements of GSL’s and ITCs.

- Identifying the most appropriate biofumigant for a particular target pathogen and evaluating its efficacy under laboratory and field conditions
- Understand the agronomy behind biofumigant crops/meals; e.g. seed rate, fertilizer applications, and machinery to optimize maceration and incorporation.
- Breeding of biofumigant crops to enhance the active agent(s)
- Collaborating with industry to develop stable, effective commercial products

4.7 Health Benefits and Therapeutic Uses

While mustard has been used as a food since ancient times, it has also been used as a medicine in Ayurvedic and traditional Chinese medicine practice to relieve joint pain, fever, alleviate cough and colds, and lessen swelling. Mustard oil has been used for the treatment of various skin diseases and wounds (Mazumbder et al, 2016) with its medicinal properties being attributed to the glucosinolates and the isothiocyanate products (Challenger, 1959).

Of the two main glucosinolates in mustard cultivars, sinigrin, from *Brassica juncea* and its ITC, allyl isothiocyanate, have been most studied. However, as noted previously, glucosinolates can be sourced from many cruciferous vegetables with more than 120 glucosinates being identified. ITCs from these various sources show interesting chemopreventative activities against several chronic-degenerative diseases including cancer, cardiovascular disease, neurodegeneration and diabetes.

The therapeutic benefits of ITCs being studied include their anti-cancer activity, their anti-inflammatory activity, antibacterial and antifungal activity, antioxidant activity, and wound healing properties.

There have been extensive studies evaluating how ITC functions in cancer cell systems. ITCs modulate a large number of cancer-related targets or pathways (Kumar, 2013; Hayes et al, 2008) including:

- Inhibition of CYP enzymes
- Induction of phase II enzymes via activation of NF-E2-related factor 2(Nrf2)-involved in expression of wide array of antioxidant genes-perhaps plays a role in all pathological conditions being investigated.
- Modulation of cell cycle regulators
- Induction of apoptosis
- Inhibition of nuclear facter kappa B-regulates expression of pro-inflammatory cytokines.- ITCs anti-inflammatory activity
- Inhibition of microtubule polymerization,
- Inhibition of metastasis

These strategies open up a new therapeutic approach from cancer prevention to cancer treatments. There has also been a focus on synergistic approaches of ITCs with other existing anti-cancer drugs; on drug delivery strategies using nanoparticle/carriers to lower dosage, and more information about the action of ITCs.

Some examples of the types of cancers being investigated are summarized below:

- Bladder cancer development and recurrence (using pure AITC from Sigma Chemicals) –*in vitro* evaluated in bladder carcinoma lines and *in vivo* using two rat bladder cancer models. Results: AITC selectively delivered to bladder cancer tissue through urinary excretion and potently inhibits bladder cancer development and invasion partly due to its ability to elicit cell cycle arrest and apoptosis (Bhattacharya et al., 2010)
- AITC may selectively target cancer cells as it has also been shown to be significantly more toxic to prostate cancer cells and colon cancer cells than to normal prostrate and colon cells (Musk et al, 1993; Xiao et al, 2003). Mechanism is unknown.
- Savio et al, (2014, 2015) studied the effect of AIT on the TP53 mutations in bladder cancer cell models. Tp53 mutations are the most common alterations in bladder cancer cells and are related to cellular transformation, malignancy and high recurrence rate of urinary bladder cancers. Found that AIT caused cell cycle arrest, increased apoptosis rates and showed varying genotoxicity depending on TP53 status. Mechanism of action also depends upon the cells lines being studied. Need more work on other molecular targets as there could be other intrinsic genetic alterations not identified in this study
- In a small human intervention trial, found that consumption of a single, diet-related amount of hot mustard (20 g of mustard preparation per day on bread) containing 25mg total ITC could strongly reduce cell sensitivity towards direct –acting genotoxins. However a larger intervention trial and validity verified by another cytogenetic marker is needed to confirm the effects (Lamy et al, 2011)

Dietary consumption of AITC appears to be several orders of magnitude lower (10ugAITC/kg bw) than doses used in animal genotoxicity studies (90 and 270 mg AITC/kg bw). The most potent products of myrosinase-mediated hydrolysis of GLS yields are the isothiocyanates which include allyl isothiocyanate (from sinigrin in cabbage, mustard and horseradish), benzyl isothiocyanate (BITC from glucotropaeolin in red cabbage), phenethyl isothiocyanate (PEITC from gluconasturtin in watercress and wasabi) , phenylhexyl isothioscyanate, erucin and sulforaphane (SFN) from glucoraphanin in broccoli, cauliflower, brassicas and kale) as chemopreventative agents. There is concern that some of these ITCs exhibit genotoxic effects (PEITC and BITC) whereas the researchers indicated that only AITC exhibits protective effects at levels of dose lower that those resulting in genotoxic effects. Further studies are needed to characterize the biological and toxicological effects of the different ITCs. Concern has been raised in the health community because the scientific literature and popular press are highlighting the benefits of increased consumption of foods and dietary supplements enhanced with ITC (Fimognari et al, 2012).

Further examples of the therapeutic benefits of mustard include:

a) White mustard (*Sinapis alba*) is a traditional Chinese medicine used widely for prevention and treatment of asthma and bronchitis by topical application administration in the summer. Studies are underway to better understand the mechanisms of action underlying its immune regulation activity (Guo et al., 2013)
b) Preliminary investigations using rat models showed that rats consuming ground mustard seed (*Sinapis alba*) fed at 5% of a standardized rat diet exhibited less severe symptoms of induced psoriasis than control group. Although this requires further work, it was suggested this could be a future treatment for psoriasis (Yang et al, 2013).
c) Eskin and coworkers (2007) at the University of Manitoba used the water soluble novel mucilaginous fraction from *Sinapis alba* to determine if it had anticancer properties by measuring the ability of mustard mucilage to prevent colonic preneoclastic lesions (aberrant crypt foci (ACF) in two rodent models associated with sporadic and obesity associated colon cancer. They found that feeding a diet containing 5% water soluble mucilage decreased the number of total and large ACF in the both rodent models. Further studies are required to determine the underlying mechanisms of action

What is less well studied are the potential health benefits of consuming mustard, mustard proteins, bran or mustard oils or other bioactives as part of balanced diet.

Mustard Oil

Dhara and coworkers (2013) evaluated the dietary effects of a diacylglycerol rich mustard oil. Diacyl glycerol (DAG) oil is effective in preventing body fat accumulation and obesity related disorder. DAG ingestion reduces postprandial hypertriglyceridemia compared with triacylglycerol (TAG) ingestion. Mustard oil (MO) (*Brassica juncea*) contains 8-9% saturated fatty acid and 88-91% unsaturated fatty acids in which 48-50% are erucic acid (C22:1). MO also contains 2 essential fatty acids (EFA) and tocopherol. MO could be a potential raw material to produce low-calorie healthful edible oil. In this study, mustard oil was converted into DAG rich mustard oil by enzymatic glycerolysis. The researchers found that DAG rich MO reduced body weight, lowered the plasma leptin content (leptin is an antiobesity hormone), and reduced atherosclerotic factors such as plasma TAG, and non-HDL cholesterol.

Mustard bran and yellow mustard mucilage

If mustard bran is to be considered as a dietary fibre, its physiological benefit must be determined. This report identified 3 studies investigating the health benefits of consuming mustard fiber or whole mustard powder:

1) Lett and coworkers (2013) evaluated the effect of consuming soluble fibers (guar gum, pectin, locust bean gum, xanthan gum, psyllium mucilage and yellow mustard bran (YMB)) in glycaemic modulation and attenuation of post prandial glycaemia. This was the first study investigating how YMB could lower the glycaemic response of carbohydrate-rich test meal and increase satiety in 10 young healthy male adults (mean age 21.1 years).The findings revealed that consumption of 5 g yellow mustard bran consumed in potato or leek soup significantly reduced peak blood glucose, significantly delayed average time for glucose curve to peak and resulted in significantly different mean blood glucose levels at 15, 30 and 90 minutes.
2) Kay (MSc thesis, 2016) investigated the effect of soluble dietary fibers such as yellow mustard mucilage (YMM), fenugreek gum (FG) and flaxseed mucilage (FM) when added to pudding on acute postprandial glycemic response in fifteen adults at risk for type 2 diabetes. Kay's study was part of a larger study to identify the physicochemical properties and *in vitro* gastroinstestinal behaviour and glycemic response of underutilized dietary fibers from agricultural by-products. YMM, FG, and FM have potential to modulate post prandial glycemic response in person at risk for type 2 diabetes in a randomized, double-blinded, cross-over intervention. Puddings matched for *in vitro* viscosity as viscosity impacts the glycemic response of a meal. Snack food was effective in improving some, but not all, acute glycemic and insulinemic markers of T2D risk.
3) Yadav et al, 2004 postulated that consumption of a diet containing 10% *Brassica juncea* seed powder could play a role in management of pre-diabetic state of insulin resistance. This research was based upon a rat model.

Mustard Protein

There are virtually no studies evaluating the nutritional value (e.g. protein quality, protein digestibility), safety (toxicology) or dietary exposure of mustard protein to humans which is important in determining its suitability as a food ingredient and how it can be differentiated from other plant proteins.

Sarker et al. (2015) evaluated the protein digestibility of three forms of protein: a) protein in raw mustard cakes, b) protein free of AITC and c) precipitated protein isolates of black and yellow mustard cakes. They found *in vitro* protein digestibility values were higher for the protein isolates than for the mustard cakes, hypothesizing that the presence of the fiber and glucosinolates may have inhibited digestibility.

The European Food Safety Authority (EFSA) evaluated the nutritive value of the *Brassica napus* protein isolate ``Isolexx`and found it sufficient for human nutrition (EFSA, 2013). Studies similar to the ones used in this canola protein safety assessment would be needed to support the nutritional value of mustard protein.

4.8 Other Research Initiatives

During this project a number of research publications exploring different attributes of mustard components and extractions were found. Brief summaries are provided below:

- Investigating methods of extraction and quantification of sinigrin and total isothiocyanates in mustard seed to determine the most efficacious method for future use in testing as an antimicrobial agent and anticancer activity (Cools and Terry, 2012)
- Developing strong-ion exchange centrifugal partition chromatography processes for purification of sinalbin from white mustard seeds. A high performance separation technique on an industrial scale is needed to obtain sinalbin which can then be used as a pure reference standard for pharmacology studies. Glucosinolates are scarce mainly because they are difficult to isolate in large quantities and high purity to examine biological activity of intact GLS in anti-cancer research (Toribio et al., 2009)
- Development of alternative processing methods to inactivate myrosinase activity in mustard seeds without affecting physiochemical or functional properties
    - Use radio frequency treatments (a non-thermal process) to treat yellow mustard flour and inactivate the myrosinase enzyme. (Cserhalmi et al., 2001; Ildiko et al., 2005.)
- Utilization of mustard waste isolates for improved production of astaxanthin by *Zanthophyllomyces dendrorhous*.
    - Astaxanthin is a valuable keto-carotenoic pigment widely used as a food colourant and in cosmetic and medical applications due to high antioxidant activity. While produced by chemical synthesis, there is demand from food industry for natural sources. However, no commercially viable processes are developed to produce significant quantities. It may be possible to use this microorganism because astaxanthin accounts for 80-90% of the total carotenoid compounds it produces. One strategy to increase yield and reduce costs is to use low-cosy by-products and residues of the agro-industrial origin (e.g. molasses, grape juice, peat hydrolysates etc). Mustard wastes from the industrial production of allyl isothiocyanate were investigated. Ten tons of mustard seed produces 70 tons of mustard waste suspension (in water) which is usually discarded. The process generates 2400 metric tons of waste per year so finding a value added use is economically beneficial, as well as reducing environmental impact. It was found that hydrolyzed mustard waste precipitate at a medium concentration of 10% was an effective substrate for microbial growth and good production of astaxanthin (Tinoi et al, 2006)
- Using plant extracts for inhibition of metallic corrosion as plant based inhibitors due to their non-toxic nature, abundant source, low cost, biodegradability and non-bioaccumulative properties.

  - Looking for eco-friendly inhibitors to HCl and H_2SO_4, acids which are used in the pickling industry to remove scale. Defatted mustard seed extract was shown to inhibit the corrosion of steel in both 2M HCl and 1 M H_2SO_4 solutions (Umoren, 2016).
- Production of oligosaccharides from canola and mustard bran (Yuan et al., 2015) for better utilization of carbohydrates (fiber) in canola meal and mustard bran. These components could be used for the production of prebiotics such as xylanoligosaccharides (XOS) and arabinoxylooligosaccharides (AXOS).Prebiotics are used to support growth of bifidobacteria, enhance immunity activation, are non cariogenic and have dietary-fiber like effects and water retention capacity.

5.0 CONCLUSIONS

Consumer interest in health and wellness and the desire for “natural” ingredients and “clean labels” on food products are prime factors driving the industry to investigate new plant-proteins as a healthy protein alternative to animal proteins and natural alternatives to chemical preservatives, including antimicrobial and antioxidant agents. These trends spur the research community to provide the evidence required to incorporate new ingredients into foods.

It appears Canada is the leader in isolating and characterizing mustard protein and yellow mustard mucilage and investigating the antimicrobial activity of mustard glucosinolates and isothiocyanates in foods and antimicrobial packaging. Researchers at Agriculture and Agri-Food Canada (AAFC) (Drs. Wanasundera and Aluko (now at University of Manitoba (UofM)) and the University of Toronto (Dr. Diosady) have done significant research on mustard protein characterization, extraction and purification. Dr. Wanasundera has been instrumental in determining some of the genes responsible for the mustard protein allergenicity. Dr. Cui (AAFC) and Dr. Eskin (UofM) have lead programs in isolating and characterizing yellow mustard mucilage. The antimicrobial activity of yellow and brown mustards has been extensively studied by Dr. Holley and his graduate students at the University of Manitoba investigating the effect of mustard on and ITC microorganisms in fermented and processed meats and chicken, evaluating methods of delivery and studying ITC mechanism of actions in bacteria. Dr. Lim’s research program at the University of Guelph has investigated how these ITCs can be incorporated into antimicrobial packaging films.

Research opportunities for mustard proteins and yellow mustard mucilage identified in a report for Mustard 21 (2009) remain valid in 2016. These include functionality characteristics and applications in foods, in-depth nutritional profiles, dietary exposure evaluations, optimization of processes to extract and concentrate the proteins or mucilage, safety assessments and defining health benefits for human consumption.

Glucosinolates from the Brassicaceae family (Brussel sprouts, turnip, radish, mustard etc) are of interest to many groups globally: investigating the antimicrobial, antioxidant, anticancer, anti-inflammatory and wound healing properties of the isothiocyanates in *in vitro* and *in vivo* experimental models.

For the food industry, the two main uses for the isothiocyanates from mustard: allyl isothiocyanate (AITC) from brown/oriental mustard and *para*-hydroxybenzyl isothiocyanate (*p*-HBITC) from yellow/white mustard will be as antimicrobial agents and antioxidants to be added to foods directly or incorporated into packaging films. The primary challenge for AITC and p-HBITC use in foods will be the impact on the sensory profile of the foods. While these ITC are effective against pathogenic microorganisms, they impart strong flavours and aromas which negatively affect consumer perception. Other factors to be considered include regulatory oversight, enhancing antimicrobial efficacy, determining validation methods to confirm antimicrobial activity, effective delivery mechanisms and keeping the antimicrobial form as close as possible to the natural source form (e.g. white mustard essential oil versus the purified ITC, *p*-HBITC).

In the last two decades there has been considerable interest in learning about phenolics in commodity crops (e.g. pulses, cereals, oilseeds) and the associated positive benefits. Research into mustard phenolics is very much in its infancy with a limited number of studies investigating the extraction, identification and quantification of phenolics and determining the antioxidant activities, including how they work in different food systems.

Mustard meals are being used as natural pest control agents, also called biofumigants, in the control of nematodes and fungi in horticultural crops and turf grass. The challenge is finding a commercial champion with the financial resources to market the products.

Regardless of the depth of research conducted on mustard proteins, mucilage, or bioactive components, the key to successful commercialization for any application will require engaging and partnering with large multinational companies with the interest, experience and financial resources to commercialize the product. Critical factors for success will include regulatory approvals (if needed), sound scientific evidence of efficacy, consumer acceptance, cost, and customer value. For the food industry part of the challenge will be the recognition that the use of ITC in food will be as a component of an overall food safety hurdle strategy and not as a single “magic bullet”.

Until industry champions emerge, the challenge for mustard ingredient companies and, indirectly mustard producers, will reaching out to new users and describing the functional benefits of using mustard in food products beyond condiments.

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APPENDIX 1 SOY AND MILK PROTEINS

Extracted, with permission, from Mustard 21 2009 Report

Models of Soy and Whey Proteins

As the development of mustard proteins is very much in the emerging stage, this report uses soy and whey proteins as examples of two successful proteins that currently dominate the food market. These proteins could be used as models to design R&D activities and fast track market opportunities for mustard protein. However, to attain this level of information will take decades of work, significant resources and industry partners.

A1-1 Soy Protein

(Decker, 2001)

Soybeans are 30 percent carbohydrate (of which 15 percent is fiber), 18 percent oil (85 percent unsaturated), 14 percent moisture and 38 percent protein. They are categorized as a high-quality, complete protein. Soy's nutrition benefits include being good sources of phosphorus, potassium, B vitamins, zinc, iron and the antioxidant vitamin E.

Soybeans are used as whole beans and full-fat flours as well as being separated into fractions of oil, protein and hulls. Figure A1-1 outlines, in broad terms, soybean processing and potential applications. All fractions have extensive use in the food and industrial sectors. Tables A1-1, A1-2 and A1-3 list the myriad of product applications for all forms of soy ingredients.

Three Major Processed Soy Products

The initial steps in soybean processing include cleaning, conditioning, cracking, dehulling and rolling into flakes. The next step is to remove the soy oil from the flakes. The flakes are then dried, creating the "defatted soybean flakes". This defatted material is the basis for the three major soy protein product categories: flour, concentrates and isolates.

Soy flour is the most basic soy protein product made by grinding dehulled, defatted soybean flakes. Soy flour is approximately 50 percent protein by weight and has the characteristic "beany" flavour associated with the carbohydrate portion of soybean.

Soy protein concentrates (approximately 70% protein) are made by removing a portion of the carbohydrates from defatted and dehulled soybeans. Alcohol extraction is the method most commonly used to manufacture soy protein concentrates even though the method results in the loss of isoflavones. The amounts of alcohol-soluble compounds, such as isoflavones and saponins, that remain in the soy concentrates depend on whether water or alcohol was used in

the extraction process. Soy protein concentrates retain most of the fiber in the original soybeans and must contain at least 65% protein on a moisture-free basis. They are often used as a functional or nutritional ingredient in a wide variety of food products.

Isolated soy proteins are prepared through a process using water extraction and minimum heat on soy flakes. The product is nearly carbohydrate and fat-free, with no characteristic "beany" flavour. Soy protein isolates prepared this way retain the naturally occurring bioactive components and are 90 % protein by dry-weight. Isolated soy proteins are used in soy-based infant formulas and are widely used as a nutritional, functional or economic alternative to traditional proteins in food bars, beverages, baked goods, breads, cereals, poultry, red meat, and seafood products.

Texturized soy proteins (TSPs) are produced from soy flour, soy protein concentrate or isolated soy proteins through extrusion of soy flour under moist heat and high pressure giving products of many sizes, shapes, colours and flavours (meat patties, soups, vegetarian meat analogs, granolas, cereals, protein bars and pet food). Combining soy proteins with starches and other powdered proteins, such as wheat gluten, produces unique textured products that simulate ground meats or meat chunks and strips.

Hydrolyzed vegetable proteins (HVPs) are soy proteins hydrolyzed with enzymes and/or acid and heat and are used as flavor enhancers in foods (Decker, 2001).

The worldwide application of soybean proteins is due to its superlative functionality. Typical functional properties include gelation, emulsification, foaming, cohesion-adhesion, elasticity, viscosity, solubility, water and fat absorption and water and flavour binding. Properties depend upon physiological properties, environmental factors and technological conditions. The soybean proteins (glycinin and β-conglycinin) show different functional properties when they interact (Lampart-Szcazapa, 2001). Table A1-4 identifies the functional characteristics of soy flours, protein concentrates and protein isolates and soy whey.

Soy Health Benefits

Studies have demonstrated that soy or its biologically active components may play a beneficial role in many health-related conditions including cardiovascular disease, certain types of cancer, osteoporosis, diabetes, hypertension, cognitive function, menopausal health and more recently, obesity (Schryver, 2002; Isanga & Zhang, 2008). Two main components in soy---protein and isoflavones---have received the majority of research dollars and media attention. In 1999, the U.S. Food and Drug Administration (FDA) approved the health claim that “soy protein, when consumed in amounts greater than or equal to 25 grams per day, may reduce the risk of heart disease (USFDA, 1999). The ability of food companies to make a soy–heart health claim resulted in a burst of new soy products in the market place.

Soy isoflavones have also been extensively studied over the past twenty years for their potential health benefit in reducing or altering some risk factors associated with cardiovascular disease, relieving menopausal symptoms, contributing to bone growth and stabilization and as a possible chemopreventative agent (Endres, 2001)

Figure A1-1 Soybean Products and Applications (Endres, 2001)

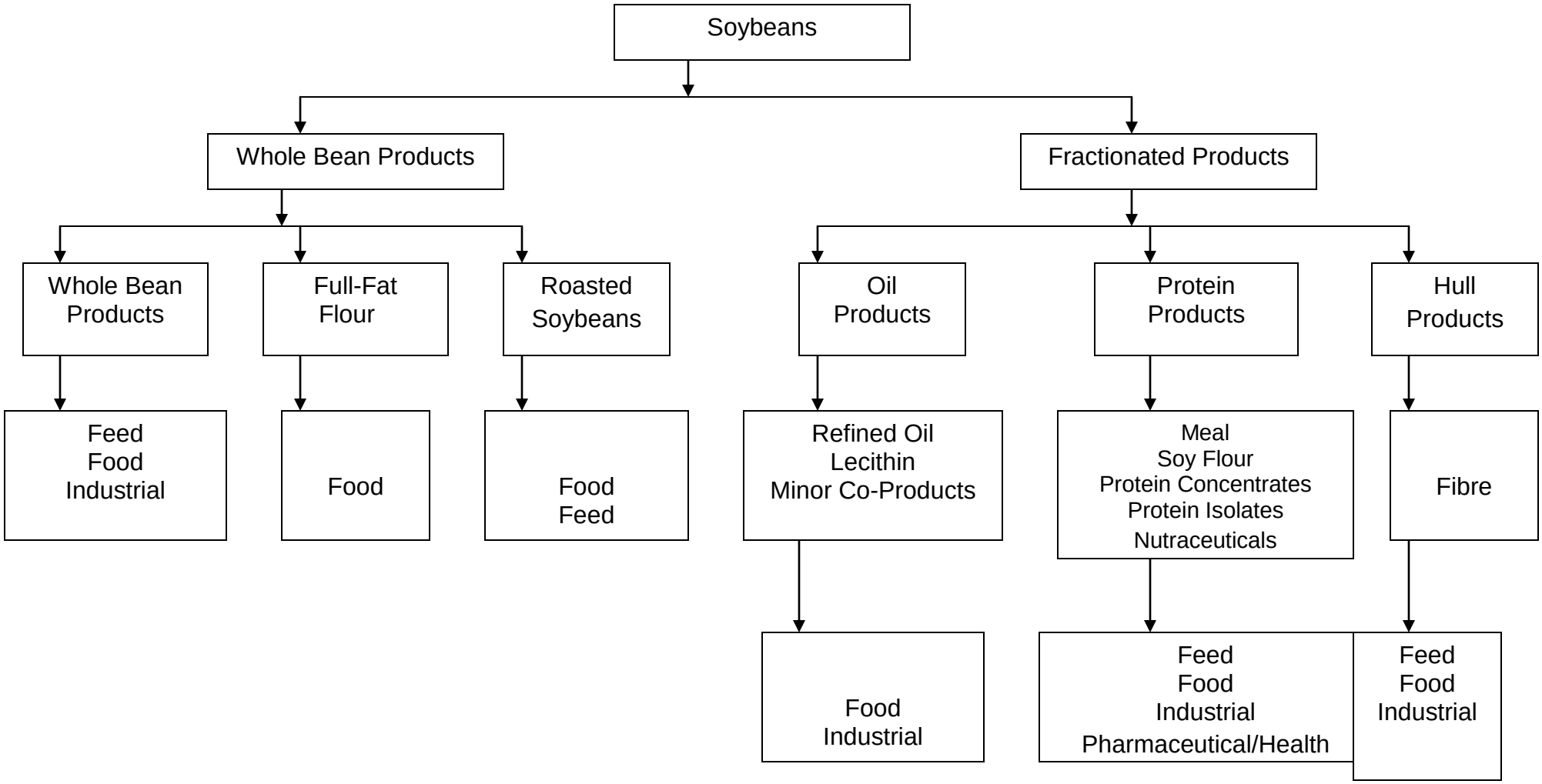


Table A1-1 Soybean Applications: Whole Form*

Soybeans					
Whole Bean Products			Full-Fat flours		Roasted Soybeans
Feed	Food	Industrial	Food	Food	Feed
Swine feed	Fresh green soybeans Baked soybeans Bean sprouts Soymilk Tofu Miso Natto Edamame Tempeh Other Asian soyfoods	Bean-stuffed objects	Bread Candy Doughnut mix Frozen desserts Pancake flour Pie crusts Sweet goods Low-cost gruels Instant milk drinks Crackers	Candy Confection Cookie mixes Cookie toppings Cracker mixes Fountain toppings Soy 'coffee' Soynut butter Diet foods Snack foods	Swine feed

*Endres, 2001

Table A1-2 Soybean Oil Product Applications*

Soybean Oil Products				
Refined Oil		Lecithin		Minor Co-Products
Food	Industrial	Food	Industrial	
Cooking oils Salad oils Mayonnaise Medicinal Pharmaceuticals Sandwich spreads Shortenings Filled milks Coffee whiteners Candy Chocolate coatings Frying fats and oils Frozen desserts Cheese dips Gravy mixes Pastry fillings Icings Whipped toppings	Anti-corrosion agents Anti-static agents Caulking compounds Soap Shampoo Detergents Solvents Core oils Lubricants Diesel fuel Hydraulic fluids Waterproof cements Disinfectants Electrical insulations Insecticides Fungicides Herbicides Linoleum backing Oiled fabrics Candles Cosmetics Crayons Printing inks Protective coatings Plastics Tin and tern plate oils Wallboard Dust suppressants Paint removers Epoxies Metal casting agents paints	Margarine Candy/chocolate coatings Dietary supplements Emulsifying agents Nutritional supplements Pharmaceuticals Shortenings Pan release agents	Anti-foam agents Anti-spattering agents Cosmetics Dispersing agents Printing inks Insecticides Paints Synthetic rubbers Stabilizing agents Wetting agents Yeast agents Yeast culture	Glycerol Chemicals Lubricants Structured lipids Antifreeze Printing acids Cements Explosives Cosmetics Fatty acids Soaps Detergents Oleochemicals Structured lipids Sterols Pharmaceuticals Tocopherols Vitamin E Antioxidants

*Endres, 2001

Table A1-3 Applications for Soybean Protein and Hull Products

Soybean Protein Products				Soybean Hull Products
Meal	Soy Flour, Protein Concentrates and Isolates		Bioactives	Fibre
Feed	Food	Industrial	Pharmaceutical/ Health	Industrial/Feed/Food
Calf milk products Swine feed Poultry feed Beef-cattle feed Dairy-cattle feed Bee foods Pet foods Furbearer diets Aquaculture diets	Bakery ingredients Alimentary pastes Beer and ale Noodles Prepared meat products Meat analogs Meat pumping solutions Breakfast cereals Prepared mixes Food drinks Baby food Hypo-allergenic milk Confections Candy products Sausage casings Yeast cultures Imitation dairy products Flavourings Infant formula Salad condiments	Adhesives Plywood Particle board Insecticides Dry-wall tape compound Texture paints Fermentation nutrients Yeast carriers Linoleum backing Antibiotics Paper coatings Fire-fighting foams Fire-resistant coatings Asphalt emulsions Cleaning compounds Cosmetics Printing inks Leather substitutes Water-based paints Plastics Textiles	Isoflavones Saponins Phytic acids Protease inhibitors	Filter material High-fibre breads Cattle roughage

*Endres, 2001

Table A1-4 Functional Properties of Soy Protein Products in Food*

Functional Property	Mode of Action	Food System Used	Product
Solubility	Protein solvation, pH dependent	Beverages	Flour, concentrate, isolate, hydrolysate
Water absorption and binding	Hydrogen binding of water, entrapment of water (no drip)	Meats, sausages, breads, cakes	Flour, concentrate
Viscosity	Thickening, water binding	Soups, gravies	Flour, concentrate
Gelation	Protein matrix formation and setting	Meats, curds, cheeses	Concentrate, isolate
Cohesion, adhesion	Protein acts as adhesive	Meats, sausages, baked goods, pastas	Flour, concentrate, isolate
Elasticity	Disulfide links in deformable gels	Meats, bakery items	Isolate
Emulsification	Formation and stabilization of fat emulsions	Sausages, bologna, soups, cakes	Flour, concentrates, isolates
Fat absorption	Binding of free fat	Meats, sausages, doughnut	Flour, concentrates, isolates
Flavour binding	Adsorption, entrapment	Simulated meats, bakery items	Concentrate, isolates, hydrolysates
Foaming	Forms film to entrap gas	Whipped toppings, chiffon desserts, angel cakes	Isolates, hydrolysates, soy whey
Colour control	Bleaching (lipoxygenase)	Breads	Flour

*Endres, 2001

A1-2 WHEY PROTEINS

Historically whey was considered a dairy waste by-product and thus a disposal problem. With the advent of strict environmental regulations worldwide, the dairy industry had to re-evaluate its waste management policies and processes and find ways to dispose of whey more effectively and economically. In the 1970's, membrane technologies became commercially available that permitted whey to be concentrated up to five fold resulting in a whey product containing 35% protein (Decker, 2001, WCDR, 2002)

Bovine milk contains two different main protein categories: caseins, making up 80% of milk's protein and whey proteins that comprise the remaining 20% of bovine milk. Caseins are defined as those proteins that precipitate at 20ºC or less and have an isoelectric point of pH 4.6. Whey proteins exhibit relative acid stability and heat sensitivity. Casein and whey protein are not interchangeable in food applications as they have different nutritional and functional properties. Figure A1-2 on the following page illustrates how various milk proteins are obtained.

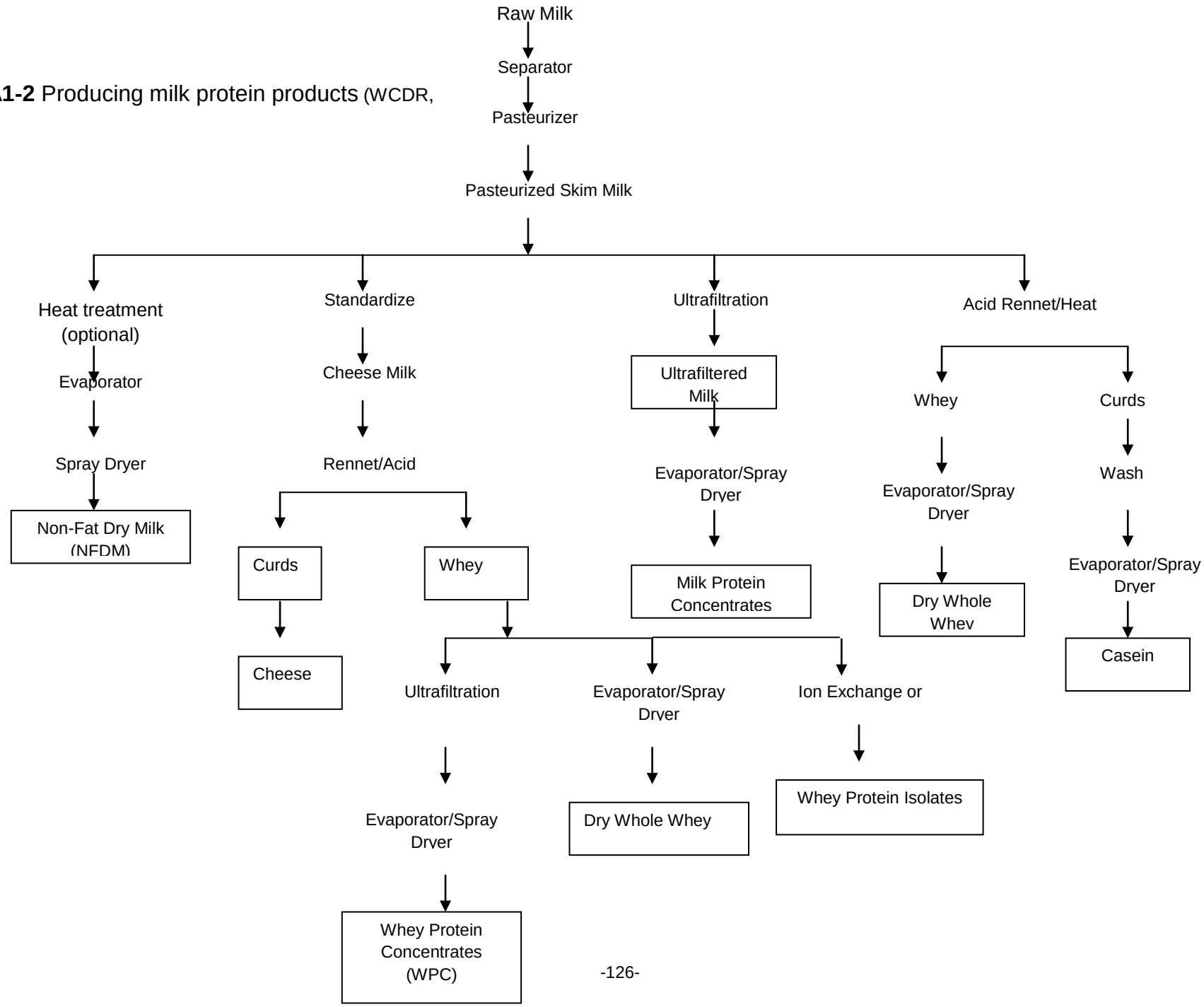
The main difference between whey protein concentrate (WPC) and whey protein isolate (WPI) is their protein concentration but they also differ in how they are processed and their effectiveness as ingredients.

Whey protein concentrates (WPC) undergo an ultrafiltration (UF) process that removes lactose, water and minerals as well as a little bit of fat that comes through in the whey stream. Depending on the amount of water removed, lactose may be left in the concentrated protein. The lower end of the spectrum sees a WPC of about 34% protein and 50% lactose. Reaching the upper end of the scale — WPCs at around 80% protein and 10% to 15% lactose — requires diafiltration in addition to the ultrafiltration. Higher-protein WPCs — at around 80% — may become ingredients in meat products where gelation properties and moisture-binding texturize the meat and improve its mouthfeel.

Whey protein isolates (WPI) are even more concentrated containing 87% to 92% protein. The WPI are processed in two ways-via an ion exchange process where the positive charges on the ion exchange resins traps the negatively charged whey proteins or using a microfiltration system where the membrane pore size permits proteins to pass through, but leaves the fat behind. Passing the WPIs through a microfiltration system retains the bioactive proteins within this fraction whereas ion exchange causes some bioactive proteins to leach through. WPIs' have a higher concentration of bioactive compounds, such as alpha-lactalbumin,

β-lactoglobulin, GMP, lactoferrin and immunoglobulins. This results in some differences in the respective protein profiles and functionalities but generally,

Figure A1-2 Producing milk protein products (WCDR, 2002)



protein isolates and concentrates are both acid-stable and soluble, with excellent foaming, gelation and emulsification capabilities.

WPI are considered to be high quality proteins that are used in products designated for the body builders, sports nutrition “devotees”, infants and hospital patients who need high protein diets.

Table A1-5 examines milk proteins and applications.

Table A1-5 Milk Proteins

Protein Type	Composition	Physical/Chemical/Functional	Applications
Caseinates (blander, more neutral flavour than whey proteins	Sodium caseinates	Good solubility at neutral pH, excellent emulsification and aeration capacity, high fat and water binding capacity and freeze/thaw stability	Coffee whiteners Baked goods Whipped toppings
	Calcium caseinates	Good dispersibility Low viscosity High opacity and whitening power Good emulsion capacity	Nutritional beverages Coffee whiteners
	Calcium sodium caseinates	Good dispersibility Controlled viscosity Suspension stability	Frozen desserts Diet beverages Infant formulas
Whey Proteins	Whey Protein Concentrates (WPC)	Heat denaturable Soluble in low-pH foods (a desired attribute) High sulfhydryl and disulfide content Heat coagulability forming irreversible gels with high water holding capacity Foaming capacity High whey protein concentrates (75-80%)	Functionalities comparable to egg proteins Replace dry milk or eggs in many applications including soft cookies, salad dressings, pasta and quiches Binders for meat- and surimi-based products
	Whey Protein Isolates (WPI)	87% to 92% protein Soluble in low-pH foods	Body building products Infant foods Medical foods with high-protein
	Hydrolysed whey proteins		Physiological effects like boosting the immune system or aiding in calcium absorption
Milk Protein Isolates (Total Milk Protein-TMP™)	Comprised of both casein and whey proteins	Highly soluble Low ash concentration minimizes off-flavour contributions Retort stable Good emulsification properties Low or high viscosity Good dispersibility Excellent binding properties	Bakery products Liquid nutritional supplements Nutrient fortification

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APPENDIX 2 XANTHAN

Extracted, with permission, from Mustard 21 Report (2009)

A2-1 XANTHAN GUM

Xanthan is the common name given to the extracellular polysaccharides secreted by bacteria of the genus *Xanthomonas*. The commercial polysaccharide is derived from *X. campestris*. It received U.S. FDA approval as a food additive in 1969.

Xanthan is produced by an aerobic fermentation in batch culture. The bacterial cells are removed prior to the gum being extracted from the broth by alcohol precipitation.

Xanthan gum is the major bacterial polysaccharide that dominates the food market in the field of thickeners, stabilizers, and gelling agents due to the high yields and low production costs (Giavasis & Biliaderis, 2007). It is a well established product (10K-20K tonnes/year) with more than 1600 patents on production and applications in the US alone.

The xanthan structure is based upon a repeating pentasaccharide unit. It is comprised of a cellulose backbone (β-(1-4)-D-glucose) substituted at C-3 on alternate glucose residues with various trisaccharide side chains containing mannose and glucuronic acids. Pyruvate and acetate are minor components, positioned at the terminal mannose residues on the side chains.

Yellow mustard mucilage has many similarities to xanthan gum, particularly its shear thinning behaviour and its ability to synergistically interact with galactomannans. The characteristics of xanthan gum are as follows:

- Produces highly viscous solutions at low gum concentrations
- Pseudoplastic rheology (shear reversible behaviour)
- Soluble in hot or cold water
- Stable over a range of pH and temperatures
- Compatible with and stable in systems containing high concentrations of salt
- Excellent suspension for insoluble solids and oil droplets
- Resistant to enzymatic degradation
- Synergistic increase in viscosity or gel strength when used with galactomannans (locust bean gum, tara gum, modified guar gums)
- Solutions of xanthan gum are not generally affected by changes in pH value. Xanthan gum will dissolve in most acids or bases.
- Xanthan’s shear thinning (thixotropy) and gelation properties are its two most important rheological properties.

Shear thinning means that at high stirring (shear) rates; the viscosity of an aqueous solution containing a low concentration of polysaccharide is reduced. At

low stir rates, viscosity increases (i.e. solutions thicken). The behaviour is reversible. Xanthan exhibits this shear thinning property. This property permits products containing xanthan to be spread, pumped, poured or sprayed.

Xanthan gum also forms thermo-reversible gums when mixed with certain plant galactomannans such as locust bean gum or tara gum, or glucomannans such as Konjac gum. Xanthan gels can also be formed by the addition of trivalent cations or borate anions.

In aqueous solutions, xanthan is used to stabilize emulsions, foams and particulate suspensions. Dry mix formulations such as sauces, gravies and desserts can be heated or refrigerated without losing desirable textural characteristics.

Xanthan-galactomannan mixtures are used to prepare gelled or thickened foods, to control particle sedimentation in juices and drinks and contribute to freeze-thaw stability and reduced syneresis in foods.

Table A2-1: Some Food Applications of Xanthan Gum (Giavasis & Biliaderis, 2007)

Industry	Applications
Dairy	Milkshakes, whipped creams, custards and puddings, yoghurts, beverages, water gels, ice creams, sorbets
Bakery	Dough improvers, pastry fillings
Meat and fish	Canned goods, pâtés
Condiments	Mayonnaise, salad dressings, sauces, soups, frozen and ready-to-eat foods, jams, desserts
Beverages	Pulp suspension, powdered beverages

Table A2-2 Non-Food Applications of Xanthan Gum (Giavasis & Biliaderis, 2007)

Industry	Applications
Animal Feed	Cattle feed supplements, calf milk substitutes,
Agriculture	Fertilizers, herbicides, pesticides, fungicides, agricultural foams
Packaging	Paper and cardboard food-packaging materials
Cosmetics	Toothpaste, creams, gels
Other	Stabilizer in ceramic glazes, mining ores, paints and polishes, non-drip paints and jet printing, explosives, photographic industries, fire fighting liquids Enhanced oil recovery

In Canada, xanthan gum is permitted in salad dressings, and unstandardized foods at levels equal to Good Manufacturing Practices (GMP), in cottage and creamed cottage cheeses, calorie reduced margarines, cream cheeses and cheese foods, mustard pickles, relishes, ice cream mixes, ice milk mixes, sherbet and cream for whipping. Usage levels range from GMP to actual maximum limits i.e. 0.02 to 0.5 %, depending upon the food.

Table A2-3 Comparison of YMM with Flax and Psyllium Mucilages (Cui et al, 2007)

Property Source	Mustard Mucilage *Sinapis alba*	Flaxseed Linum usitatissimum	Psyllium Plantago ovato, P. psyllium
Regulatory Approval	Mustard bran approved as novel fibre only at condimental levels; not approved as dietary fibre	Flax as a milled cereal permitted as dietary fibre in Canada- must not be finely ground	Approved for cholesterol lowering claim in US Approved as dietary fibre in Canada
Production	Epidermal layer of seed. Highly soluble in water and extracted from whole seed or seed coat (bran) Whole seed: Snow white fibrous material with yield of ~5%-not economically feasible as there is no use for seed after water extraction Bran: by product of mustard flour milling- extraction yield 15-20%	Seed coat contains thick mucilaginous layer. Gum extracted directly from seed, from flaxseed meal (byproduct of linseed oil production) or hull	Mechanical milling/grinding of seed coat, Yield ~ 25% seed weight. White fibrous material absorbs water quickly to give clear, colourless mucilaginous gel
Structure	Complex polysaccharides: cellulosic backbone with 6 neutral sugars(glucose, mannose, rhamnose, arabinose and xylose) and 2 uronic acids.	Wide chemical composition depends on variety, environment and material used for extractions (seed, meal or hull) Gum has 2 major fractions: neutral polysaccharide comprised of xylose, arabinose and galactose and an acidic polysaccharides comprised of D-galactose, L-rhamnose and D-galacturonic acid.	Heteroxylan consisting of (1-3) and (1-4) mixed link β-D-xylopyranosyl backbone chains with side chains of xylopyranosyl and arabinofuranosyl residues.
Functional Properties	Properties resemble xanthan gum Exhibits shear thinning behaviour at low concentrations (0.3% and above) Forms weak gel structure Interacts synergistically with galactomannans.	Newtonian flow behaviour at low concentrations and shear thinning behaviour at high concentrations Broad variation in chemical composition- Gums with high neutral polysaccharides are more viscous and exhibit Newtonian	Does not dissolve completely in water Swells to mucilaginous gel with wallpaper paste appearance 2% dispersion exhibits weak gel-like structure (like xanthan gum) Newtonian flow properties at low shear rate

	Salt concentration and pH affect viscosity: viscosity increases at low or high pH Emulsifying capacity and emulsion stability higher than most commercial gums Good interactions with starches	flow behaviour, Gums with high acidic polysaccharides show lower viscosity and Newtonian flow behaviour. Range from viscoelastic fluid to real gel (1-3% concentration) pH affects flow behaviour and viscosity	Syneresis upon aging Stable in high salt solutions
Food Applications	Expect similar applications to xanthan particular its interactions with galactomannans-salad dressings, sauces, gravies etc Currently, ground mustard products at ~5% used in processed meats as condiments or bulking agents	Used as thickener and stabilizer Affects pasting, dough rheology and baking of breads 0.5% flax gum could replace 0.1% xanthan in some bakery applications Oil/water stabilizer at 0.5 – 1.5% concentration Use as egg white substitute Strong buffering action for beverage manufacture	Ice creams, frozen desserts as stabilizer and thickener Used with other gum to replace wheat gluten Used in preparation of highly soluble fiber foods e.g. Ready-To-Eat breakfast cereals and nutrition bars
Non-Food Applications	Cosmetics: Lotions, liquid soap, sunscreen, self tanning solutions and creams, anti-aging products	Printing, textile, cigar industries Hair dressing applications Cosmetics-hand cream Denture adhesive Saliva substitute	Landscaping as water binding agent Environmentally friendly binder for fixing joints of interlocks in landscape industry
Health benefits	Initial studies in rats show decreased postprandial insulin levels Could reduce glycemic index of foods May have anticancer properties	Reduce levels of LDL cholesterol Treatment of skin ailments Bulk laxative, cough emollient, stabilizer in barium sulfate suspensions for x-ray diagnostic disintegration Table formation-slow rate of drug release	Long history of medical use-husk is used by pharmaceutical firms to make fiber-based laxative products Reduces total and LDL cholesterol in animals and humans May protect against colon cancer Used as demulcent in dysentery, erosion of intestines, dry coughs, burns, excoriations, and inflammation of eyes. Bulk laxative May delay allergic reactions due to water holding capacity and gelling property

A2-2 Regulatory Approval of Yellow Mustard Mucilage

Future regulatory approvals of yellow mustard mucilage or its isolated fractions will depend upon its form and intended use. Even though mustard seed, flour, and mustard bran are approved for human food consumption, the processed YMM, as a concentrated fraction, could be considered a novel ingredient and thus follow the Novel Food Regulations (Division 28, FDAR) for approval as a new food ingredient.

Hydrocolloid

If the intention is to have yellow mustard mucilage used as a hydrocolloid, similar to xanthan gum and at similar usage levels, then it will need to be approved as a “food additive”. Division 16 of the FDAR outlines the process to gain regulatory approval (Figure 5) for hydrocolloids.

Approved hydrocolloids can be found under Table IV “Food additives that may be used as emulsifying, gelling, stabilizing and thickening agents” of Food Additives, Division 16 (FDAR, 2016). The table outlines the foods in which the ingredient is permitted and the maximum levels of use.

As examples, some of the hydrocolloids permitted in foods in Canada include acacia gum, agar, algin, algin, alginic acid, ammonium alginate, carrageenan (ammonium, calcium), furcellan (ammonium, calcium), arabinogalactan, carob bean gum, carboxymethylcellulose, carrageenan, cellulose gum, gelatin, gellan, guar, gum arabic, karaya gum, Irish moss gelose, locust bean gum, oat gum, pectin, tragananth gum, and xanthan gum. Some gums have miscellaneous uses i.e. as fining agents for beer, alcohols and wine (acacia gum, agar) and are included in other Tables in Division 16.

Each ingredient is permitted in specified foods and/or unstandardized foods at the designated maximum levels permitted. An unstandardized food is one which no standard is prescribed (through regulation) —there is no standard of identity.

Figure A2-1 Regulatory Approval of a Food Additive

Food Additives

B.16.002, FDAR

A request that a food additive be added to or a change made in the Tables following section B.16.100 shall be accompanied by a submission to the Minister in a form, manner and content satisfactory to him and shall include

(*a*) a description of the food additive, including its chemical name and the name under which it is proposed to be sold, its method of manufacture, its chemical and physical properties, its composition and its specifications and, where that information is not available, a detailed explanation;

(*b*) a statement of the amount of the food additive proposed for use, and the purpose for which it is proposed, together with all directions, recommendations and suggestions for use;

(*c*) where necessary, in the opinion of the Director, an acceptable method of analysis suitable for regulatory purposes that will determine the amount of the food additive and of any substance resulting from the use of the food additive in the finished food;

(*d*) data establishing that the food additive will have the intended physical or other technical effect;

(*e*) detailed reports of tests made to establish the safety of the food additive under the conditions of use recommended;

(*f*) data to indicate the residues that may remain in or upon the finished food when the food additive is used in accordance with good manufacturing practice;

(*g*) a proposed maximum limit for residues of the food additive in or upon the finished food;

(*h*) specimens of the labelling proposed for the food additive; and

(*i*) a sample of the food additive in the form in which it is proposed to be used in foods, a sample of the active ingredient, and, on request a sample of food containing the food additive.

Dietary Fibre

If the intention is to have mustard bran, yellow mustard mucilage or the derived β-glucans be considered as dietary fibre, then another regulatory process must be followed.

Many hydrocolloids used in the food industry are not considered as dietary fibres because their usage levels are too low to have a physiological health benefit. In Canada, hydrocolloid food additives not recognized as dietary fibres include pectin, carrageenan, guar gum, methylcellulose, carboxymethylcellulose, microcrystalline cellulose and other cellulose derivatives. Wood celluluse (powdered cellulose) is currently allowed under an Interim Marketing Authorization.

Since 2009, newly approved dietary fibres include beta-glucans derived from oats and barley and partially hydrolyzed guar gum.

To be considered a dietary fibre, the ingredient must follow the guidelines outlined by Health Canada (1997, 2012) and the Canadian Food Inspection Agency (2016).

Firstly, the ingredient must meet all the conditions of the Canadian definition of a dietary fibre which is as follows:

"Dietary fibre[16] consists of:

1. Carbohydrates with a degree of polymerization of 3 or more that naturally occur in foods of plant origin and that are not digested and absorbed by the small intestine ; and
2. Accepted novel fibres

Novel fibres are ingredients manufactured to be sources of dietary fibre and consist of carbohydrates with a degree of polymerization of 3 or more that are not digested and absorbed by the small intestine. They are synthetically produced or are obtained from natural sources which have no history of safe use as dietary fibre or which have been processed so as to modify the properties of the fibre contained therein. Accepted novel fibres have at least one physiological effect demonstrated by generally accepted scientific evidence.

[16] Canadian Food Inspection Agency. 2016. Elements within the Nutrition Facts Table: Carbohydrates: Dietary Fiber (http://www.inspection.gc.ca/food/labelling/food-labelling-for-industry/nutrition-labelling/elements-within-the-nutrition-facts-table/eng/1389206763218/1389206811747?chap=5#s6c5

The substances in part 1 of this definition are all edible plant materials that have a history of use as food and have been processed or cooked using conventional processes. They include fruits, vegetables, pulses, seeds, nuts, cereals, legumes, etc.

Substances in part 2 of the definition include substances obtained from agricultural crop by-products and from raw plant materials, substances of animal or bacterial origin, chemically modified substances, synthetic products, etc. These substances are not historically used as food fibre sources. In addition, novel fibres may also include products used at higher than traditionally used levels in the diet.

If the physiological effect of a novel fibre source has not been demonstrated, the ingredient is considered an unproven novel fibre. If safe, it may be used in foods but it cannot be claimed to be a source of dietary fibre, and its amount of dietary fibre must not be included as part of the total dietary fibre declaration in the Nutrition Facts table."

Physiological effects that are acceptable for dietary fiber include:

- improves laxation or regularity by increasing stool bulk;
- reduces blood total and/or low-density lipoprotein cholesterol levels;
- reduces post-prandial blood glucose and/or insulin levels;
- provides energy-yielding metabolites through colonic fermentation.

Brans that have been accepted as a dietary fibre include barley bran, corn bran, oat bran and wheat bran.

There could be an opportunity to have mustard bran declared a dietary fibre if there is enough clinical evidence to support the claim and if it can be added to a food at a level to have a physiological effect, without compromising the integrity of the food product. This issue needs more investigation.

At the current time, industry appears to be interested in pursuing this avenue provided there is sufficient supply of mustard bran to meet demand and not take away from the use of mustard flour, a higher value product.

A2 References

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Health Canada. 2012. Policy for labeling and advertising of dietary-containing food products. http://www.hc-sc.gc.ca/fn-an/legislation/pol/fibre-label-etiquetage-eng.php Accessed July 2016.

APPENDIX 3: PATENTS RELATED TO MUSTARD

Table A3-1 Patents Related to Mustard

Canadian Patent No.	Holder/Status	Title	Description
CA2250620 Issued: 2001-02-27	Sakai, S; Sakai, M.; Tanaka, H. Japan	Novel method of production of mustard powder	A process is provided for the production of a pressed mustard cake from mustard seed, as well as mustard powder yielded from the milling of the mustard cake of the process. Employing strict temperature controls at key points in the process, this process yields a mustard cake and mustard powder of increased pungency, good flavour, enhanced protein content and enhanced preservability.
2449007 2009-05-19	Diosady, L.L.; Chen, B-K; Xu, L Canada, University of Toronto	Production of high-quality protein isolates from defatted meals of Brassica seeds	The present invention provides a method for processing defatted oil seeds, comprising the steps of: (a) solubilizing at least a portion of the protein contained in the oil seeds to produce suspended residual solids and a first solution comprising protein, phenolic-protein complexes, and free phenolic compounds; (b) separating at least a portion of the free phenolic compounds from the first solution and recovering a free phenolic reduced solution; and (c) treating the free phenolic reduced solution to precipitate at least a portion of the protein as a precipitated protein isolate and recovering a treated solution containing a soluble protein isolate. Novel protein products are also disclosed. Food and drink products containing the novel protein products are also disclosed. Method can be applied to ricebran, sunflower, cotton seed, Brassica seeds and/or mixtures. Method to remove phenolics To be used in processed meat products (meat binder, extender), vegetarian meat substitute, bakery, infant formulation, nutritional supplements, bar, beverage, carbonated beverage as substituts for gluten, casein and soy proteins

			Utilizes membrane filtration techniques: ultrafiltration, diafiltration and reverse osmosis
CA13119877 Issued: 1992-12-22 Expired: 2009-12-22	Diosady, L.L. Rubin, L.J. Tzeng, Y.-M.	Production of rapeseed protein materials	A process of treating meal containing vegetable proteins is disclosed. This process includes the step of extracting the meal with a suitable aqueous solvent in which the vegetable proteins are soluble to obtain an extraction solution. The solubility of the dissolved protein in the extraction solution is then adjusted to precipitate at least some of the protein and therefore obtain a precipitated protein fraction and an unprecipitated protein fraction in solution. The precipitated protein fraction is then separated from the protein fraction in solution, and the unprecipitated protein fraction is separated from the undesirable components in the solution by membrane processing. Each of the protein fractions is then suitably dried to recover the proteins.
CA 2270750 Issued: 2003-10-14	Cui, W.S, Eskin, M., Duan, Z, Zhang, Z Han N.M Owners; Cui & Eskin, Natunola Health	Extraction process and use of yellow mustard gum	The present invention provides for optimal extraction conditions for extracting yellow mustard gum from yellow mustard bran to provide for high yield. The four variables examined were extraction temperature, pH, water:solid ratio and extraction time. Of these variables, temperature and pH had a much greater influence on the yield and rheological properties of the extracted gum compared to water/solid ratio and extraction time. Optimum extraction conditions were temperatures between 50-70°C, pH 7-10, water/solid ratio of 40:1 - 60:1 and extraction time of 2-2.5 hr. The yield of gum obtained under the optimum extraction conditions was 30% of bran weight. The extracted gum exhibited maximum shear thinning flow behaviours. It can be incorporated into cosmetic products and skin lotions.
CA 2029770 Issued: 2005-03-15	Sharafabadi, S.K. (Canada)	Pseudoplastic yellow mustard gum	An improved process of gum extraction from whole yellow mustard seed is described. The process is time temperature interdependent. In a first step the seed is treated in water, preferably at elevated temperature, the extract is then separated, preferably mechanically, more preferably by a perforated centrifuge, or even more preferably by a slitted centrifuge. The aqueous extract on precipitation and drying gives a gum with pseudoplastic properties similar

			to those of xanthan gum. The extraction using mechanical separation, especially by a perforated bowl centrifuge, which can be slitted, is suitable for industrial scale extraction of the yellow mustard seed. A centrifuge adapted for juice extraction is suitable. The gum produced under these conditions exhibits unique properties dramatically different from those reported from other previous processes, in both composition and viscosities.
CA 2724391 2009-05-15	Tang, Q.N. BioExx Specialty Proteins Ltd (Canada) US application no: 61/053858 and 61/099783	Oilseed protein concentrates and isolates, and processes for the production thereof	Protein concentrates and protein isolates, in addition to processes for the production of protein concentrates and protein isolates, are disclosed. In particular, the disclosure relates to a process for removing fiber from an oilseed meal, comprising: i) mixing an oilseed meal with a blending solvent, optionally water, saline solution, polysaccharide solution or protein containing solution, to form a mixture; ii) optionally adjusting the pH of the protein slurry to a pH of about 2 to about 10; and iii) separating the mixture to form a protein slurry comprising soluble and insoluble proteins and an insoluble fiber fraction.
CA 2820392 Filed: 2011-10-14 US Patent: 8450244 Issued: May 28, 2013	Robinson, J MPT Mustard Products and Technologies Inc (Canada)	Mixtures of mustard plant material for the control of pests and methods of making	A composition for controlling pests comprising a mixture of plant material obtainable from a mustard plant of the species *Sinapis alba* and plant material obtainable from a mustard plant of the species *Brassica juncea* is described. The mixture contains an effective amount of a glucosinolate breakdown product (nitrite, thiocyanate, isothiocyanate) in a mustard meal
CA 2794578 Filing Date:	Wagner, N MPT Mustard Products and	Compositions comprising plant material and sugar for	Compositions and methods for controlling pest are described. The compositions comprise (a) a material obtainable from a plant comprising an effective amount of a glucosinolate breakdown product and (b) a sugar. The novel compositions exhibit improved properties over known formulations, for example with respect to their

2011-03-25	Technologies Inc (Canada)	the control of pests and methods of making	potency, ease of manufacture and ease of application.
2915941 Filed 2014-06-25	Robinson, J MPT Mustard Products and Technologies Inc (Canada)	Compositions comprising pesticide precursors and methods of making and use	Disclosed is a two-part pesticide formulation and methods of making and using. The pesticide formulation comprises a first part comprising a glucosinolate concentrate, and a second part comprising a plant material comprising myrosinase. The parts may be stored and transported in inactive form, and are activated upon application to a substrate requiring pesticide treatment.
CA2920061 Filed: 2014-08-01 Issued US Patent 9258999 February 16, 2016.	Heatherington,M; Robinson, J MPT Mustard Products and Technologies Inc (Canada)	Biopesticide compositions comprising water soluble polyols	Provided are liquid compositions comprising inactive biopesticide precursors comprising a glucosinolate concentrate, a plant material comprising a myrosinase enzyme complex, and a water soluble polyol. Further provided are methods of making and using such compositions.
CA 2688464 Issued: 2016-04-12	Wanasundera, J and McIntosh, T	A process of aqueous protein extraction from Brassicaceae oilseeds	A process of aqueous protein extraction from Brassicaceae oilseed meal, such as canola, commercial canola meal or yellow mustard, to obtain a napin-rich protein extract, a cruciferin-rich protein extract, and a low-protein residue. The process comprising the steps of performing aqueous extraction of the Brassicaceae oilseed meal at a pH of from about 2.5 to about 5.0 to obtain a soluble napin-rich protein extract and a cruciferin-rich residue followed by performing aqueous extraction of the cruciferin-rich residue to obtain a soluble cruciferin-rich protein extract and a low-

			protein residue. The cruciferin-rich residue may be treated with cell wall degrading enzymes to obtain a cruciferin-rich fraction The cruciferin-rich protein products may be substantially free of napin protein and may be useful as a non-allergenic food product for human consumption.
US 8048463 Issued Nov 1, 2011	Diosady, LL; Xu, Lei, Chen, B.-K. Toronto	Production of high-quality protein isolated from oil seeds	The present invention provides a method for processing defatted oil seeds, comprising the steps of: (a) solubilizing at least a portion of the protein contained in the oil seeds to produce suspended residual solids and a first solution comprising protein, phenolic-protein complexes, and free phenolic compounds; (b) treating at least a portion of the phenolic-protein complexes in the first solution to liberate at least some phenolic compounds from the phenolic-protein complexes thereby producing a second solution; (c) separating at least a portion of the free phenolic compounds from the second solution and recovering a free phenolic reduced solution; and (d) treating the free phenolic reduced solution to precipitate at least a portion of the protein as a precipitated protein isolate and recovering a treated solution containing a soluble protein isolate. Novel protein products are also disclosed. Food and drink products containing the novel protein products are also disclosed.
US6905713 Issued: June 14, 2005	Diosady, LL; Xu, Lei, Chen, B.-K.	Production of high-quality protein isolates from defatted meals of Brassica seeds	The present invention provides a method for processing defatted oil seeds, comprising the steps of: (a) solubilizing at least a portion of the protein contained in the oil seeds to produce suspended residual solids and a first solution comprising protein, phenolic-protein complexes, and free phenolic compounds; (b) separating at least a portion of the free phenolic compounds from the first solution and recovering a free phenolic reduced solution; and (c) treating the free phenolic reduced solution to precipitate at least a portion of the protein as a precipitated protein isolate and recovering a treated solution containing a soluble protein isolate. Novel protein products are also disclosed. Food and drink products containing the novel protein products are also disclosed.
US 4,889, 921 Issued: Dec. 26, 1989.	Diosady, L.L.; Rubin, L.J.	Production of rapeseed protein materials	A process of treating meal containing vegetable proteins is disclosed. This process includes the step of extracting the metal with a suitable aqueous solvent in which the vegetable proteins are soluble to obtain an extraction solution. The solubility of the dissolved protein in the extraction solution is then adjusted to precipitate at least some

	Tzeng, Y.-M. University of Toronto Innovations Foundation		of the protein and therefore obtain a precipitated protein fraction and an unprecipitated protein fraction in solution. The precipitated protein fraction is then separated from the protein fraction in solution, and the unprecipitated protein fraction is separated from the undesirable components in the solution by membrane processing. Each of the protein fractions is then suitably dried to recover the proteins.
Patent application #: 20070237877 Filed: March 13, 2007	Diosady, LL; Xu, Lei, Chen, B.-K.	PRODUCTION OF HIGH-QUALITY PROTEIN ISOLATED FROM OIL SEEDS	The present invention provides a method for processing defatted oil seeds, comprising the steps of: (a) solubilizing at least a portion of the protein contained in the oil seeds to produce suspended residual solids and a first solution comprising protein, phenolic-protein complexes, and free phenolic compounds; (b) treating at least a portion of the phenolic-protein complexes in the first solution to liberate at least some phenolic compounds from the phenolic-protein complexes thereby producing a second solution; (c) separating at least a portion of the free phenolic compounds from the second solution and recovering a free phenolic reduced solution; and (d) treating the free phenolic reduced solution to precipitate at least a portion of the protein as a precipitated protein isolate and recovering a treated solution containing a soluble protein isolate. Novel protein products are also disclosed. Food and drink products containing the novel protein products are also disclosed.
Patent Application: 20050202154 Filed: September 15, 205	Diosady, LL; Xu, Lei, Chen, B.-K.	Production of high-quality protein isolated from defatted meals of Brassica seeds	The present invention provides a method for processing defatted oil seeds, comprising the steps of: (a) solubilizing at least a portion of the protein contained in the oil seeds to produce suspended residual solids and a first solution comprising protein, phenolic-protein complexes, and free phenolic compounds; (b) treating at least a portion of the phenolic-protein complexes in the first solution to liberate at least some phenolic compounds from the phenolic-protein complexes thereby producing a second solution; (c) separating at least a portion of the free phenolic compounds from the second solution and recovering a free phenolic reduced solution; and (d) treating the free phenolic reduced solution to precipitate at least a portion of the protein as a precipitated protein isolate and recovering a treated solution containing a soluble protein isolate. Novel protein products are also disclosed. Food and drink products containing the novel protein products are also disclosed.

20030060607 Filed: March 27, 2003	Diosady, LL; Xu, Lei, Chen, B.-K.	Production of high-quality protein isolated from defatted meals of Brassica seeds	The present invention provides a method for processing defatted oil seeds, comprising the steps of: (a) solubilizing at least a portion of the protein contained in the oil seeds to produce suspended residual solids and a first solution comprising protein, phenolic-protein complexes, and free phenolic compounds; (b) separating at least a portion of the free phenolic compounds from the first solution and recovering a free phenolic reduced solution; and (c) treating the free phenolic reduced solution to precipitate at least a portion of the protein as a precipitated protein isolate and recovering a treated solution containing a soluble protein isolate. Novel protein products are also disclosed. Food and drink products containing the novel protein products are also disclosed.