

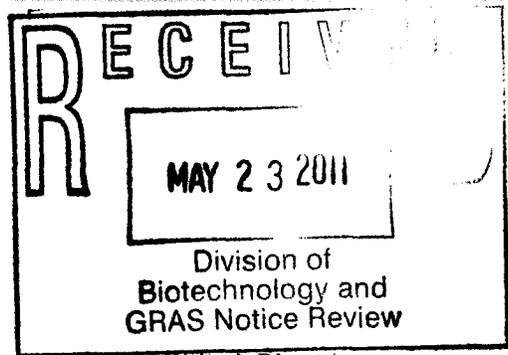
GR



ORIGINAL SUBMISSION

000002

1001 G Street, N.W.
Suite 500 West
Washington, D.C. 20001
tel. 202.434.4100
fax 202.434.4646



Writer's Direct Access
Melvin S. Drozen
(202) 434-4222
drozen@khlaw.com

May 20, 2011

Via Overnight Mail

Office of Food Additive Safety (HFS-225)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Pkwy
College Park, MD 20740

Re: GRAS Notification for Canola Protein Isolate (Isolexx[™]) and
Hydrolyzed Canola Protein Isolate (Vitalex[™])

Dear Sir or Madam:

We respectfully submit the attached GRAS Notification on behalf of our client BioExx Specialty Proteins, Ltd. for Canola Protein Isolate (Isolexx[™]) and Hydrolyzed Canola Protein Isolate (Vitalex[™]) for use as food ingredients. We have determined that these canola proteins are generally recognized as safe (GRAS), consistent with section 201(s) of the Federal Food, Drug and Cosmetic Act. This determination is based on scientific procedures and has been evaluated by experts qualified by scientific training and experience to assess the safety of the canola proteins under the conditions of their intended use in food. Therefore, the use of Canola Protein Isolate (Isolexx[™]) and Hydrolyzed Canola Protein Isolate (Vitalex[™]) in food as described in this GRAS Notification are exempt from the requirement of premarket approval.

The attached GRAS Notification provides a review of the information related to intended uses and manufacturing and safety of the ingredients. We have included four (4) hard copies of the GRAS Notification and all Appendices including the GRAS Expert Panel Opinion.

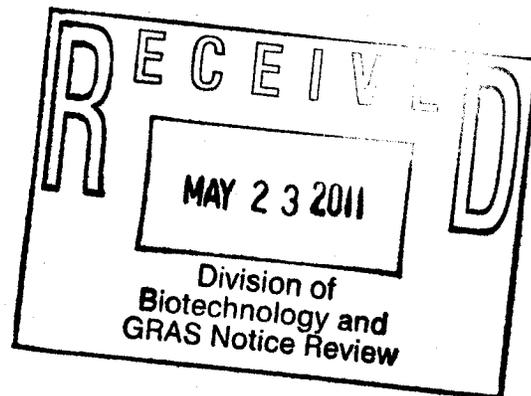
We look forward to the Agency's review of this submission and would be happy to provide the Agency with any information they need to complete their review.

Sincerely,

(b) (6)

Melvin S. Drozen

000003



**GRAS Notification for BioExx Canola Proteins:
Canola Protein Isolate (Isolexx™) and
Hydrolyzed Canola Protein Isolate (Vitalex™)**

Prepared for:

U.S. Food and Drug Administration
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
5100 Paint Branch Parkway
College Park, MD 20740-3835

Prepared by:

Keller and Heckman LLP
1001 G Street, NW
Suite 500W
Washington, DC 20001

000004

Table of Contents

I.	Introduction	1
II.	Administrative Information	2
	A. Claim Regarding GRAS Status.....	2
	B. Name and Address of the Notifier.....	2
	C. Common or Usual Name of GRAS Substance.....	2
	D. Intended Use.....	2
	E. Self-Limiting Levels of Use.....	3
III.	Product Identity and Specifications	3
	A. Product Specifications.....	3
	B. Data on Representative Lots.....	4
	C. Protein Identification.....	6
	1. <i>Protein Analyses</i>	6
	2. <i>Amino Acid Analyses</i>	6
IV.	Manufacturing Process	7
	A. Manufacturing Process for the Canola Protein Products.....	7
	B. Safety of Substances Used in the Manufacture of the Canola Protein Products.....	10
	1. <i>Enzymes</i>	10
	2. <i>Solvents, Acids, Bases, and Salts</i>	11
V.	Consideration of Potential Anti-Nutrient Factors	13
	A. Erucic Acid.....	13
	B. Tannins.....	14
	C. Phenolic acids (Sinapine).....	14
	D. Glucosinolates & Allyl Isothiocyanate (AIT).....	15
	E. Phytates.....	17
VI.	Consideration of Potential Contaminating Materials	18
	A. Pesticide Residues.....	18
	B. Dioxin Residues.....	18
	C. Polycyclic Aromatic Hydrocarbons.....	19
	D. Acrylamide.....	19
	E. Aflatoxin.....	19
	F. Residual Solvent.....	19
	G. Heavy Metals.....	19
VII.	Nutrition – Protein Digestibility	20
VIII.	Basis for GRAS Determination	22
	A. Safety Overview.....	22
	1. <i>Taxonomy of the Oilseed Brassica Species</i>	22
	2. <i>Human Food Use</i>	23
	3. <i>Animal Use of B. juncea Pressed Cake</i>	24
	4. <i>Safety Studies on Canola Protein Products</i>	24
	5. <i>Summary of Farm Animal Studies of Canola Meal</i>	27
	6. <i>Mutagenicity Studies</i>	27
	7. <i>Allergenicity of B. juncea</i>	28
	B. Estimated Consumption of Canola Proteins Derived from <i>B. juncea</i> and <i>B. Napus</i> from Proposed Food Uses.....	29

IX. Summary of Basis for GRAS Determination 30
X. Appendices 30
XI. Tables 31
XI. References 32

I. Introduction

Keller and Heckman LLP submits the enclosed information on behalf of our client, BioExx Specialty Proteins, Ltd. (BioExx), in support of this notification that canola proteins derived from *Brassica juncea* (*B. juncea*) and *Brassica napus* (*B. napus*) are generally recognized as safe (GRAS) for use in multiple food applications. Specifically, this notification covers two canola protein products—canola protein isolate (Isolexx™) and hydrolyzed canola protein isolate (Vitalex™). The canola proteins are intended for use as food ingredients in foods where protein is used for functional or nutritional purposes such as bakery products, snack foods, beverages (including nutritional beverages), soups, dairy products, dry instant milkshake mixes and protein drinks, instant powdered nutritional beverages, processed meat products, vegetarian food products/meat analogues, and meal replacement/nutritional bars.

The determination of GRAS status is based on scientific procedures, in accordance with 21 C.F.R. § 170.30(b) and conforms to the guidance issued by the Food and Drug Administration (FDA) under *proposed* 21 C.F.R. § 170.36, 62 Fed. Reg. 18938 (Apr. 17, 1997).

We submit information in the following areas:

- Identity and specifications for the canola protein products;
- The production of the canola protein products;
- Safety of any anti-nutrient components or impurities in the canola protein products;
- Digestibility and nutritional quality of the canola protein products;
- Intended uses and an estimation of consumption of canola proteins;
- Relevant safety data on canola proteins;
- External panel reviewers' evaluation and conclusion that the canola proteins are GRAS for their intended uses.

The canola protein products are highly purified protein products that do not have toxic properties. Further, the protein products are of a high quality, as indicated by their high Protein Digestibility Corrected Amino Acid Score (PDCAAS) scores.

The analytical data, published studies, and information that are the basis for this GRAS determination are available for FDA review and copying at reasonable times at the notifier's address below or will be sent to FDA upon request. It is our expectation that FDA will concur that the information presented fully supports the determination that canola proteins as produced by BioExx are GRAS for use as food ingredients.

000007

II. Administrative Information

A. Claim Regarding GRAS Status

BioExx hereby notifies the agency of its determination that canola proteins derived from *B. juncea* and *B. napus* are GRAS based on scientific procedures for use as food ingredients in certain specific categories of food where protein isolates are commonly used.

B. Name and Address of the Notifier

BioExx Specialty Proteins Ltd.
219 (North) Dufferin Street
Suite 100 B
Toronto, Ontario
M6K 3J1

All communications on this matter are to be sent to Counsel for the Notifier:

Melvin S. Drozen
Keller and Heckman LLP
1001 G Street, NW
Suite 500W
Washington, DC 20001
Telephone: (202) 434-4222
Facsimile: (202) 434-4646
Email: drozen@khlaw.com

C. Common or Usual Name of GRAS Substance

The ingredients determined by BioExx to be GRAS are a canola protein isolate and a hydrolyzed canola protein isolate. The canola protein isolate product contains at least 90% protein on a dry weight basis and will be marketed under the tradename Isolexx™. The hydrolyzed canola protein isolate is a highly soluble almost fully hydrolyzed mixture of peptide oligomers and amino acids with a small amount of protein, which will be marketed under the tradename Vitalexx™. The ingredients will be referred to as canola protein isolate, or Isolexx™, and hydrolyzed canola protein isolate, or Vitalexx™ or collectively as canola protein products throughout the document.

D. Intended Use

The canola protein products each have distinguishing ingredient functionality properties leading to preferred applications. For example, the canola protein isolate (Isolexx™) contains intact protein with excellent water solubility and emulsification and foaming properties. It compares well to soy, pea, whey and egg proteins and would be used in similar applications. The hydrolyzed canola protein isolate (Vitalexx™) is highly hydrolyzed, leading to greater solubility across a range of pHs, with a much smaller average molecular weight and intended for use in applications where absorption, ease of digestion, and high solubility are important.

000008

Therefore, depending on the particular food application, the canola protein products will be used as food ingredients in the various food categories at levels up to those outlined in **Table 14**.

Foods containing the canola protein products will be consumed by the general population. Infant foods or formula are not included in the scope of this GRAS Notification. Information on estimated exposure levels for all age groups, excluding children younger than 3 years, is set forth in **Section VIII.B**.

E. Self-Limiting Levels of Use

The use of the canola protein products as food ingredients is limited by the level that can technically be added to a given food without jeopardizing its quality and consumer acceptability. In addition, use is limited by cost of the canola protein ingredients.

III. Product Identity and Specifications

A. Product Specifications

Canola protein isolate (Isolexx™) has a protein content of greater than or equal to 90% on a dry basis. The following chemical and microbiological specifications have been established for canola protein isolate:

**Table 1
Specifications Canola Protein Isolate - Isolexx™**

	Test Method	Limits (dry weight basis)
Protein (nx6.25)	AOCS Ba 4e-93	≥90%
Soluble protein	Roe, M.B., Sniffen, C.J. and Chase, L.E. 1990. Techniques for measuring protein fractions in feedstuffs. Proc. Cornell Nutr. Conf. p. 81. Ithaca NY	>85%
Moisture	AOCS Ba 2a-38	<7% (as is)
Carbohydrate	By difference	2-7%
Fat	AOCS Ba 3-38	<2.0%
Ash	AOCS Ba 5a-49	<4%
Fiber	AOCS Ba 6-84	<0.5%
Total glucosinolates	Method of the CGC, Grain Research Lab.[Duan and MacGregor, Glucosinolate analysis of Rapeseed(Canola) December 15, 1981)]	<1 µmol/g
Total phytates	Gao et al 2007	<1.25%
Aerobic plate count	MFHPB-18	<10,000 cfu/g
<i>E. Coli</i>	MFHPB-34	Negative/10g
<i>Salmonella</i>	MFHPB-20	Negative/25g
<i>Staphylococcus aureus</i>	MFHPB-21	Negative/10g

000009

Hydrolyzed canola protein isolate (Vitallexx™), is a highly soluble, almost fully hydrolyzed mixture of peptide oligomers and amino acids with a small amount of protein. It is hydrolyzed using the protease enzymes, Alcalase and Flavourzyme, which are discussed further in **Section IV. B. 1**. The following chemical and microbiological specifications have been established for hydrolyzed canola protein isolate:

Table 2
Specifications Hydrolyzed Canola Protein Isolate - Vitallexx™

	Test Method	Limits (dry weight basis)
Peptides and Amino Acids (nx6.25)	AOCS Ba 4e-93	≥80%
Solubility	Roe, M.B., Sniffen, C.J. and Chase, L.E. 1990. Techniques for measuring protein fractions in feedstuffs. Proc. Cornell Nutr. Conf. p. 81. Ithaca NY	>98%
Moisture	AOCS Ba 2a-38	<9% (as is)
Carbohydrate	By difference	5-15%
Fat	AOCS Ba 3-38	<2.0%
Ash	AOCS Ba 5a-49	<7%
Fiber	AOCS Ba 6-84	<0.5%
Total glucosinolates	Method of the CGC, Grain Research Lab.[Duan and MacGregor, Glucosinolate analysis of Rapeseed(Canola) December 15, 1981]	<1 µmol/g
Total phytates	Gao et al 2007	<1%
Aerobic plate count	MFHPB-18	<10,000 cfu/g
<i>E. Coli</i>	MFHPB-34	Negative/10g
<i>Salmonella</i>	MFHPB-20	Negative/25g
<i>Staphylococcus aureus</i>	MFHPB-21	Negative/10g

B. Data on Representative Lots

Nine production lots, four of Isolexx™ and five of Vitallexx™, were analyzed with respect to the different parameters indicated in the specifications for the canola protein products derived from *B. juncea*. The corresponding data are presented below in **Tables 3 and 4**. The results of the analyses indicate consistency in production and compliance with relevant specifications. In addition, the results of the analyses when compared to the results of other canola protein products derived from *B. napus* as well as two production lots of Vitallexx™ derived from *B. napus*, as shown in **Table 5**, demonstrate that canola protein products derived from *B. juncea* and *B. napus* are similar.

000010

Table 3
Analysis of representative lots of Canola Protein Isolate - Isolexx™ from *Brassica Juncea*

	Specification (dwb)	BIOBPCI 20100323-A	BIOBPCI201 00614-B	BIOBPCI201 00705-B	BIOBPCI20 100705-C
Protein (nx6.25) (%)	≥90	95.3	91.7	90.7	93.3
Soluble protein (%)	>85	87.9	85.2	85.0	88.7
Moisture (as is) (%)	<7	3.3	3.7	3.2	1.5
Carbohydrate (%)	2-7	2.3	6.1	6.8	4.3
Fat (%)	<2.0	0.3	0.2	0.2	0.1
Ash (%)	<4	2.2	2.0	2.3	2.3
Fiber (%)	<0.5	0.00	0.00	0.06	0.05
Total glucosinolates (μmol/g)	<1	0.15	0.06	0.05	0.08
Total phytates (%)	<1.25	0.44	0.83	1.14	1.0
Aerobic plate count (cfu/g)	<10,000	410	80	6700	9800
<i>E. Coli</i>	Negative/10g	<5	<5	<5	<5
<i>Salmonella</i>	Negative/25g	Neg	Neg	Neg	Neg
<i>Staphylococcus aureus</i>	Negative/10g	<5	<5	<5	<5

Table 4
Analysis of representative lots of Hydrolyzed Canola Protein – Vitalexx™ from *Brassica Juncea*

	Specification (dwb)	BIOBPCV 20101107-B	BIOBPCV 20101107-C	BIOBPCV 20101107-D	BIOBPCV 20101107-E	BIOBPCV 20101107-F
Peptides and Amino Acids (nx6.25) (%)	≥80	86.6	88.5	89.0	88.7	86.9
Solubility (%)	>98	99.99	100.00	99.74	99.94	99.99
Moisture (as is) (%)	<9	8.4	8.1	8.2	7.9	6.6
Carbohydrate (%)	5-15	8.8	7.5	5.4	5.7	8.5
Fat (%)	<2.0	0.12	0.14	0.10	0.10	0.07
Ash (%)	<7	4.44	3.81	5.56	5.56	4.46
Fiber (%)	<0.5	0.05	0.03	<0.01	<0.01	<0.01
Total glucosinolates (μmol/g)	<1	0.20	0.25	0.22	0.20	0.16
Total phytates (%)	<1	0.72	0.38	0.30	0.63	0.49
Aerobic plate count (cfu/g)	<10,000	2200	2200	5100	1200	1230
<i>E. Coli</i>	Negative/10g	<5	<5	<5	<5	<5
<i>Salmonella</i>	Negative/25g	Neg	Neg	Neg	Neg	Neg
<i>Staphylococcus aureus</i>	Negative/10g	<5	<5	<5	<5	<5

000011

Table 5
Analysis of representative lots of Hydrolyzed Canola Protein – Vitalex™ from *Brassica Napus*

	Specification (dwb)	BIOBPCV 20101115A	BIOBPCV 20101115B
Peptides and Amino Acids (nx6.25) (%)	≥80	81.4	83.9
Solubility (%)	>98	99.99	99.95
Moisture (as is) (%)	<9	7.9	8.7
Carbohydrate (%)	5-15	12.6	10.4
Fat (%)	<2.0	0.10	0.11
Ash (%)	<7	5.95	5.65
Fiber (%)	<0.5	<0.01	<0.01
Total glucosinolates (µmol/g)	<1	0.15	0.24
Total phytates (%)	<1	0.40	0.71
Aerobic plate count (cfu/g)	<10,000	870	600
<i>E. Coli</i>	Negative/10g	<5	<5
<i>Salmonella</i>	Negative/25g	Neg	Neg
<i>Staphylococcus aureus</i>	Negative/10g	<5	<5

C. Protein Identification

1. *Protein Analyses*

Sedimentation velocity analyses of the three canola protein products from *B. juncea* was conducted to determine the protein profile of the products and to determine the levels of the anticipated major proteins, 2S, 7S and 12S. The full report for this analysis is set forth in **Appendix 1**. The two major peaks for the proteins napin (2S) and cruciferin (12S) comprise from 70-85% percent of the protein in the unhydrolyzed product Isolexx™. This protein composition is consistent with the protein composition reported in the literature for *B. napus* and confirms the similarity of the *B. juncea* and *B. napus* species. In the hydrolyzed protein, Vitalex™, the amount of intact 2S and 12S protein remaining is approximately 1/100th of that of that in Isolexx™.

2. *Amino Acid Analyses*

The amino acid compositions of the BioExx protein products Isolexx™ and Vitalex™ are substantially similar as they are both derived from the same primary fraction of the seed protein. The differences can be attributed to the mode of action of the hydrolysis which allows recovery of the more insoluble proteins by cleaving them from the fibrous material whereas the isolate is composed of only the proteins that readily dissolve. In addition, the amino acid profile will vary due to lot to lot variation in the seed. **Table 6** below provides average amino acid

content values for the lots of BioExx products Isolexx™ and Vitalexx™ reported in Tables 3, 4 and 5 above.

Table 6
Amino Acid Profile of BioExx Canola Protein Products

Amino Acid	Gram Amino Acid/100 Grams of Protein		
	Isolexx™	Vitalexx™	
		Average	Average Juncea
Alanine	4.5	4.7	5.1
Arginine	7.6	6.7	4.8
Aspartic Acid	8.8	7.0	7.3
Cysteine (sulfur containing amino acid)	2.0	3.0	2.4
Glutamic acid	19.8	19.4	18.8
Glycine	5.4	5.2	5.2
Histidine*	3.1	3.7	3.2
Isoleucine*	4.2	4.5	4.6
Leucine*	7.8	7.6	7.8
Lysine*	5.5	5.6	5.7
Methionine* (sulfur containing amino acid)	2.0	2.2	2.5
Phenylalanine*	4.4	4.2	4.1
Proline	5.8	6.8	6.4
Serine	4.9	5.3	6.0
Threonine*	4.5	3.9	4.3
Tryptophan*	1.5	1.6	1.6
Tyrosine	3.3	3.4	4.1
Valine*	5.0	5.3	6.0

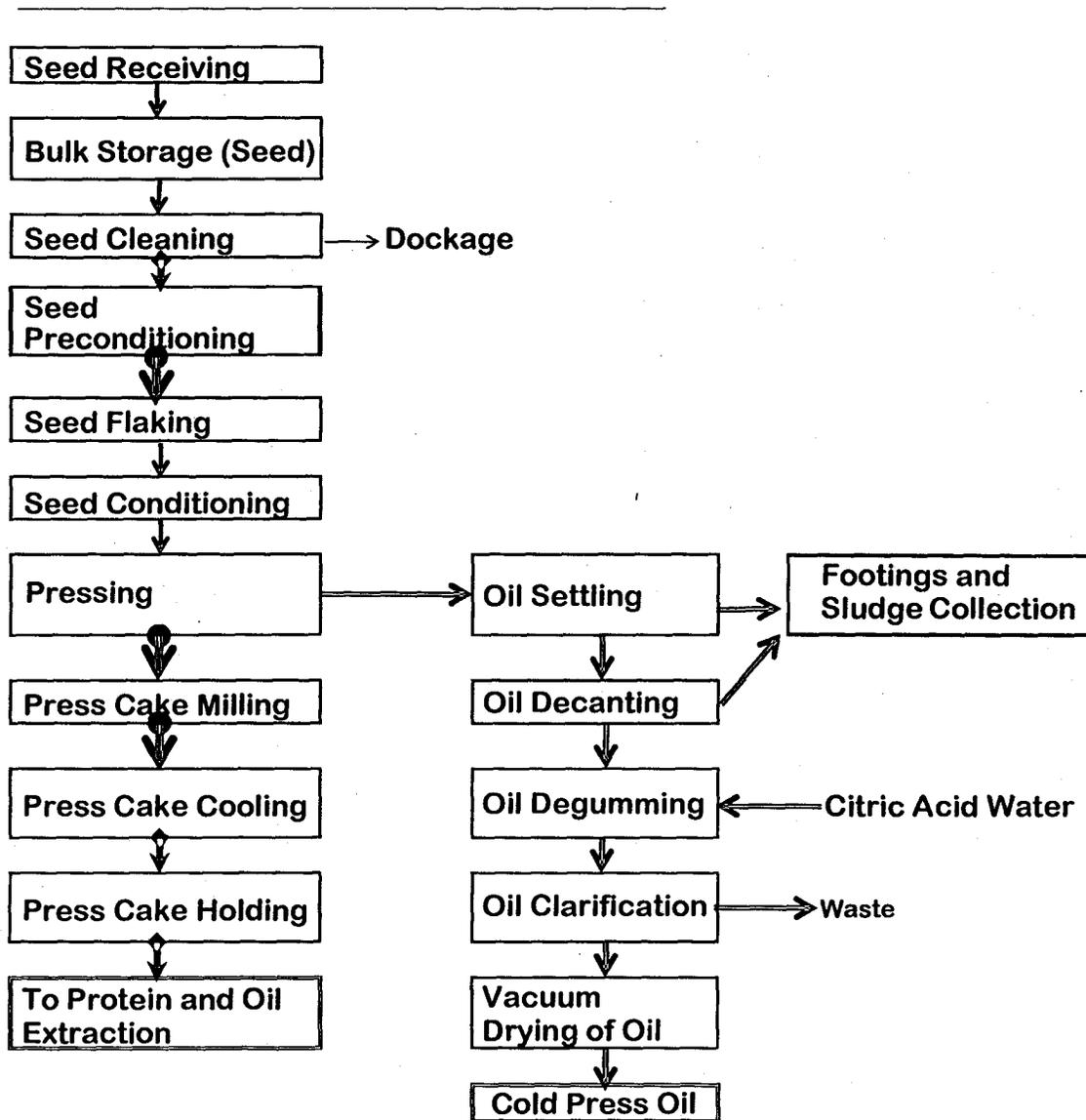
* Essential Amino Acids

IV. Manufacturing Process

A. Manufacturing Process for the Canola Protein Products

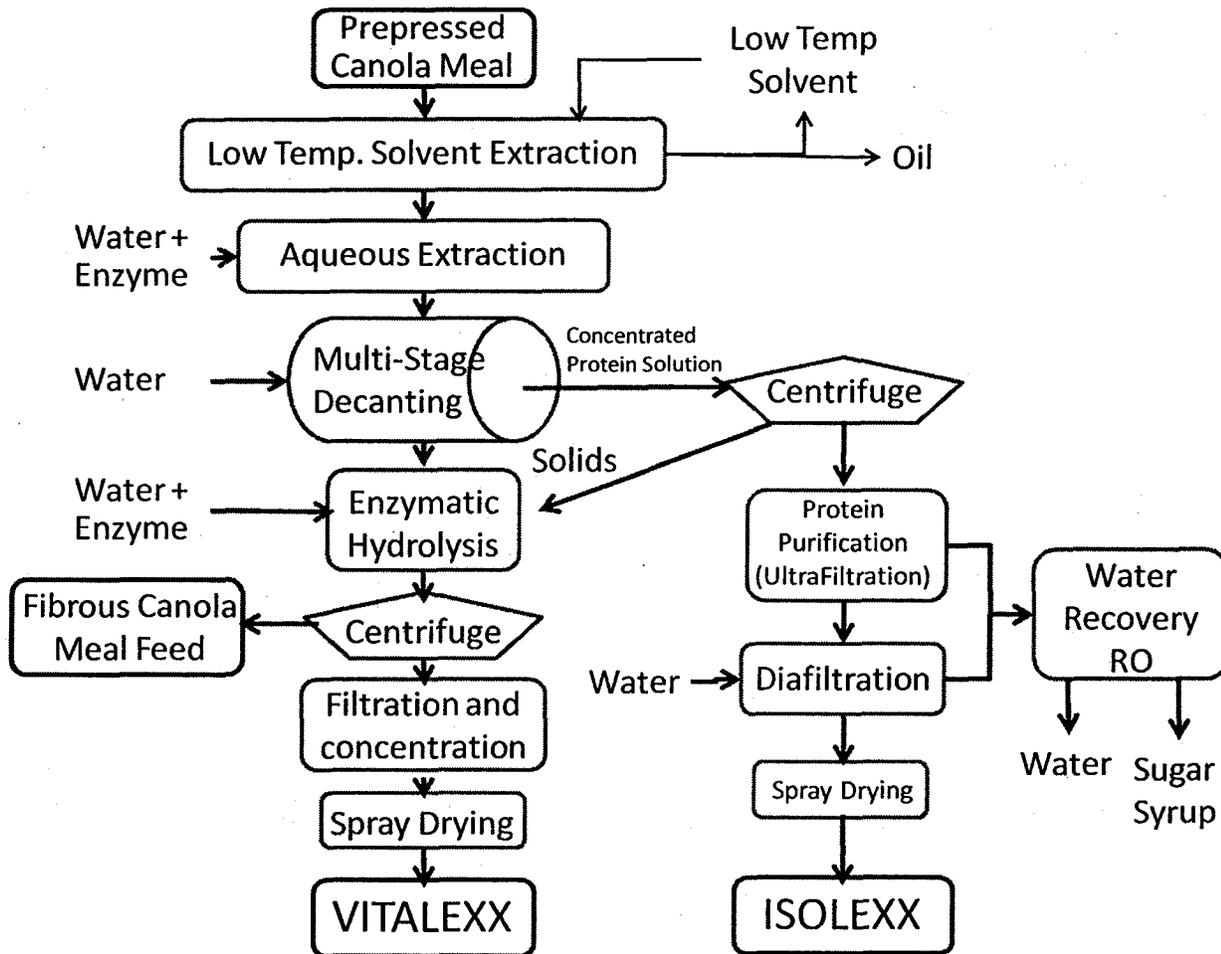
The starting material for the isolation of the canola protein products is raw canola *B. juncea* or *B. napus* seed. The manufacturing process begins with steps leading to the separation of the canola oil from the canola *B. juncea* or *B. napus* seed. The pressed canola oil is filtered, degummed, dried and removed for storage and shipping. These manufacturing process steps are illustrated in the process diagram Figure 1 below.

Figure 1. BioExx Oil Pressing from Canola Seed



The pressed cake (Prepressed Canola Meal) is extracted using butane at high pressure (150 PSI) to remove the remaining oil. The solvent is flashed off and the remaining oil is bleached and removed for storage and shipping. The last traces of solvents are then removed from the meal by the following steps. Water with added phytase enzyme is introduced into the extractor to contact the solvent-extracted meal and is then maintained at 55°C under high pressure (150 PSI). Subsequently, the pressure is released and then the vacuum is applied to remove all traces of the solvent which is re-compressed and stored.

Figure 2. Production of Isolexx™ and Vitalexx™ from Prepressed Canola Meal



The slurry of water and protein-containing defatted meal is the starting material for the production of the canola proteins. These manufacturing process steps are illustrated in the process diagram Figure 2. The meal–water slurry from the oil extraction process is then treated in several steps to isolate and concentrate the different protein fractions. A centrifugation step produces a fibrous solid fraction which contains the source of the hydrolyzed canola protein isolate (Vitalex™) and a water fraction from which the canola protein isolate (Isolexx™) is recovered, purified and spray dried.

The fibrous solids from the protein isolation process are redispersed and enzymatically treated to liberate the remaining protein from the fiber, and the slurry separated into a fiber stream and a protein rich stream. The protein-rich stream consisting of hydrolyzed protein is microfiltered, concentrated and spray-dried to produce the final product, hydrolyzed canola protein isolate (Vitalex™).

While BioExx intends to manufacture canola protein products derived from both *B. juncea* and *B. napus* seeds, with the exception of the batch analyses discussed in **Section III.B.**, the canola protein products used for the analyses discussed in this document were manufactured from *B. juncea*.

B. Safety of Substances Used in the Manufacture of the Canola Protein Products

1. *Enzymes*

a) *Phytase*

During the process of meal desolventizing and solvent recovery, water at 55°C is introduced to displace the solvent. The water contains about 300 parts per million (ppm) of phytase, an enzyme that can destroy phytates naturally present in the canola. Phytates are anti-nutritional factors responsible for complexing metals, impacting availability of minerals and lowering the solubility of proteins thus impacting the extraction process. Phytases break down the indigestible phytic acid (phytate) portion of the grains and oil seeds, resulting in the release of digestible phosphorus, calcium and other nutrients.

**Table 7
Residual Phytase Enzyme Activity in BioExx Canola Protein Products**

Sample	Total Phytase Enzyme by activity predryer (%w/w)	Total Phytase Enzyme by Activity after dryer (%w/w)
Isolexx™ from <i>B. Juncea</i>		
Composite Sample		<0.5
Vitalexx™ from <i>B. Juncea</i>		
BIOBPCV 20101107-B	<0.5	<0.5
BIOBPCV 20101107-C	<0.5	<0.5
BIOBPCV 20101107-D	<0.5	<0.5
BIOBPCV 20101107-E	<0.5	<0.5
BIOBPCV 20101107-F	<0.5	<0.5
Composite	<0.5	<0.5
Vitalexx™ from <i>B. Napus</i>		
BIOBPCV 20101115A	<0.5	<0.5
BIOBPCV 20101115B	<0.5	<0.5
Composite	<0.5	<0.5

* Method : Phytase- Manual Vanadate Method DL= 0.5%w/w

The enzymes comply with the recommended purity specifications for food-grade enzymes in the Food Chemicals Codex (FCC, 7th Ed., p1207). As shown in **Table 7** above, Phytase residues are below detection limits in the final products.

b) *Alcalase and Flavourzyme*

The hydrolyzed canola protein isolate (Vitalexx™) is produced using the food grade protease enzymes, Alcalase 2.4 L FG and Flavourzyme 1000 L. Alcalase and Flavourzyme are both aqueous protease enzyme solutions with excipients. Alcalase is produced from *Bacillus licheniformis* and is composed of 10-15% protease, 45-50% glycerin and 35-40% water. Flavourzyme is produced from *Aspergillus oryzae* and is composed of 15-20% aminopeptidase, 20-30% sucrose and 5-10% potassium chloride.

Table 8
Residual Protease Enzyme Activity in Final Vitalexx™ Products

Sample	Total Proteolytic Enzyme(ppm)	Absorbance (nm)
Vitalexx™ BIOBPCV2010107B –Juncea	<0.4	0.04
Vitalexx™ BIOBPCV2010107C –Juncea	<0.4	0.03
Vitalexx™ BIOBPCV2010107D –Juncea	<0.4	0.03
Vitalexx™ BIOBPCV2010107E –Juncea	<0.4	0.04
Vitalexx™ BIOBPCV2010107F –Juncea	<0.4	0.04
Vitalexx™ BIOBPCV2010115A –Napus	<0.4	0.07
Vitalexx™ BIOBPCV2010115B –Napus	<0.4	0.04

* Method: Protease Activity, endo-Protease Assay by Spectrophotometer

Both enzymes comply with the purity specifications for food-grade enzymes of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the FCC. The FCC indicates that protease enzyme preparations that are derived from any of the following source organisms are acceptable for use in food processing: *Bacillus licheniformis*, *Bacillus subtilis*, *Aspergillus niger*, or *Aspergillus oryzae* (FCC, 7th Ed., p1185). Because the enzymes used in the manufacture of the hydrolyzed canola protein isolate, Vitalexx™, are both proteases, one derived from *Bacillus licheniformis* and one from *Aspergillus oryzae*, we conclude that both of these protease enzymes are safe and suitable for production of BioExx's protein hydrolysates. Testing for residual protease activity has been below the detection limit of 0.4 ppm for all samples produced from either *B. Juncea* or *B. Napus*.

2. *Solvents, Acids, Bases, and Salts*

The following solvents, acids, bases, and salts are used in the manufacturing process for the BioExx canola proteins—butane, citric acid, nitrogen, sodium hydroxide (diluted caustic soda), and phosphoric acid. All of these are GRAS substances for the uses described as explained further below.

a) *Butane*

Butane (as *n*-butane and iso-butane) is GRAS affirmed in 21 C.F.R. §184.1165 for use in food as a propellant, aerating agent, and gas, at levels not to exceed good manufacturing practice

000017

(GMP). Under 21 C.F.R. §170.3(o)(25), propellants, aerating agents, and gases are defined as “[g]ases used to supply force to expel a product or used to reduce the amount of oxygen in contact with the food in packaging.” Butane has also been cleared by the European Union for use as an extraction solvent for use consistent with GMP for all food uses (European Council, 2009).

Butane or *n*-butane is a saturated open chain hydrocarbon (alkane) and like all alkanes, it is practically non toxic for single exposures below the lower flammability limit (1.9% or 19,000 ppm). The boiling point is - 0.5°C and butane is a highly volatile gas under standard conditions. The alkanes from propane through the octanes show increasing narcotic properties at longer inhaled exposures at high concentrations; the greater the chain length the greater the dermal and pulmonary irritancy. In humans, the inhalation of 10,000 ppm butane for ten minutes can result in CNS depression, but produces no systemic effects. The NIOSH Recommended Exposure Limit and the ACGIH TLV are 800 ppm or 1900 mg/m³ (Carréon, 2001).

Pure instrument grade butane (no mercaptanes added) is used to extract the residual canola oil from the pressed cake. Subsequently, the solvent is flashed off and the remaining oil is bleached and removed for storage and shipping. The solvent is then removed from the meal by a series of steps, utilizing water under pressure in a closed system to remove all traces of the solvents. Several different production lots of the pressed cake were tested for residual butane levels, and in each instance residual butane was not detected at a limit of detection of 10 ppm. Accordingly, residual butane has been reduced to less than 10 ppm in the pressed cake, and is expected to be reduced to even lower negligible levels in each of the BioExx protein products. In particular, as indicated above, the final protein is spray dried, which volatilizes the butane leaving virtually no residue. We conclude that only trivial levels could be present in the final BioExx canola protein products and that the use of butane in this application is GRAS. As shown in **Table 11** residual solvent levels of both VitalexTM and IsolexTM are below detection limits.

b) Citric Acid

Citric acid is affirmed as GRAS at 21 C.F.R. § 184.1033 for general use in foods with no limitation other than compliance with good manufacturing practice (GMP) and Food Chemicals Codex (FCC) specifications. Accordingly, the use of citric acid in the manufacture of the canola protein products is GRAS.

c) Nitrogen

Nitrogen is GRAS affirmed at 21 C.F.R. §184.1540 as a propellant, aerating agent and gas (gases used to supply force to expel a product or used to reduce the amount of oxygen in contact with the food in packaging) and may be used in food at levels not to exceed current good manufacturing practice (GMP). Accordingly, the use of nitrogen in the manufacture of the canola protein products is GRAS.

d) Sodium Hydroxide

Sodium hydroxide is GRAS affirmed at 21 C.F.R. §184.1763 as a pH control agent and processing aid when used at levels consistent with GMP. Processing aids in this context are

defined as “substances used as manufacturing aids to enhance the appeal or utility of a food or food component, including clarifying agents, clouding agents, catalysts, flocculents, filter aids, and crystallization inhibitors, etc.” (See 21 C.F.R. §170.3(o)(24)) and pH control agents as “substances added to change or maintain acidity or basicity, including buffers, acids, alkalies, and neutralizing agents.” (See 21 C.F.R. §170.3(o)(23)). Because this clearance covers the use of sodium hydroxide as a pH correcting agent in the manufacture of the canola protein products, the use of sodium hydroxide in the manufacture of the canola protein products is GRAS.

e) Phosphoric Acid

Phosphoric acid is listed as a multiple purpose GRAS food substance at 21 C.F.R. §182.1073 when used in accordance with good manufacturing practice (GMP). This clearance covers the use of phosphoric acid as a pH correcting agent in the manufacture of the canola protein products. Accordingly, the use of phosphoric acid in the manufacture of the canola protein products is GRAS.

V. Consideration of Potential Anti-Nutrient Factors

BioExx tested composite lots of Isolexx™ and Vitalexx™ to confirm that the following potential anti-nutrients were not present at levels that would raise any safety issues—Erucic Acid, Tannins, Phenolic Acids (Sinapine), Glucosinolates (including Allyl Isothiocyanate) and Phytates.

A summary of the results for Erucic Acid, Tannins, and Phenolic Acids (Sinapine) is provided in **Table 9** below; the data on Glucosinolates is provided in **Table 10** below; the data on Phytates is provided in **Tables 3, 4, & 5** above.

A. Erucic Acid

Erucic acid is a fatty acid in the oil (lipid or fat) of cruciferous plants including rapeseed, mustard and canola. Selective breeding resulted in the development of canola grade varieties with drastically lower erucic acid levels; modern varieties having lower than 0.1% erucic acid in the oil. In addition, effective protein/oil separation procedures ensure that the erucic acid levels in the proteins are at trace levels and not a toxicological concern. As noted in the specifications, the level of fat in the protein is less than 2% and the level of erucic acid in the fat is less than 0.1%. The maximum erucic acid level in the two BioExx products is therefore $0.02 \times 0.001 = 20$ ppm. These values are well below the 2% maximum limit set by FDA for canola oil. Thus, the erucic acid content in the canola protein products does not present a toxicological concern.

Table 9
Potential Anti Nutrients in Composite Lots of BioExx Canola Protein Products

	<u>Isolexx™</u> <u>Composite from</u> <u>B. Juncea</u>	<u>Vitalexx™</u> <u>Composite from</u> <u>B. Juncea</u>	<u>Vitalexx™</u> <u>Composite from</u> <u>B. Napus</u>
Erucic Acid [% product]	0.002	0.002	0.003
Total Phenolics [% of product]*	0.14%	0.39%	0.48%
Condensed Tannins	0.005%	0.013%	0.024%
Sinapic Acid Derivatives†	0.08%	0.14%	

* Expressed on basis of gallic acid, as tested by University of Nebraska Lincoln

† based representative lots, expressed in Sinapic Acid equivalent.

Testing on composite lots and representative lots has shown that erucic acid levels are less than 0.0002 mg/g of the product and would represent significantly lower intakes than would be expected from oil consumption and would be expected to have no physiological effect.

B. Tannins

Tannins are astringent and bitter plant polyphenols capable of binding with proteins and reducing their digestibility (Glick et al., 1970). Examples of tannins are the gallotannins that produce gallic acid and sugars upon hydrolysis, and the proanthocyanidins that are resistant to hydrolysis. Tannins are pervasive in edible plants and fruits. Tea typically contains 4-12% catechol tannins in the dried leaves. A single cup of tea can contain from 50-150 mg of tannins, and typical consumption of 3 cups per day corresponds to 150-450 mg of tannins. Several adverse nutritional effects may be associated with high levels of tannins, including depression of food intake, complexation with digestive enzymes, thus interfering with normal digestion, and local and systemic toxicity (Fahey et al., 1989). Several studies have shown that such effects require significant amounts of dietary tannin, far more than the small amounts found in dietary levels in tea. As seen in **Table 9** above, the total phenolics content including both tannins and phenolic acids for representative lots of Isolexx™ and Vitalexx™ is 0.14% and 0.39% respectively. The tannins content is approximately 0.005% (50 ppm) and 0.014% (140ppm). With a daily intake of 50 grams canola protein (which as noted above is likely to be exaggerative), the tannin intake for Isolexx™ is $50 \mu\text{g/g} \times 50 \text{ g} = 2.5 \text{ mg/p/day}$, which is less than the intake associated with much less than 1 cup of tea per day. Thus, the tannin content in the canola protein product does not present a toxicological concern.

C. Phenolic acids (Sinapine)

The class of phenolics includes not only polymeric polyphenols such as tannins but also small molecular weight phenolic acids which contribute to the color and astringent flavor of the seed. Sinapinic or sinapic acid (3,5-Dimethoxy-4-hydroxycinnamic acid) or Sinapine (its choline ester) are the principal phenolic acids in various rapeseed products and have been reported at levels of 1.1-1.8%. (Naczki et al., 1998). BioExx has developed a process to minimize the presence of these compounds due to deleterious effect on the organoleptic properties of the finished products. The presence of phenolics in food products is ubiquitous and

they are responsible for many of the anti-oxidant effects of traditional products such as blueberries and wine.

Analysis of total phenolics for representative samples of both Isolexx™ and Vitalex™ show the levels to be quite low, 0.14% and 0.38% respectively. Analysis for sinapic acid and derivatives shows levels in the region of 800 ppm for Isolexx™ and 1400 ppm for Vitalex™.

The analysis of both tannins and phenolics are interfered with by the presence of phenolic mono amino acids such as tryptophan and are thus overestimated in the Vitalex™ samples. In spite of this, the artificially high results are still on par with other highly purified canola proteins coming to market and similar levels were reviewed by FDA in GRAS Notification 327. They do not pose a health hazard at the low levels shown.

D. Glucosinolates & Allyl Isothiocyanate (AIT)

Glucosinolates are a class of water soluble, sulfur or nitrogen-containing glucosides that occur as secondary metabolites in virtually all species of *Brassica*. These plants also contain the enzyme myrosinase, which in the presence of water, cleaves of the glucose group from the glucosinolates, resulting in an isothiocyanate, thiocyanate, or nitrile. The signature product, allyl isothiocyanate, (AIT) or oil of mustard is produced from the glucosinolate, Sinigrin, which is present in several *B. species* (SCOGS, 1975). The specific selective breeding of canola species, including *B. juncea*, markedly reduced the level of glucosinolates and reduced the potential for AIT formation in canola protein. As shown in **Table 10**, glucosinolates are present in BioExx's proteins from *B. juncea* are at levels less than 0.2 -1.0 $\mu\text{mole/g}$ or equivalent to a maximum isothiocyanate level less than 27-138 ppm. The molecular weight of allyl isothiocyanate is 99.15 g/mole while the average molecular weight of the aglycones of the glucosinolates is of the order of 138 g/mol; thus if they are present in the finished product at a level of 0.20-1.0 $\mu\text{mole/g}$ = 20-138 $\mu\text{g/g}$ = 20-138 ppm. Thus, if all glucosinolates present were able to be converted to allyl isothiocyanate, it would be present in the finished product at a level of up to 0.20 $\mu\text{mole/g}$ = 20-100 $\mu\text{g/g}$ = 20-100 ppm. However, since allyl isothiocyanate is only derived from allyl glucosinolate, which has never been detected in the sample (LOD 0.05 $\mu\text{mole/g}$), the true Allyl Isothiocyanate level derived from the glucosinolate would be less than 5 ug/g.

The 1975 SCOGS Panel evaluated the safety of AIT as the major component and the defining ingredient in mustard oil, made from the seeds of *B. nigra*. The Panel concluded that, based on the available toxicological information, AIT was safe at its estimated maximum average intake of approximately 12 mg/person per day. The safety data included short-term studies, teratology studies, and mutagenicity studies, but no long-term or cancer studies.

Subsequently, the National Institute of Health/National Toxicology Program (NIH/NTP) conducted a long-term bioassay on AIT, which began in 1978. A single-dose study, a 14-day study, and a 13-week study were performed before the chronic study was conducted. Pathologic findings seen in the 14-day study at 50 mg/kg included a thickened mucosal surface of the stomach in rats and mice and a thickened urinary bladder wall in male mice. No gross or microscopic lesions were seen at the highest dose level (25 mg/kg) in the 13-week study. Following the 14-day and 13-week study, a 2-year carcinogenesis study was conducted by administering 12 or 25 mg/kg AIT in corn oil five times per week by gavage to groups of rats

and mice of each sex for 103 weeks (NTP, 1981). AIT was found to be non-carcinogenic in both species of mice and female rats, and weakly carcinogenic in male rats, producing transitional-cell papillomas in the urinary bladder ($P < 0.05$; controls 0/49, 0%; low dose, 2/49, 4%; high dose, 4/49, 8%). Under NTP criteria, AIT was considered carcinogenic in male rats. There was also an increased incidence of cytoplasmic vacuolization in the liver of male mice: controls 2/49, 4%; low-dose, 8/49, 16%; high dose, 13/50, 26%. Based on the non-neoplastic lesion, the NOEL for the long term study was found to be below 12 mg/kg bw/day or less than 840 mg/p/day. This study provides further support (that apart from the controversial carcinogenicity) for the earlier conclusion by SCOGS that AIT is safe at 12 mg/p/day.

Table 10
Glucosinolate Analysis of Representative Lots of BioExx Canola Protein Products

Glucosinolate [$\mu\text{mol/g}$]	Total	Total Aliphatic	Allyl	3-butenyl	2-OH-3-butenyl-	3-CH3-indolyl-	Phenyl ethyl-	Allyl ITC [mg/kg]
Isolexx™ from B. Juncea								
BIOBPCI20100323A	0.15	0.15	<0.05	0.15	<0.05	<0.05	<0.05	<100
BIOBPCI20100614B	0.06	0.06	<0.05	0.06	<0.05	<0.05	<0.050	<100
BIOBPCI20100705B	0.05	0.05	<0.05	0.05	<0.05	<0.05	<0.05	<100
BIOBPCI20100705C	0.08	0.08	<0.05	0.08	<0.05	<0.05	<0.05	<100
Vitalexx™ from B. Juncea								
BIOBPCV 20101107-B	0.18	0.18	<0.05	0.15	<0.05	<0.05	<0.05	<100
BIOBPCV 20101107-C	0.23	0.18	<0.05	0.15	<0.05	<0.05	0.05	<100
BIOBPCV 20101107-D	0.2	0.2	<0.05	0.16	<0.05	<0.05	<0.05	<100
BIOBPCV 20101107-E	0.18	0.18	<0.05	0.14	<0.05	e<0.05	<0.05	<100
BIOBPCV 20101107-F	0.15	0.15	<0.05	0.12	<0.05	<0.050	<0.05	<100
Vitalexx™ from B. Napus								
BIOBPCV 20101115A	0.14	0.14	<0.05	0.07	0.07	<0.05	<0.05	<100
BIOBPCV 20101115B	0.22	0.17	<0.05	0.09	0.08	0.05	<0.05	<100

*<0.05 represents below detection limit

The entire database relevant to the carcinogenicity of AIT was also reviewed by the International Agency for Research on Cancer (IARC) in 1999 (IARC, 1999). IARC concluded that animal data on AIT, including the NTP study, was insufficient. IARC determined that AIT is not classifiable as to its carcinogenicity to humans (Group 3).

In 2003, a GRAS Notification (GRN 133) was presented to FDA regarding the use of AIT (volatile oil of mustard) as an additive for use in packaging to aid in prolonging shelf life and retarding spoilage of the contained food. The level of AIT was stated to be below the level required to impart flavor, estimated in the range below 10-30 ppm in the food. The FDA did not comment specifically on the carcinogenicity of AIT. According to FDA, the scientific support for GRN 133 principally rests on the conclusion of the SCOGS Report and the determination that the levels used in the packaging application would be below those already approved as GRAS for flavors. FDA had "no questions" regarding the proposed GRAS status of AIT under the

proposed conditions of use. In addition, in 2005, FDA received GRAS Notification 180 (GRN 180) for the use of AIT in a similar packaging application. The human exposure to AIT from the proposed packaging applications was estimated between 0.68 and 1.46 mg/person/day. Again, FDA, recognizing the existence of the NTP study, had “no questions” regarding its proposed GRAS status under the conditions of use.

Subsequent to 1980, several reports have downplayed the significance of the NTP study as implying a human risk and questions remain over whether there is significant data that AIT is an animal carcinogen. First, there was IARC’s conclusion that the NTP study is not by itself conclusive. Second, there are several other scientific studies in the literature arguing that the male rat is not a good animal model for AIT. One of these is based on the fact that the principal metabolite of AIT in rats is the glutathione metabolite, which appears to be a minor metabolite in mice and humans (Bollard et al. 1997). Another study found that transitional cell papillomas in rats were secondary to hyperplasia, and would not be produced at lower non-irritant doses (Jiao et al. 1994). The lack of mutagenicity for AIT supports the view that AIT lacks the ability to directly induce cancer through a genotoxic mechanism, which provides support for the existence for a dose threshold in the experimental range.

Based on the SCOGS Panel review, and in light of the several reports that have downplayed the significance of the NTP study, AIT at intake levels of up to 12 mg/p/day would be considered safe. As discussed previously, the estimated daily intake of the BioExx protein products is not expected to exceed FDA’s Daily Reference Value for protein of 50 g/day (See 21 C.F.R. § 109(c)(9)). Accordingly, we estimate the maximum daily intake of Isothiocyanate from the BioExx isolates as follows: (maximum isothiocyanate contamination in protein) × (daily intake BioExx protein) = (20 – 150) µg/g × 50 g/d = 1000-7500 µg/d = 1-7.5 mg/p/d. The true allyl Isothiocyanate intake is estimated at less than 5 µg/g × 50 g/d = 0.25 mg/p/d. Therefore, the potential daily intake of AIT from the proposed use of BioExx’s protein products does not present a toxicological concern.

E. Phytates

Phytic acid (known as inositol hexakisphosphate (IP6)) or phytate when in salt form) is the principal storage form of phosphorous in many plant tissues especially bran and seeds (NRC, 1973). Phytic acid accounts for 50–80% of the total phosphorus in different cereals. Phosphorous in phytate form is, in general, not bioavailable to non-ruminant animals because they lack the digestive enzyme, phytase, which is required to separate phosphorus from the phytate molecule. Phytate is also a strong chelator of important minerals such as calcium, iron and zinc, and, therefore, can contribute to mineral deficiencies in people by the sequestration of these minerals thus reducing their bioavailability. Phytate also acts as an acid, chelating the vitamin niacin (B₃), which is basic, and may contribute to vitamin B₃ deficiency (pellagra) (Reddy and Sathé, 2002). As shown in **Tables 3, 4, & 5**, the level of phytates in the BioExx canola protein products are specified to be ≤ 1.25% for Isolexx™ and ≤ 1.0% Vitalexx™, with the specific levels found to be between 0.4% and 1.1% for Isolexx™ and 0.3% and 0.7% for Vitalexx™. These are well below the 5.25% phytate level found to cause adverse effects in rats fed rapeseed flours from early rapeseed cultivars (Anderson, *et al.* 1976). The fact that the protein quality was high in digestibility studies of the BioExx proteins containing such levels of phytate indicates also shows that these phytate levels are too low to interfere with protein

digestion. Accordingly, the phytate concentrations in the BioExx canola protein products are not likely to be of toxicological or nutritional concern.

VI. Consideration of Potential Contaminating Materials

A variety of contaminants may be present in the seed that could potentially end up in the final protein products. Table 11 shows the results of testing composite samples of Isolexx™ and Vitalexx™ for some of the more regulatory significant compounds.

A. Pesticide Residues

A variety of agricultural pesticides can be present in canola production. Composite lots of both Isolexx™ and Vitalexx™ were tested for a broad spectrum of 307 potential pesticides by an outside lab. They include the classes of OrganoPhosphates, OrganoNitrogens, Organochlorinated and N-methylcarbamates. No pesticide residues were detected in any sample.

Table 11
Contaminant Analysis of Composite Lots of BioExx Canola Protein Products

	<u>Isolexx™</u> <u>Composite</u> <u>from B. Juncea</u>	<u>Vitalexx™</u> <u>Composite</u> <u>from B. Juncea</u>	<u>Vitalexx™</u> <u>Composite</u> <u>from B. Napus</u>
Pesticides (307 varieties) [ppb]	ND	ND	ND
Dioxins and Dioxin-like substances [TCDD eq ppq]	ND	ND	ND
Polycyclic Aromatic Hydrocarbons	ND	ND	ND
Benzo(a)pyrene [ppb]	ND(<2.0)	ND(<2.0)	ND(<2.0)
Benzo(a)anthracene [ppb]	ND(<2.0)	ND(<2.0)	ND(<2.0)
Acrylamide [ppb]	ND(<10.0)	124	67.3
Total Aflatoxins [ppb]			
B1 [ppb]	ND(<0.50)	ND(<0.50)	ND(<0.50)
B2 [ppb]	ND(<0.50)	ND(<0.50)	ND(<0.50)
G1 [ppb]	ND(<0.50)	ND(<0.50)	ND(<0.50)
G2 [ppb]	ND(<0.50)	ND(<0.50)	ND(<0.50)
Residual Solvent [ppm]	ND(<10.0)	ND(<10)	ND(<10.0)

ND – Not detected

B. Dioxin Residues

Dioxins are a variety of compounds similar in structure that have a variety of toxic effects in animals and humans. They have also been seen to be carcinogenic and can bioaccumulate due to long persistence. They are widely distributed in the environment due to long range transport. A battery of testing for Dioxin and Dioxin-like substances was performed on composite lots of both Isolexx™ and Vitalexx™. No reportable levels of Dioxin or Dioxin-like materials were detected as reported in Table 11.

C. Polycyclic Aromatic Hydrocarbons

Isolexx™ and Vitalexx™ were also both tested for the presence of persistent polycyclic aromatic hydrocarbons including Benzo (a) anthracene and Benzo (a) pyrene. All composite samples tested below detection limits for all PAHs.

D. Acrylamide

Acrylamide is a consequence of the reaction of the amino acid Asparagine with reducing sugars at elevated temperature such as during baking or frying. The Isolexx™ isolation specifically avoids high temperature and as such the risk for production of acrylamides is very low. A composite sample of Isolexx was shown to be free of Acrylamides. Composite samples of Vitalexx™ were shown to be low in acrylamides. See **Table 11**.

E. Aflatoxin

Aflatoxins are compounds produced by fungal growth during storage of the seed and/or product. Presence of these compounds is common throughout much of the world including the United States and Canada. They are a consequence of warm moist storage conditions in the supply chain and are of concern as potential carcinogens. **Table 11** shows the tabulated results from testing of composite lots of Isolexx™ and Vitalexx™. The level set by the FDA in CPG 555.400 for total aflatoxins in food (B1, B2, G1 & G2) is 20 µg/kg. At less than 0.5 µg/kg, the aflatoxins levels are an order of magnitude lower than the safe limits specified and thus should be considered safe. Individual analysis of the aflatoxins for both Isolexx™ and Vitalexx™ showed each specific type to be below detection limits.

F. Residual Solvent

The light weight solvent employed in the oil extraction process is hydrocarbon based and is thoroughly stripped during the process. In some oilseed extraction processes, the solvent such as hexane has an affinity for the solids and may be left behind. All testing of the meals and protein products of the BioExx process have been shown to be below detection limits of the solvent. As seen in **Table 11**, no residual solvents were detected on composite samples of Isolexx™ and Vitalexx™. In addition, each individual lot of Vitalexx™ was tested and no residual solvent was detected in any lot.

G. Heavy Metals

The concentration of arsenic, mercury, lead and cadmium was determined via ICP-MS. As shown in **Table 12**, all lots were well within compliance of U.S. and European regulations.

Table 12
Heavy Metals Analysis of Representative Lots of BioExx Canola Protein Products

Heavy Metals [ppm]	Arsenic	Cadmium	Lead	Mercury	Total Heavy Metals
Isolexx™ from <i>B. Juncea</i>					
BIOBPCI20100323A	<0.05	0.28	<0.01	0.007	<0.34
BIOBPCI20100614B	<0.05	0.19	0.02	0.012	<0.27
BIOBPCI20100705B	<0.05	0.25	0.02	<0.005	<0.32
BIOBPCI20100705C	<0.05	0.16	0.02	<0.005	<0.23
Vitalexx™ from <i>B. Juncea</i>					
BIOBPCV 20101107-B	<0.05	<0.01	0.02	<0.002	<0.08
BIOBPCV 20101107-C	<0.05	<0.01	<0.01	<0.002	<0.07
BIOBPCV 20101107-D	0.06	0.01	0.04	<0.002	<0.11
BIOBPCV 20101107-E	<0.05	<0.01	0.02	<0.002	<0.08
BIOBPCV 20101107-F	<0.05	<0.01	0.01	<0.002	<0.07
Vitalexx™ from <i>B. Napus</i>					
BIOBPCV 20101115A	<0.05	<0.01	0.01	<0.002	<0.07
BIOBPCV 20101115B	<0.05	<0.01	<0.01	<0.002	<0.07

VII. Nutrition – Protein Digestibility

The nutritional value of dietary proteins is dependent on digestibility. Accordingly, BioExx conducted protein digestibility studies on the two canola protein products to investigate the quality of the canola proteins derived from *B. juncea*. The digestibility studies on the canola proteins, Isolexx™ and Vitalexx™, included a complete Protein Digestibility Corrected Amino Acid Score (PDCAAS) analysis based on the measured protein digestibility of the protein isolates and the amino acid requirements in rats. The studies were conducted at the Division of Animal Nutrition Physiology of Goettingen University in Germany under the direction of Prof. Dr. Frank Liebert. The complete studies are attached in **Appendix 2** and include several Tables in the appendix to the studies.

The digestibility studies conducted at Goettingen University used male Wistar rats that were fed approximately 15% of Isolexx™, Vitalexx™, Dunasoy (a type of soy protein), or Casein (milk protein). Rats were fed three times a day on a restrictive feeding level to avoid feed losses. Daily feed supply was mostly fixed during the collecting period and based on the feed intake in the pre-period. Feed losses were recorded and taken into account when daily feed intake was calculated. Individual body weight (BW) of the animals was measured at the beginning of the pre-period and at the start as well as at the end of the collecting period. The average BW applied for calculating the metabolic BW was the mean value of BW at the beginning and at the end of the collection period.

As discussed above, Isolexx™ and Vitalexx™ are both highly purified protein canola isolates derived from *B. juncea*, and as discussed more fully in **Section V** above, these products are essentially free of anti-nutrients. Dunasoy and Casein are standard protein sources. The

design of the studies permitted a comparison of the quality of the four protein sources. Since there can be variation between laboratories, accurate comparisons require that studies be done by the same investigators using the same methods for each protein. The digestibility studies provide experimental data about basal endogenous nitrogen (N) losses via gut and urine, respectively. These experimental data are provided in Tables 2 and 3 in Appendix 2.

The calculated true digestibility of the canola isolates are both above 90%. Lysine was the limiting amino acid for both Isolexx™ and Vitalexx™, and the PDCAAS value for Isolexx™ and Vitalexx™ were 1.04 and 1.08 respectively based on the amino acid analysis performed on the sample and the Gassman 2006 scoring pattern. Because lysine was the limiting amino acid, to ensure that it was accurately measured, duplicate assays for the amino acids were run. These are shown in the Report Appendix Tables 9 and 10). Using the Average amino acid analysis shown in Table 6, the calculated digestibilities and the FAO 2007 scoring pattern for ages 3-10, the PDCAAS values are 1.11 and 1.14, respectively for the Juncea Isolexx™ and Vitalexx™. The studies show that the canola proteins, Isolexx™ and Vitalexx™, are highly digestible proteins for the rat. Overall, the studies show that the quality of the canola proteins were superior to Dunasoy, as derived from the PDCAAS of lysine (for the Isolexx™ and Vitalexx™) or methionine+cysteine (for the Dunasoy). Appendix 2-Table 9B from the Study Report is reproduced below:

Appendix 2-Table 9b: PDCAAS of Canola proteins according to AA-analysis of LUFA 9/2010

Reference protein humans								
AA	His	Ile	Leu	Lys	Met+Cys	Phe+Tyr	Thr	Trp
mg AA/g protein	-	-	-	51	25	-	27	7
Test protein (Isolexx™)								
AA	His	Ile	Leu	Lys	Met+Cys	Phe+Tyr	Thr	Trp
mg AA/g protein	-	-	-	55.75	23.00	-	40.65	14.29
AAS	-	-	-	1.09	1.77	-	1.51	2.04
TPD (%)*	94.83							
PDCAAS	-	-	-	1.04	1.68	-	1.43	1.93
Test protein (Vitalexx™)								
AA	His	Ile	Leu	Lys	Met+Cys	Phe+Tyr	Thr	Trp
mg AA/g protein	-	-	-	56.21	50.21	-	49.51	13.95
AAS	-	-	-	1.10	2.01	-	1.83	1.99
TPD (%)*	98.00							
PDCAAS	-	-	-	1.08	1.97	-	1.79	1.95

(*) true protein digestibility

It should also be noted that the calculated protein quality values were derived from rat studies that utilized the tested feed canola proteins as single protein sources, without any

supplementation with other proteins. Typically, single protein diets suffer from an unbalanced amino acid profile, especially diets based on vegetable proteins. Often there is less lysine than the other amino acids, which can affect protein quality. Here, even though the tested material was the only protein source in the diet, the protein quality was adequate. Under typical use conditions of use the presence of other proteins in the diet would only further improve the protein quality of the canola protein products.

In addition to the sponsor's studies, several studies on protein utilization of *Brassica* species protein in animals exist, including a published review on rapeseed meal (*B. napus*) (Bell, 1984). It is generally reported that canola protein from *B. napus* is also of similar high quality.

VIII. Basis for GRAS Determination

A. Safety Overview

1. *Taxonomy of the Oilseed Brassica Species*

As sources of common vegetables *Brassica* species have been in use for thousands of years: (1) *B. oleracea* (kale, cabbage, cauliflower, collard greens, broccoli), (2) *B. napus* (rape kale, rutabaga), and (3) *B. rapa* (bok choy, turnip, napa cabbage, turnip, turnip rape). (4) *B. juncea* (mustard greens, Indian mustard). Over the past 50 years the oilseed *Brassica* crops have become internationally important. The expanded use of the crops occurred when plant breeders transformed the chemistry of the seeds to reduce the level of erucic acid in the oil and then the level of glucosinolates in the meal. The seeds are now widely harvested and crushed to release the oil (~40% by weight) and the remaining meal, largely protein also approximately 40% by weight, is primarily blended into animal feed (Lamb, 1989). In Europe, the crop is known as "00 rape," signifying reduction of erucic acid and glucosinolates to near zero. In Canada, the crop is referred to as canola and the oil is known as Canola oil (Canadian oil, low acid) (initially it was referred to as LEAR oil (Low Erucic Acid Rapeseed) in Canada and the U.S.).

Although agriculture and the food industries often treat rapeseed as a single commodity as reported by Lamb (1989), the crop is actually a composite of seed from two or three species (Downy, 1983; Prakash and Hinata, 1980). In Asia, *B. campestris* and *B. juncea* are widely grown. In Europe, Canada, Australia, and New Zealand, *B. napus* and to a lesser extent *B. campestris* are grown. These species are closely related. *Brassica juncea* is thought to be a natural hybrid between *B. campestris* and *B. nigra*, and *B. napus* arose as a natural hybrid between *B. campestris* and *B. oleracea*. Plant breeders in the 1960s used selective breeding to develop rapeseed varieties low in erucic acid. During the 1970s, the Canadian government, jointly with Canadian farmers, developed low glucosinolate levels as well as low erucic acid lines of rapeseed (canola) and eventually developed cultivars of *B. juncea* with the same impurity and fatty acid profile as canola (The Canola Council of Canada changed the definition of canola to include *B. juncea*. The first *B. juncea* varieties, Arid and Amulet, received contract registration in April 2002). The production of canola seed is now well established in several countries, notably in Canada and Australia, where the utilization of canola protein for human food is being actively investigated (Tan, et al. 2010).

Brassica species rank third in oil-seed production after soybean and palm. *B. napus*, *B. campestris* and *B. juncea* are currently used in oil-seed production in Canada, with *B. juncea* of

more recent vintage. *B. juncea* is reportedly more disease resistant and better adapted to the semi-arid conditions of the Canadian prairie than *B. napus* (Woods et al. 1991). As indicated earlier, BioExx's canola protein products will be derived from both *B. juncea* and *B. napus*.

2. Human Food Use

a) History of Use

Brassica species, as noted above, are one of the most widely cultivated species of plant for human food. *B. juncea* is the plant source of edible mustard greens and mustard seed. Mustard is typically made from three principal types of seed: black mustard (*B. nigra*), white mustard (*B. alba*) (White mustard is correctly *Sinapis alba*, but is commonly referred to as *Brassica. alba. Sinapis* is a closely related species in the *Brassicaceae* family), and brown mustard (*B. juncea*). Mustard seeds of each variety can be traced back to different areas of Europe and Asia, with the black variety originating in the Eastern Mediterranean regions, the brown from India, and the white from the Middle East. White mustard was used as a spice in ancient Greece, while the Romans used a paste from the ground seeds, which was probably the ancestor of our modern day mustard condiment (Ensminger et al. 1986).

In the U.S, brown mustard from *B. juncea* (L.) Coss. is listed as a GRAS spice/other natural seasoning and flavoring (21 C.F.R. §182.10). The FDA has not issued definitions or standards of identity for spices including prepared mustard or mustard seed. In lieu of standards, FDA has issued guidance in the form of two Compliance Policy Guides (CPG) that provide information on the definitions for mustard (FDA, 1980). The seeds of *B. juncea* are recognized as "mustard seed" in these two CPGs. The majority of mustard seeds are grown in India, Canada, the U. S., Hungary, and Great Britain (World's Healthiest Foods, 2001-2011). India exceeds the world in per capita consumption of mustard seed, as it is widely used both as an ingredient in curry (masala) as well as a directly added spice.

b) Estimated U. S. Intake of *B. juncea* Proteins from Condiment Mustard

Condiment mustard is prepared from mustard seeds or mustard flour (ground seeds), vinegar, water, and other spices. According to the 1975 Select Committee on GRAS Substances (SCOGS) Report, the maximum possible daily intake of brown mustard flour is about 6.02 grams/day (SCOGS, 1975). Approximately 48% or 2.9 g/day is brown mustard flour from *B. juncea*. The mustard flour consists primarily of the ground mustard seeds, which contain approximately 25% protein. Accordingly, the maximum possible average daily intake of *B. juncea* protein from condiment mustard is $2.9 \text{ g} \times 0.25 = 725 \text{ mg/p/day}$. A more realistic estimate, also provided in the SCOGS report, based on the amount of brown mustard actually used by the food industry, rather than the amount produced, is 18 mg/p/day or 4.5 mg/p/day of mustard protein. Current USDA Tables indicate an average intake of prepared mustard of 1.0 g per day, which includes both brown and yellow mustards (USDA, 1994-1996). Assuming prepared mustards can contain as much as 15% solids (assumed to be 100% mustard seeds of seed flour), and brown mustard is 48% of the total, the estimated intake of protein from brown mustard is $1.0 \text{ g} \times 0.15 \times 0.48 \times 0.25 = 18 \text{ mg/B. juncea protein /person/day}$. Doubling this amount to estimate the 90th percentile, we obtain 36 mg *B. juncea* protein/person/day. This amount, 36 mg *B. juncea* protein/person/day, is a reasonable estimate for the 90th percentile

amount consumed on a chronic basis, but the maximum acute level, or maximum possible serving size, is certainly much larger and closer to the SCOGS estimate of 725 mg/p/day.

c) *Comparison of Intakes of B. juncea proteins: India vs. the U.S.*

As indicated above, India probably uses more mustard seeds in food than any other country, and consumption in India certainly vastly exceeds the consumption in the U.S. Although the data is variable, we can make a rough estimate of per capita consumption of mustard seed in India as follows. The "Mustard Seeds Outlook Report" from Karvy Comtrade Limited gives the total world production of mustard seed (2007-08) as 49.82 million metric tons or 49.8×10^9 kilograms (Mustard Seeds Outlook Report, 2008). India produces about 14% of the world crop and virtually all of it is used in processed foods as mustard flour or as a condiment. We assume that amount of mustard seed making its way into the food supply after correcting for other uses and losses is 50% of this amount or about 25×10^9 kilograms. Assuming the dry solid is 60% of the total seed and 25% of this is protein, the amount of mustard protein consumed in India is:

$(0.14 \times 0.6 \times 0.25) \times 25 \times 10^9$ kilograms = $0.021 \times 25 \times 10^9$ kilograms = 5.3×10^8 kilograms.

Dividing by the Indian population of 1.2×10^9 persons, the per capita consumption of mustard protein is estimated to be 0.44 kg/person/year or 442 g/365 days or 1.2 g/person/day. This is approximately 65 times the average per capita estimate for the U.S. (1,200 mg/18 mg = 66.6).

3. *Animal Use of B. juncea Pressed Cake*

As discussed more completely in Section VII.A.3. on the animal field studies on canola seed, tens of thousands of food producing animals in Canada and the U.S., (including cattle, chickens and swine), have thrived on the pressed cake from the production of canola oil including *B. juncea*. The pressed seed cakes from the varieties of canola seed are rich in protein and are blended with feed to improve the nutritional value of the ratio.

4. *Safety Studies on Canola Protein Products*

The rapeseed species *B. napus*, *B. juncea* and *B. campestris* each contain the same two major proteins: a 12S, globular protein, cruciferin and a 2S albumin, napin. The minor proteins and the ratios of the two major ones depend on the species, the growing season, the cultivars and other factors. Both proteins are storage proteins. Compared to other plant proteins, cruciferin is rich in lysine, which contributes to the quality of canola proteins (Bos et al. 2007). There are three conventional published animal safety studies on the protein isolates from rapeseed that has been treated to reduce or eliminate erucic acid and glucosinolates:

- (1) Loew, F.M., et al. Evaluation of a dietary rapeseed protein concentrate flours in rats and dogs. *Toxic Applied Pharma.* 35: 257-267, 1976.
- (2) Plass, R., et al. Toxicological evaluation of rapeseed products in a subacute feeding study in rats. *Die Nahrung* 36: 248-252, 1992.

- (3) Mejia, L.A., et al. A 13-week sub-chronic dietary toxicity study of a cruciferin-rich canola protein isolate in rats. *Food Chem. Tox.* 47: 2645-2654, 2009.

We discuss each of these studies in turn below, and, because it is the most recent and most relevant study, the Mejia *et al.*, 90-day feeding study will be discussed last and in more detail than the others.

a) *Lowe et al. (1976)*

In the 1976 Loew *et al.* study, two different diets containing protein isolates from rapeseed flour (RSF) were fed to growing dogs and rats for 90-days. In the first experiment both rats and dogs were fed a protein isolate containing 930 ppm total glucosinolates, including 529 ppm total isothiocyanates (determined by GLC). The diets were semi-synthetic and 20% and 40% of the protein was supplied by RSF. Control groups received only casein as the source of protein.

No antithyroid treatment effects or any other treatment related effects were noted in the dogs. There were no significant effects on weight gain or food consumption. No significant differences in either iodine uptake or in serum thyroxin in the dogs were observed. No significant histological effects were observed in the dogs, including cellular morphological measurements indicative of thyroid status.

Slight anti-thyroid effects were noted in the rats. Both 20% and 40% RSF groups appeared to have thyroid glands that were more cellular and had more follicles than in the control group. There were statistically significantly lowered serum thyroxin levels at 30 days in the 40% RSF-treated group and the average thyroid to body weight ratio of the rats was slightly greater in the 40% RSF treated females than in the other groups.

In the second experiment only rats were used, and they were exposed to the same 20% and 40% RSF-diets, but this time the diets were specially purified to virtually eliminate the glucosinolates. In particular, the glucosinolates were reduced to 30 ppm total glucosinolate, containing only 6 ppm total isothiocyanates. Iodine uptake was slightly lower in the controls than in the RSF-treated groups but there were no differences in serum thyroxin concentration and thyroid-to-body-weight ratios, and thyroid histology remained essentially normal. There was some detectable improvement compared to earlier-fed diets that were 30 times higher in total glucosinolates. The authors concluded that in neither diet were the changes remarkable, with only slight histological changes and no biologically significant alterations in uptake or serum thyroxin concentrations.

b) *Plass et al. (1992)*

In the Plass *et al.* study, a rapeseed protein isolate (RPI) and rapeseed extraction residue (RER) were fed to male Wistar rats at levels of 2.5%, 5%, and 10% for 28 days. The glucosinolate levels in both test materials were less than 10 ppm. The RPI was between 85.9-88.2% protein, whereas the RER was only 26.3% protein with approximately 74% uncharacterized material. Throughout the study the mean food consumption did not differ between the groups. No deaths, treatment-related effects in weight, or changes in appearance or behavior were observed. Weights and histology of the thyroid did not reveal any effects on

thyroid activity, which is considered the main effect in animals fed rapeseed containing glucosinolates. The two higher dose levels for RER produced mild liver hypertrophy when measured by absolute liver weights, but no effects on relative liver weight. The relative kidney weight was reduced slightly in all the RER groups, but the absolute weight was not affected. At the 10% dose of RPI there was a small affect on absolute liver weight. The authors proposed a preliminary no-observed effect level (NOEL) of 5% RPI and 2.5% for RER in male Wistar rats.

c) *Mejia et al. (2009)*

In the 2009 *Mejia et al.* study, the test article (Puratein™), a cruciferin rich canola protein, was fed to rats ad libitum at levels of 5%, 10% and 20% for 90-days. Four groups of Crl:CD Sprague Dawley rats (20/sex/group) were used in the study, following FDA Red Book Guidelines. The animals were fed an AIN-93 diet, with the lower doses adjusted with casein to the required level of at least 18% protein in rodent diets. Puratein™ is a purified protein isolate from *B. napus*, containing a minimum 80% of the protein cruciferin with lower levels of the proteins, napin and albumen. The level of total glucosinolates was reported to be 0.24 µmole/g or approximately 30 ppm. The major isothiocyanates found in rapeseed by *Lowe et al* were: Goitrin (MW-129.2), pentenyl isothiocyanates (MW-127.2), and butenyl isothiocyanates (MW-113.2). We assume an average MW for the mixture of 125 daltons. According to *Lowe et al*, the ratio of the weight of the glucosinolates to isothiocyanates in rapeseed was ~1.8. The concentration of the glucosinolates in the animal's diet fed Puratein™ was $0.24\mu\text{mole/g} = 125 \times 0.24 = 30$ ppm isothiocyanates or $30 \times 1.8 \sim 54$ ppm on a glucosinolate basis. However, the toxic derivatives of the glucosinolates, the isothiocyanates and nitriles were not detected in the test article. This, presumably, is because of the destruction of the enzyme myrosinase during heat processing or attributable to the absence of bacterial enzymes that can affect the conversion. The level of erucic acid was reported as less than the detection limit of 25 ppm. Pesticides, heavy metals, mycotoxins, solvent residues, PAHs, dioxins and acrylamide were report as either below detection limits or substantially below toxicological limits.

There were no treatment related effects in the animals fed Puratein® at any dose, even at 20%. These included survival, clinical, and functional observations. Food consumption was equivalent at all doses and body weight gains were the same for all animals of the same sex at all doses. While there were sporadic changes in neutrophil counts in 10% and 20% -treated females on study day 45, a similar trend was not seen for males, and the neutrophil counts for the females returned to normal on study day 91. There were no treatment related changes in serum chemistry or urinalysis. There were some non-dose related differences in some serum chemistry parameters (potassium, sorbitol dehydrogenase, alkaline phosphatase). These differences were very small and were not considered toxicologically significant. There were no treatment-related changes. The NOAEL for the dietary administration of Puratein® was the highest dose tested, 20% in the diet, equivalent to 11.24 g/kg bw/day for males and 14.1 g/kg bw/day for females.

This study is applicable to the BioExx protein products for two reasons. First, the species *B. juncea*, *B. napus*, and *B. campestris*, all rapeseed (or canola) species, contain the same major storage proteins, cruciferin and napin plus smaller amounts of related proteins. In the paper by *Mejia et al.*, the Puratein® composition was stated to be at least 80% cruciferin, with the remainder being the storage protein napin and the remainder being the albumin storage protein, napin. The major differences are in the different ratios of these proteins in the difference species,

not in their qualitative make-up. The evidence from hundreds of thousands of animals fed the pressed cake at high levels demonstrates that there are no significant levels of unusual toxic proteins in these *Brassica* species. Therefore, there is no reason to expect that the systemic toxicity of the protein(s) is a factor in the safety assessment. Second, and more importantly, as summarized in **Table 13**, the levels of potentially toxic anti-nutrients in both Puratein® and the BioExx proteins are well below the level of toxicity.

Table 13
Comparison of Anti-Nutrient Levels in Puratein® and BioExx Canola Protein Products

	Puratein®	Isolexx™	Vitalexx™
Total Glucosinolates	1.22 µmole/g	<1.0 µmole/g	< 1.0 µmole/g
Erucic acid	25 ppm	<3 ppm	3 ppm
Phytic acid	0.3%	< 1.25 %	<0.7 %
Phenolics	0.40%	0.15%	0.5%
Aflatoxin B1, B2, G1, G2	ND	ND	ND

5. *Summary of Farm Animal Studies of Canola Meal*

While few conventional animal toxicology studies have been conducted on canola or *B. juncea* meal, it has been fed in dozens of controlled experiments with field animals to determine its influence on growth and development. While the purpose of these studies was not to study the potential effects of exaggerated doses of the canola or *B. juncea* meal over a lifetime, any adverse effects experienced at conventional use levels were recorded. Many of the studies were conducted in young animals. The studies show that the early rapeseed (*B. Napus* or *B. juncea*) meal contained significant levels of anti-nutrients, which impaired the nutritional quality of the meal and in some cases, harmed the animals. As the *B. Napus* and *B. juncea* cultivars were bred to reduce and essentially eliminate the anti-nutritional factors, the quality of the meal improved to the extent that young and developing animals thrived. Thus, while the studies do not determine the conventional “no observed adverse effect level” (NOAEL), they do reveal whether the growth, development of the animals, or health were impaired at doses of the protein in the meal considerably higher than those contemplated for the BioExx protein products. The current canola or *B. juncea* meal is essentially a refined protein mixture, a macro nutrient, which has no measurable toxicity at typical food protein levels.

The feeding studies on canola or *B. juncea* meal pressed cake in field animals are summarized in **Appendix 3**, which is attached to this notification.

6. *Mutagenicity Studies*

We are of the opinion that there is no scientific justification for conducting mutagenicity tests on purified proteins. Proteins, including food-borne enterotoxins and neurotoxins produced by some bacteria, are not genotoxic (Pariza and Johnson, 2001). The history of such testing has

been reviewed by Pariza and Johnson, who conclude that not a single mycotoxin or clastogen has ever been detected that would not have been detected by properly conducted analytical chemistry and limited animal feeding studies. In any event, ADM has conducted a series of mutagenicity and clastogenicity studies on their canola proteins, Puratein® and Supertein®, as reported in GRAS Notification No. 327. All of these studies demonstrated negative results for canola proteins.

7. *Allergenicity of B. juncea*

Mustard seed is a known allergen (Figueroa et al. 2005; Morisset et al. 2003). Allergenic food proteins in mustard have been identified and characterized (Asero et al. 2002; Menendez-Aria et al. 1988). There are three main types of mustard seeds produced worldwide: pale yellow or white mustard (*Sinapis alba*, formally classified as *Brassica alba*), black mustard (*Brassica nigra*), and brown or oriental mustard (*Brassica juncea*). The *Brassica* and *Sinapis* genera share close botanical lineage including 2S albumin seed storage proteins which have been shown to contain sensitizing protein sequences (Monsalve et al. 1993). Thus, individuals that are known to be sensitive to one species of mustard are likely to show sensitivity to other species as indicated in IgE immunoblotting experiments (Monsalve et al., cited). Although mustard is not a widely recognized allergen in the U.S., it is one of the 12 recognized major food allergens in Europe

Dr. Joseph Baumert, an Assistant Professor at the Food Allergy Research & Resource Program at the University of Nebraska, evaluated the effect of introducing BioExx protein isolates into the U.S. diet at levels corresponding to the proposed uses. We have attached his report as **Appendix 4**. The population in the U.S. is being exposed daily to several milligrams of *B. juncea* and other closely related mustard proteins and has been for generations, without significant reported allergenicity (Baumert, 2009; Robotham et al. 2005).

One critical question is whether the higher *B. juncea* protein exposures will significantly increase the prevalence of allergenicity in the population. It is difficult to predict with certainty whether or not exposure to the higher levels of these specific proteins would result in increased sensitization. However, previous experience with the introduction of protein isolate and concentrate products into the human diet from known allergenic sources such as soy or whey protein concentrates and isolates would suggest that these products do not increase the prevalence of sensitization of humans (Goodman et al. 2007). The prevalence rates of both soybean allergy and milk allergy have not been shown to have increased dramatically as a result of the use of these products. The proposed levels of BioExx protein to be used in finished products (0.5 – 10%) and their intended uses are not unlike the levels and uses of whey protein isolates and dairy product solids which obtained GRAS notification by the FDA in 2000 (GRN No. 37). As shown in **Section VIII.A.2**, recent consumption of mustard seed in India is estimated to exceed that in the U.S. by over 65 times. Yet, there are very few reported cases on mustard seed allergy from India. While not conclusive, this observation lends support to the expectation that higher levels of mustard protein consumption will not lead to a significant increase in the sensitized population.

Dr. Baumert has concluded that the introduction of protein isolate from BioExx products is unlikely to significantly increase the prevalence of allergy already existing from *B. juncea* and related protein sources.

B. Estimated Consumption of Canola Proteins Derived from *B. juncea* and *B. Napus* from Proposed Food Uses

The typical proposed food uses of BioExx’s canola protein products in food are as follows:

Table 14
Application Usage Estimates

Food Category	Maximum Use Level in Canola Protein Products (%) as consumed	
	Isolexx™	Vitalexx™
Bakery products (e.g., breads, rolls, doughnut, cookies, cakes, pies, batters, muffins, pasta, and cereal bars, etc.)	3	--
Snack foods (e.g., crackers, cookies, candy ingredients, breakfast/energy bars, snack chips, etc.)	20	--
Beverages, soups, nutritional beverages (e.g., protein fortified soft drinks, fruit juices, high protein drinks)	5	5
Dairy products (e.g., cheese, frozen dairy dessert, whipped topping, yogurt, coffee whiteners, etc.)	4	--
Dry instant milkshake mixes and protein drinks	9	--
Instant powdered nutritional beverages	--	15
Processed Meat products (where the addition of vegetable proteins are acceptable, such as unspecified products or those where they are included within the Standard of Identity) (subject to USDA approval)	2	--
Vegetarian food products and meat analogues	20	--
Meal replacement/nutritional bars	30	25

As shown in Table 14, the BioExx protein products will be used in a number of food products. Furthermore, as noted previously, FDA has established a DRV of 50 g/day for protein. In addition, the Institute of Medicine (IOM) has established a Recommended Dietary Allowance (RDA) of 56 g/day for adult males and 46 g/day for adult females. Given the variety of food uses in the major food categories listed above, the large average daily consumption of these foods, and the maximum proposed concentration of the additives, it is readily seen that the calculated daily intake of additives can clearly be a substantial fraction of the RDA, or even exceed it at the 90th percentile. This was the case for GRAS Notification 327 which covers the use of cruciferin-rich and napin-rich protein isolates in a variety of foods. Because BioExx’s proposed uses and maximum concentrations are similar, it is also the case for the BioExx proteins.

We do not realistically expect that the actual consumption of foods containing BioExx's canola protein products would result in a daily consumption of greater than the DRV or RDA for protein. Most of the population's intake of protein is, and will remain, in the form of unprocessed foods, including meat, poultry, fish and legumes. Moreover, as noted above, for the processed foods to which the proteins will be added, there are competitive products in the market. Only the inherent conservatism in the typical intake calculations suggests the possibility of exceeding the RDA at the 90th percentile.

IX. Summary of Basis for GRAS Determination

BioExx Specialty Proteins Ltd. has determined the Generally Recognized as Safe (GRAS) status of canola protein isolate (Isolexx™), and hydrolyzed canola protein isolate (Vitalex™) based on the following:

- The published toxicological studies by Lowe et al. (1976), Plass et al. (1992), and Mejia et al. (2009) where material similar to BioExx's canola proteins were fed to rats and dogs. In these studies, no toxicologically relevant effects were observed at the highest doses tested.
- The history of safe use of canola meal (*B. juncea* or *B. napus* meal) in animals, including cattle, swine, poultry and fish.
- The canola protein products are manufactured under good manufacturing practices (GMPs) and meet appropriate food grade specifications. Potential contaminants such as glucosinolates, phytates, erucic acid, and tannins are either absent (not detected) or below toxicological and regulatory allowed limits.
- The fact that even though mustard proteins are also common in canola, mustard allergy is not common in the U.S. and the increased introduction of mustard protein through the canola proteins is unlikely to significantly increase the prevalence of mustard allergy in the U.S.
- The unanimous conclusions reached through scientific procedures, by a panel of experts, qualified by scientific training and experience, that the canola protein products are GRAS for the intended uses when manufactured and used in accordance with GMPs and meeting appropriate food grade specifications.

X. Appendices

- Appendix 1 Philo, J. Sedimentation Velocity Analysis of Three Canola Proteins, Report No. POS102609, October 26, 2009.
- Appendix 2 Protein Digestibility Studies including Protein Digestibility Corrected Amino Acid Score (PDCAAS) analysis conducted at the Division of Animal Nutrition Physiology of Goettingen University, Germany (Director: Prof. Dr. Frank Liebert.)
- Appendix 3 Feeding studies on canola or *B. juncea* meal pressed cake in field animals

Appendix 4 Baumert, J. (2009) Analysis of the potential allergenicity of novel canola protein isolates and concentrates using *Brassica juncea* and *Brassica napus* as source materials. Food Allergy Research & Resource Program (FARRP), 2009.

Appendix 5 Conclusion of the GRAS Expert Panel Review

XI. Tables

Table 1	Specifications Canola Protein Isolate - Isolexx™
Table 2	Specifications Hydrolyzed Canola Protein Isolate - Vitalexx™
Table 3	Analysis of representative lots of Canola Protein Isolate - Isolexx™ from <i>Brassica Juncea</i>
Table 4	Analysis of representative lots of Hydrolyzed Canola Protein – Vitalexx™ from <i>Brassica Juncea</i>
Table 5	Analysis of representative lots of Hydrolyzed Canola Protein – Vitalexx™ from <i>Brassica Napus</i>
Table 6	Amino Acid Profile of BioExx Canola Protein Products
Table 7	Residual Phytase Enzyme Activity in BioExx Canola Protein Products
Table 8	Residual Protease Enzyme Activity in Final Vitalexx™ Products
Table 9	Potential Anti Nutrients in Composite Lots of BioExx Canola Protein Products
Table 10	Glucosinolate Analysis of Representative Lots of BioExx Canola Protein Products
Table 11	Contaminant Analysis of Composite Lots of BioExx Canola Protein Products
Table 12	Heavy Metals Analysis of Representative Lots of BioExx Canola Protein Products
Table 13	Comparison of Anti-Nutrient Levels in Puratein® and BioExx Canola Protein Products
Table 14	Application Usage Estimates

XI. References

1. Anderson, G.H. *et al.* (1976) Trace mineral deficiencies in rats caused by feeding rapeseed flours during growth, gestation and lactation. *J Nutr.* 106:1166-1176.
2. Asero, R., Mistrello, G., Roncarbolo, D., Amoto, S. 2002. Allergenic similarities of 2S albumins. *Allergy.* 57:62-3.
3. Bell, J.M. (1984) Nutrients and Toxicants in Rapeseed Meal: a Review. *J. Anim Sci* 58:996-1010.
4. Bollard *et al.* (1997). The disposition of allyl isothiocyanate in the rat and mouse. *Food and Chemical Toxicol.* 35: 933-943.
5. Bos, C. Airinei, G. Mariotti, F. *et al* (2007). The poor digestibility of rapeseed protein is balanced by its very high metabolic utilization in humans. *J. Nutr.* 137:594-600.
6. Carréon, T. (2001). Aliphatic Hydrocarbons, Chapter 49 In Patty's Toxicology, 5th Ed, Vol. 4, Ed by Bingham, Cohrssen, B., and Powell C.H., John Wiley & Sons, Inc. New York.
7. European Council. 2009. Directive 2009/32/EC of the European Parliament and of the Council of 23 April 2009, Annex I, Part 1.
8. Downy, R.K. (1983) The origin and description of the *Brassica* oilseed crops. *In High and Low Erucic Acid Rapeseed Oils: Production, Usage, Chemistry, and Toxicological Evaluation.* Ed. J.C.G Kramer, F. D. Sauer, W. J. Pigden, pp 1-20 New York Academic Press, 582 pp.
9. Ensminger, A.H., Esminger, M.K.J., *et al*, Food for Health: A Nutrition Encyclopedia Pegus Press, Clovis California, 1986.
10. Fahey Jr., G. C. and H. G. Jung. (1989) Phenolic compounds in forages and fibrous feedstuffs. p. 123-190 In: P. R. Cheeke (ed). Toxicants of plant origin. Vol. IV Phenolics. CRC Press, Inc. Florida.
11. Food and Drug Administration, 1980. Compliance Policy Guide 525.575, Prepared Mustard-Composition, available at, <http://www.fda.gov/ICECI/ComplianceManuals/CompliancePolicyGuidanceManual/ucm074463.htm> and CPG 525.750, Spices-Definitions, available at, <http://www.fda.gov/ICECI/ComplianceManuals/CompliancePolicyGuidanceManual/ucm074468.htm>.
12. Figueroa, J.C., Blanco, A.G., Dumpiérrez, L., Almeida, N., Ortega, R., Castillo, *et al.* 2005. Mustard allergy confirmed by double-blind placebo-controlled food challenges: clinical features and cross-reactivity with mugwort pollen and plant-derived foods. *Allergy* 60:48-55.
13. Foods Chemicals Codex (FCC), 7th Edition, Appendix V, p. 1185.
14. Foods Chemicals Codex (FCC), 7th Edition, Appendix V, p. 1207.
15. Glick, Z. and Joslyn, M.S. (1970). Effect of tannic acid and related compounds on the absorption and utilization of protein in the rat. *J Nutr.* 100: 516-520.

16. Goodman, R.E., Taylor, S.L., Yamamura, J., Kobayashi, T., Kawakami, H., Kruger, C.L., Thompson, G.P. 2007. Assessment of the potential allergenicity of milk basic protein fraction. *Food Chem. Toxicol.* 45:1787-94.
17. IARC, (1999). Allyl isothiocyanate. Summary of Data Reported and Evaluation, Vol. 73, p.37.
18. Jiao et al. (1994). Identification and quantification of the N-acetylcysteine conjugate on allyl isothiocyanate in human urine after injection of mustard. *Cancer Epidemiology, Biomarkers and Prevention*, 3(6) 487-492. IARC, (1997).
19. Lamb, J.L. (1989) Entomology of Oilseed Brassica Crops. *Ann. Rev. Entomol.* 34: 211-209
20. Loew, F.M., et al. Evaluation of a dietary rapeseed protein concentrate flours in rats and dogs. *Toxic Applied Pharma.* 35: 257-267, 1976.
21. Mejia, L.A., et al. 2009. A 13-week sub-chronic dietary toxicity study of a crucifer-rich canola protein isolate in rats. *Food Chem. Tox.* 47: 2645-2654.
22. Menendez-Aria, L., Moneo, I., Dominguez J., Rodrigues, R. 1988. Primary structure of the major allergen of yellow mustard (*Sinapis alba* L.) seed, Sin a I. *Eur J Biochem.* 177:159-66.
23. Monsalve, R.I., Gonzalez de la Pena, M.A., Menendez-Arias, L., Lopenz-Otin, C., Villabla, M., Rodrigues R. 1993. Characterization of a new oriental-mustard (*Brassica juncea*) allergen, Bra j IE: detection of an allergenic epitope. *Biochem. J.* 293:625-32.
24. Morisset, M., Moneret-Vautrin, D.A., Maadi, F., Fremont, S., Guenard, L., Croizier, A., Kanny, G. 2003. Prospective study of mustard allergy: first study with double-blind placebo-control food challenge trials. *Allergy* 58:295-299.
25. Mustard Seeds Outlook Report. 2008
http://www.karvycomtrade.com/downloads/karvySpecialReports/karvysSpecialReports_20080221_03.pdf
26. Naczek, M. and Amarowicz, R., Shahadi, F. (1998) Role of phenolics in flavor of rapeseed products. *Developments in Food Science, Vol. 40*, 597-613. Elsevier.
27. National Research Council. (1973). Committee on Food Protection, Food and Nutrition Board., "Phytates" *Toxicants Occurring Naturally in Foods.*" National Academy of Sciences. pp. 363-371.
28. NTP, (1981). TR-234 Carcinogenesis Bioassay for Allyl Isothiocyanate (CAS No.57-06-7) in F344/N Rats and B6C3F1 Mice (Gavage Study).
29. Pariza, M.W. and Johnson, E.A. (2001) Evaluating the safety of microbial enzyme preparations used in the food processing industry: Update for a new century, *Reg. Toxicol. And Pharmacol.* 33:173-186.
30. Plass R., et al. Toxicological evaluation of rapeseed products in a sub-acute feeding study in rats. *Die Nahrung* 36: 248-252, 1992.

31. Prakash, S. Hinata, K. (198) Taxonomy, cytogenetics and origin of crop *Brassica*, a review. *Opera Bot.* 55: 1-57.
32. Reddy, N.R., Sathe, S.K. (2002). Food Phytates. Boca Raton, CRC Press.
33. Robotham, J.M. *et al* (2005) Ana o 3, an important cashew new (*A. occidentale* L.) allergen of the 2S albumin family. *J. Allergy Clin. Immunol.* 115: 1284-1290.
34. SCOGS. 1975. Select Committee on GRAS Substances (SCOGS) Report No. 16 (1975). Evaluation of the Health Aspects of Mustard and Oil of Mustard as Food Ingredients. Contract No. FDA 72-85, Life Sciences Research Office, Federation of American Societies for Experimental Biology.
35. Tan, S.H., et al., Canola Proteins for Human Consumption: Extraction, Profile, and Functional Properties, *Journal of Food Science*, 2010.
36. The World's Healthiest Foods (2001-2011), Mustard Seeds, available at, <http://www.whfoods.com/genpage.php?tname=foodspice&dbid=106>.
37. USDA (1994-1996) Continuing Survey of food Intakes by Individuals (CSFII), Food Commonly Eaten by Individuals, USDA Table Appendix B.
38. Woods, D.L., Capcara, J.J., and Downey, R.K. (1991) The potential of mustard (*Brassica juncea*)(L.) (Coss) as an edible oil crop on the Canadian Prairies *Can J. Plant Sci.* 71: 195-198.

000040

000041

000042

APPENDIX 1
Sedimentation Velocity Analysis

000043

REPORT: Sedimentation Velocity Analysis of Three Canola Protein Samples

Report # POS102609 prepared by John Philo October 26, 2009

Purpose

To measure the content of the 2 S, 7 S, and 12 S proteins.

Samples

Three stocks of protein powder were provided, described as follows:

1. canola protein isolate
2. canola protein concentrate
3. hydrolyzed canola protein

Aliquots of each protein stock were initially dissolved in 3% NaCl at a weight concentration of 10 mg of powder per mL. After sitting overnight to allow more complete dissolution¹ a small portion of each was diluted 10-fold to make a solution at an appropriate concentration for sedimentation velocity analysis.

Method Background

Sedimentation velocity, as measured in an analytical ultracentrifuge, is an excellent method for obtaining information about heterogeneity of protein mixtures and the state of association or aggregation of purified proteins. Different proteins, or different oligomers of a single protein, can be detected on the basis of their different sedimentation coefficients. This method can detect minor components at a level below 1% by weight. Sedimentation velocity gives good quantitation of relative amounts of species, and will usually give accurate sedimentation coefficients for all species at a level above ~2%.

Sedimentation velocity is an absolute method, based on simple physical principles. Its calibration is based on fundamental units of length and time, requiring no standard molecules as references.

This method is also sensitive to differences in conformation. Conformational changes in proteins or protein complexes alter their sedimentation coefficients because they alter the amount of hydrodynamic friction (the frictional coefficient). The frictional coefficient is quite sensitive to the presence of any flexible disordered (unfolded) regions in the protein and is also sensitive to the overall shape (extended versus compact and globular). Sedimentation coefficients can be measured with high accuracy ($\pm 0.5\%$ or better), and thus they provide a sensitive and quantitative means to demonstrate comparability of molecular conformation.

¹ Note that the canola protein concentrate did not completely dissolve---after shaking a considerable portion of this material settled to the bottom of the tube within 1-2 min. The canola protein isolate sample was quite turbid but did not show obvious settling under gravity. The hydrolyzed canola protein formed a clear (but distinctly colored) solution.

Buoyancy and other solvent effects

The net force driving the sedimentation of the macromolecules is determined by the so-called *buoyant* molecular mass, M_b , rather than the true molecular mass, M . The buoyant mass is simply the true mass less the mass of the solvent it displaces (from Archimedes' principle). For molecules such as lipoproteins which are less dense than water, their buoyant mass is negative and they float toward the center of the rotor rather than sediment toward the outside.

The buoyant mass can be calculated from the formula $M_b = M(1 - \bar{v}\rho)$, where ρ is the solvent density and \bar{v} is the partial specific volume of the macromolecule (the inverse of the hydrated density).

The observed sedimentation coefficient (the so-called 'raw' value) thus depends on the solvent (buffer) density, and is also inversely related to its viscosity, both of which depend on temperature. Often these raw sedimentation coefficients are standardized to remove this dependence on the buffer properties and temperature, yielding the value that would be observed at 20 °C in water (the so-called $s_{20,w}$ value).

Relations between radius, sedimentation coefficient, and molecular mass for spherical particles

For spherical particles of radius R or molecular mass M the sedimentation coefficient, s , increases as R^2 or $M^{2/3}$, and is given exactly by

$$s = \frac{2R^2(1 - \bar{v}\rho)}{9\bar{v}\eta}$$
$$s = \frac{M^{2/3}(1 - \bar{v}\rho)}{6\pi\eta N_0^3 \sqrt{\frac{3\bar{v}}{4\pi N_0}}}$$

where η is the solvent viscosity, and N_0 is Avogadro's number.

Predicted sedimentation coefficient for aggregates based on monomer value

As noted above, sedimentation coefficients depend on molecular shape as well as molecular mass, and thus it is not possible to uniquely predict the sedimentation coefficient for an oligomer even when the monomer sedimentation coefficient is known. If we assume the aggregate shape is similar to that of the monomer then its stoichiometry, N , will be given by $(s_N/s_1)^{3/2}$, where s_N is the sedimentation coefficient of the N -mer and s_1 is the sedimentation coefficient of monomer. This assumption may be reasonable for larger aggregates; for small oligomers calculations based on oligomers of spheres² provide some useful "rule of thumb" values as well as an indication of the range of values for different shapes:

² Garcia de la Torre, J. and V. A. Bloomfield (1981). Hydrodynamic properties of complex, rigid, biological macromolecules: Theory and applications. *Q. Rev. Biophys.* 14: 81-139.

Oligomer	Ratio of oligomer sedimentation coefficient to monomer sedimentation coefficient
dimer	1.45
trimer (linear)	1.75
trimer (triangular)	1.86
tetramer (linear)	2.00
tetramer (square planar)	2.20
tetramer (tetrahedral)	2.26
pentamer (pentagon)	2.45
pentamer (bipyramid)	2.60
hexamer (hexagon)	2.67
hexamer (trigonal prism)	2.90
hexamer (octahedron)	2.97
octamer (cube)	3.46

Methods

Samples were loaded into cells with 2-channel charcoal-epon centerpieces with 12 mm optical pathlength. The 3% NaCl dilution buffer was loaded into the reference channel of each cell (the instrument functions like a dual-beam spectrophotometer, measuring the net difference in signal between the sample and the reference channel at each radius). Those loaded cells were then placed into an AN-60Ti analytical rotor, loaded into a Beckman-Coulter ProteomeLab XL-I analytical ultracentrifuge equipped with both absorbance and Rayleigh interference (refractive index) optical detection, and brought to 20 °C. The rotor was then brought to 3,000 rpm and the samples were scanned (using both absorbance scans 280 nm and refractive index scans) to confirm proper cell loading. The rotor was then brought to the final run speed of 55,000 rpm. Scans were recorded at this rotor speed approximately every 3.2 min for ~6.2 hr (114 total scans from each optical system for each sample), and then the scan rate was dropped to every 20 min for an additional 15 hr (30 additional scans).

Only the refractive index (RI) scans were analyzed. They were analyzed using the $c(s)$ method developed by Peter Schuck at the N.I.H. and implemented in his analysis program SEDFIT (version 11.3).³ In this approach many raw data scans are directly fitted (~195,000 data points for each sample in this case) to derive the distribution of sedimentation coefficients, while modeling the influence of diffusion on the data in order to enhance the resolution. The method works by assigning a diffusion coefficient to each value of sedimentation coefficient based on an assumption that all species have the same overall hydrodynamic shape (with shape defined by the frictional coefficient ratio relative to that for a sphere, f/f_0). The f/f_0 values were varied to find the best overall fit of the data for each sample. A maximum entropy regularization probability of 0.683 (1 σ) was used, and both time-independent and radially-independent noise were removed.

To convert the raw sedimentation coefficients to approximate standardized values all proteins in the samples were assumed to have a partial specific volume (\bar{v}) of 0.73 mL/g. A density of

³ Schuck, P. (2000). Size-distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and Lamm equation modeling. *Biophys. J.* 78, 1606-1619.

1.01919 g/mL and viscosity of 1.0503 cp at 20 °C were calculated for 3% NaCl using the program SEDNTERP by John Philo, David Hayes, and Tom Laue.⁴

Results and Discussion

One difficulty in characterizing samples containing a mixture of different protein species is that the different components may be detected with different sensitivity. For example when absorbance detection at 280 nm is used the sensitivity to different proteins depends on their extinction coefficients (which vary widely), and small peptide fragments that contain no aromatic amino acids would not be detected at all. One great advantage of refractive index detection is that it detects all polypeptides with nearly equal sensitivity (they are all the same within ~3%), and therefore refractive index detection was used for this analysis.

The high-resolution sedimentation coefficient distributions for the canola protein isolate and canola protein concentrate samples are shown below in Figs. 1 and 2, respectively. These graphs are much like chromatograms, with the vertical axis giving the concentration and the horizontal axis showing the separation on the basis of sedimentation coefficient. Each distribution has been normalized to account for any concentration differences among the samples, by setting the total area under the curve to 1.0 (100%) so the area for each peak gives the fraction of that species. There is a break in the vertical axis scale to allow the minor peaks to be seen. The fractions and peak positions (top of peak) for the various peaks are noted on the graph, as is the total signal from all detected species shown on the graph.

Note that the sedimentation coefficients have been approximately converted to standard conditions (adjusted for the fact that the density and viscosity of 3% NaCl are greater than those for pure water). This conversion can only be approximate because we do not have the information needed to make a precise buoyancy correction for each individual component, and therefore a typical value was used for all components.⁵

The size distribution for the canola protein isolate sample is shown in Fig. 1. The main component (largest fraction by weight) is a species at 12.3 S which is 56.7% of the total. Clearly this species corresponds to the nominal "12 S" protein. The two next-most abundant species are at 1.7 S (16.7%) and 0.75 S (9.6%). Five additional minor peaks or shoulders occur between those species and the main 12.3 S peak, and three minor peaks sedimenting faster than the main peak are also present.

Note that the half-peak at 22.2 S is at the upper limit covered by this analysis, and thus it is likely that some or all of this 2.8% is actually sedimenting faster than 22.2 S. It is also quite possible that the sample contained some very large aggregates or incompletely-dissolved components that pelleted during the rotor acceleration to 55,000 rpm and therefore were not detected at all. Indeed the total signal of 2.21 interference fringes is significantly less than the 3.3 ± 0.1 fringes

⁴ Laue, T.M., Shah, B.D., Ridgeway, T.M., and Pelletier, S.L. (1992). In: *Analytical ultracentrifugation in biochemistry and polymer science*. S.E. Harding, A.J. Rowe, and J.C. Horton, eds. Royal Society of Chemistry, Cambridge, pp. 90-125.

⁵ The as-measured "raw" sedimentation coefficients were multiplied by 1.1108 to convert to $s_{20,w}$ values.

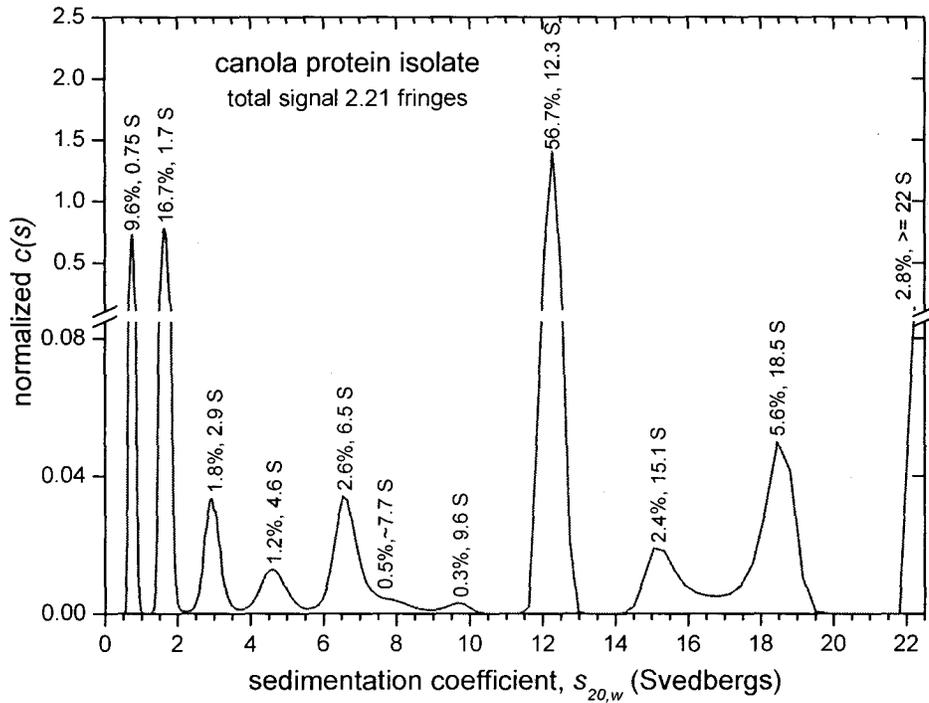


Fig. 1. Normalized sedimentation coefficient distribution for sample 1, canola protein isolate.

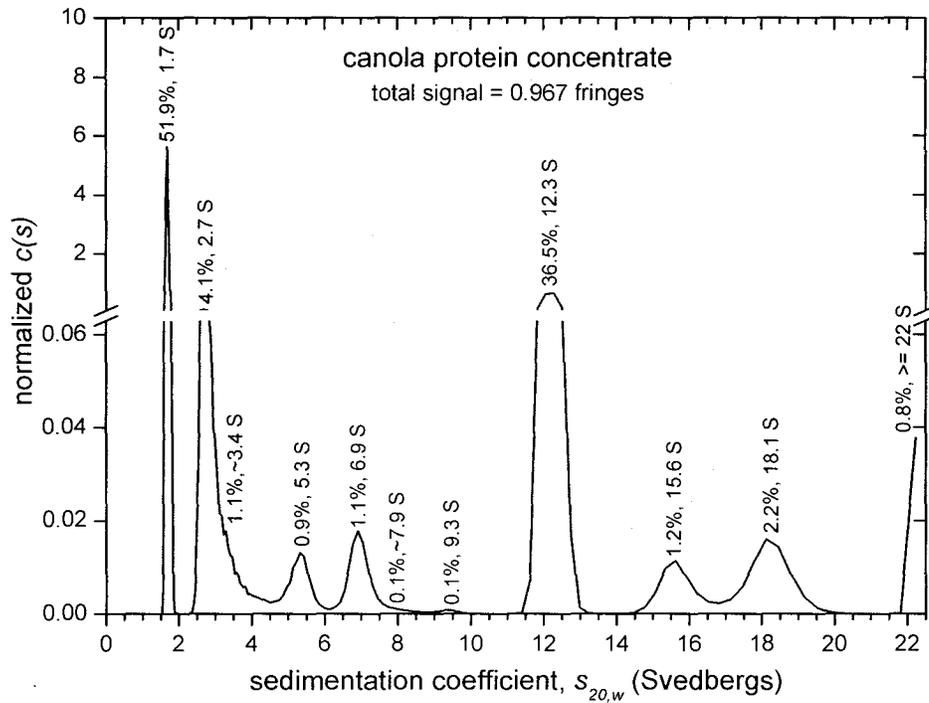


Fig. 2. Normalized sedimentation coefficient distribution for sample 2, canola protein concentrate.

expected for a protein concentration of 1 mg/mL.⁶

Note that it may be difficult to make an exact correspondence between the species detected by this modern high-resolution approach and older sedimentation velocity data taken using different instruments and data analysis approaches. For example, we can be quite certain that the shoulder at ~7.7 S could not have been resolved from the peak at 6.5 S by any measurements made prior to 2000, and hence that both of these components (and perhaps the peaks at 4.6 S and 9.6 S also) would have been counted as "7 S". Similarly it is unclear whether the peaks at 2.9 S or 0.75 S would have been resolved from the nominal "2 S" component in older measurements.

It is important to clarify that while these various peaks *probably* represent distinct, independent species, it is possible that they represent fairly stable reversible complexes between two or more different proteins, or stable non-covalent oligomers of one protein. A single measurement at a single concentration cannot rule out that some or all of these species exist in reversible association equilibrium with the corresponding monomer(s). A dilution series would be required to distinguish which species are in reversible mass-action equilibrium and will consequently dissociate at lower total protein concentrations.

The results for the canola protein concentrate sample (sample 2) are shown in Fig. 2. The protein concentration (total signal) of this sample is more than 2-fold lower than that of sample 1, presumably due to loss of insoluble material. In this case the principal component is the peak at 1.7 S (presumably the nominal "2 S" protein), which is 51.9% of the total, with the 12 S protein still a major component (36.5%). This sample appears to contain rather little of a "7 S" component. Note that equivalent peaks will not necessarily appear at exactly the same sedimentation coefficient. With this method it is normal for the positions of minor peaks to shift somewhat from one sample to another---the sedimentation coefficients for species at levels of a few percent or less cannot be determined with high precision (there is noise on the x-axis).

The results for the hydrolyzed canola protein sample (sample 3) are shown below in Fig. 3. The normalization to give percentages of the total was handled differently for this sample. It was not possible to measure the total signal for this sample because some of the peptide fragments are so small that even after over 21 hr at 55,000 rpm they have not sedimented sufficiently to deplete the concentration to zero at the inner regions of the cell.⁷ Therefore the total signal was estimated based on the weight concentration (1 mg of powder per mL), a peptide content for the powder of 82% by weight, and the nominal detector sensitivity of 3.3 ± 0.1 fringes per (mg/mL). The vertical scaling in Fig. 3 is identical to that for Fig. 2.

Therefore in Fig. 3 the area under each peak measures the fraction of that protein species remaining after hydrolysis (fraction of the total, not fraction of that individual species), and the total area under the curve is less than 100%. The actual total area is 12.0% (that is, peptides or

⁶ The actual protein concentration of this sample will of course be less than the nominal 1 mg/mL (based on weight of powder) if the isolate powder contains any moisture, salts, or other non-protein components.

⁷ Refractive index detection measures only concentration *differences*, not absolute concentrations (the zero signal level is arbitrary, unlike absorbance where zero OD has a real physical meaning). Thus the total concentration can only be determined if there is some position in the cell where the concentration of all sedimenting species falls to zero.

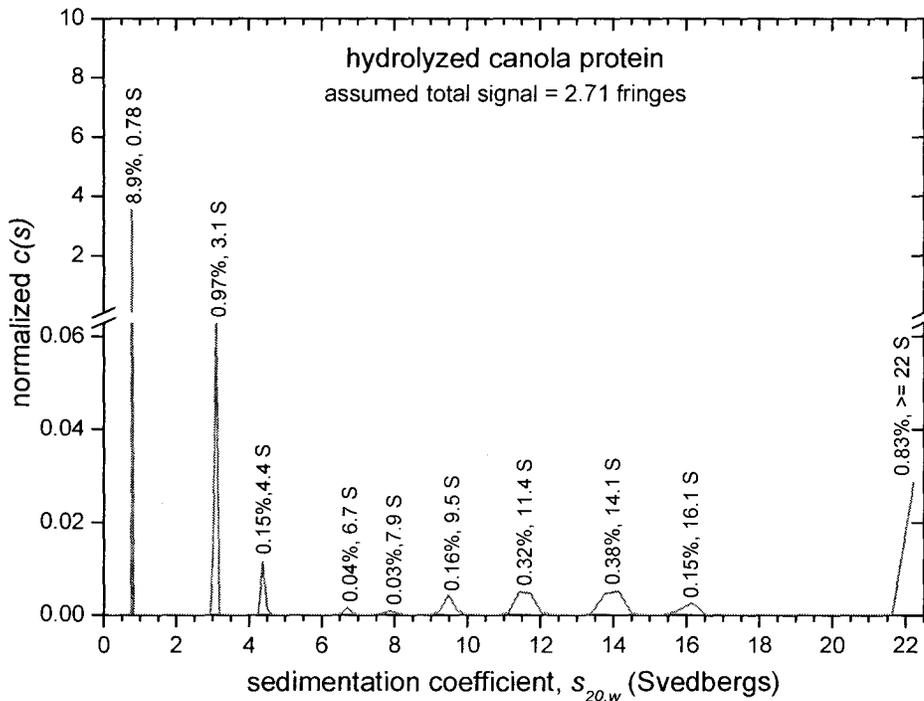


Fig. 3. Normalized sedimentation coefficient distribution for sample 3, hydrolyzed canola protein.

proteins that are still large enough to sediment significantly represent 12.0% of the total expected signal). Note that the peak at 0.78 S represents nearly $\frac{3}{4}$ of that total of 12.0%, and this peak could represent a fragment of one of the larger proteins. Because the areas for many of the other peaks in this sample are so small the ones below 1% are listed to the nearest 0.01% to limit the round-off error. The peak at 1.7 S was not detected in this sample. The peak at 11.4 S (0.32%) may represent a partially-digested (clipped) form of the 12.3 S species; whether or not that is correct, it is clear that species at 11-13 S are at least 100-fold less abundant than in samples 1 and 2.

000050

APPENDIX 2
Protein Digestibility Study

000052

Final Report, revised

Comparative evaluation of protein quality parameters of Canola protein isolates as single protein sources in the laboratory rat

The studies were conducted at Division Animal Nutrition Physiology of Goettingen University, Germany (Director: Prof.Dr.Frank Liebert)

Aim of the experiments

The study aims to evaluate protein quality of different plant protein sources (Isolexx, Vitalexx, Dunasoy 90) as related to an animal protein source (Casein) by application of different procedures for feed protein evaluation in the laboratory rat. Based on these data, the protein value for human nutrition will be derived according to the PDCAAS (Protein digestibility corrected amino acid score) procedure. For this purpose, the study provides experimental data about basal endogenous nitrogen (N) losses via gut and urine, respectively. Two experiments were conducted to yield the needed metabolic data for the rat and to achieve an *in vivo* comparison of different protein sources as well.

Material and Methods

The experiments (N-balance studies) were conducted according to the following design:

Exp.I: Direct comparison of the protein sources canola protein-isolate (Isolexx) and hydrolyzed canola protein-concentrate (Vitalexx) at similar protein level in the diet. Additionally, three different dietary protein levels of Isolexx were utilized to derive a regression function for estimating the endogenous N-losses by simulation of N-free feeding conditions.

Experimental factor:	1	Protein source
	2	Protein supply (3 graded levels Isolexx as separate factor for one way ANOVA)

Dietary treatments:

Diet 1: n=8	15% Isolexx
Diet 2: n=8	17.48% Vitalexx
Diet 3: n=8	30% Isolexx
Diet 4: n=8	7.5% Isolexx

Diets 1, 3 and 4 are utilized for conclusion of basal endogenous N-losses via faeces by means of non-linear regression. These data are applied for assessing the true protein digestibility on faecal level for application with PDCAAS. In addition, basal endogenous

losses via urine were derived to conclude the N-maintenance requirement (NMR) as sum of endogenous losses via faeces and urine, respectively. The NMR data are needed for application of protein evaluation standards like Biological Value (BV) or Net Protein Utilization (NPU)

Exp.I utilized male rats of the genotype WISTAR from reproduction unit of the University Medicine Goettingen.

Exp.II: Direct comparison of Isolexx and Vitalexx with the soybean protein Dunasoy 90 or casein as standard protein sources

Experimental factor: 1 Protein source

Dietary treatments:

Diet 1: n=8	15% Isolexx
Diet 2: n=8	17.48% Vitalexx
Diet 3: n=8	14.59% Dunasoy
Diet 4: n=8	14.23% Casein

Exp.II utilized male WISTAR rats (HsdHan WIST) of the company Harlan (An Venray, The Netherlands). The nutrient composition of the protein sources is summarised in appendix table 1. The composition of the experimental diets was very similar to Exp.I (A-table 2), but Dunasoy (14.59%) or casein (14.23%) were utilized as single protein source in exchange with starch.

For the balance studies, traditional metabolic cages were utilized according to approach of Horszczaruk and Bock (1963).

Rats were fed three times a day on restrictive feeding level to avoid feed losses. Daily feed supply was mostly fixed during the collecting period as based on the feed intake in the pre-period. Feed losses were recorded and taken into account when daily feed intake was calculated. Individual body weight (BW) of the animals was measured at beginning of pre-period, at start as well as at end of the collecting period. The average BW as applied for calculating the metabolic BW was the mean value of BW at beginning and at end of the collection period. Lighting was regulated according to 12 hours light and 12 hours dark.

Experimental parameters as derived in Exp.I and Exp.II:

- Feed intake
- N-intake
- N-excretion faeces

- N-excretion urine
- N-balance
- Crude protein digestibility (apparent)
- True digestibility of crude protein
- N-utilization parameters

Classical: PPV, NPU, BV, PDCAAS

Developed: N-utilization model (exponential function), describes N-utilization parameters by elimination of feed intake effects

(Samadi and Liebert, 2008; Liebert, 2008; Wecke and Liebert, 2009)

Details about calculation of the individual N-utilization parameters are given in the tables summarizing the experimental results.

According to Gaßmann (2006, Ernährungs-Umschau 53, 5, 176-181), the amino acid (AA) composition of reference protein for humans was applied for PDCAAS calculations as given in table 1.

Table 1: AA-requirement ratios (mg AA/g CP) for humans ≥ 1 year old (Gaßmann 2006)

AA	His	Ile	Leu	Lys	Met+Cys	Phe+Tyr	Thr	Try	Val
mg/g	18	25	55	51	25	47	27	7	32

Prior to mixing of diets, protein sources are analysed for their amino acid composition (Annex table 1). The common structure of the experimental diets was as follows:

Feed mixture (g/kg):

Test protein	150
Soybean oil	60
Premixes*	80
Titanium dioxide	3
Cellulose powder	50
Sucrose	100
Wheat starch	ad 1000

*60g/kg Mineral mixture ALTROMIN, 20g/kg Vitamin mixture ALTROMIN

In Exp.I, diets 3 and 4 contained 7.5% or 30% of the protein source Isoexx to achieve a graded dietary protein supply for estimating the endogenous faecal losses by regression analysis.

In Exp.II, different feed protein sources were directly compared at similar dietary protein supply. The protein sources Dunasoy and Casein were utilized as plant resp. animal standard protein source in the experimental diets for laboratory rat.

All chemical analyses conducted in ingredients, final diets and excreta were in accordance with German standard procedures of VDLUFA (Naumann and Bassler, 1976-1997). Statistical data analyses utilized one-way ANOVA ($p < 0.001$), making use of Tukey or Games-Howell post-hoc test according to equality or non-equality of variances (verified by Levene-test) within the program package SPSS 17.0 for Windows.

Results

Experiment I

The results of Exp.I are summarized in table 2.

Table 2: Summary of N-balance studies examining Isolexx and Vitalexx as single protein source in experimental diets for rats

	DIET 1	DIET 2	DIET 3	DIET 4
	15% Isolexx	17.48% Vitalexx	30% Isolexx	7.5% Isolexx
Initial BW (g)	94.8 ^a ± 3.4	94.0 ^a ± 6.2	96.8 ^a ± 6.3	85.5 ^a ± 8.1
Final BW (g)	119.5 ^a ± 3.1	118.9 ^a ± 5.6	126.5 ^a ± 6.1	99.5 ^b ± 9.3
DM-intake (g/d)	10.23 ^{ab} ± 0.07	10.28 ^b ± 0.03	10.37 ^a ± 0.02	10.16 ^{ab} ± 0.1
N-intake ¹⁾ (mg/d)	1074 ^b ± 21	1058 ^b ± 40	2107 ^a ± 80	593 ^c ± 36
N-excretion faeces ¹⁾ (mg/d)	114 ^b ± 13	79 ^b ± 15	247 ^a ± 34	99 ^b ± 14
N-excretion urine ¹⁾ (mg/d)	278 ^b ± 26	337 ^b ± 19	915 ^a ± 56	139 ^c ± 24
N-balance ¹⁾ (mg/d)	682 ^b ± 37	642 ^b ± 41	945 ^a ± 72	355 ^c ± 39
N-digestibility, apparent (%)	89.41 ^b ± 1.26	92.51 ^a ± 1.38	88.30 ^b ± 1.41	83.30 ^c ± 1.54
N-digestibility, true ²⁾ (%)	94.83 ^{ab} ± 1.21	98.00 ^a ± 1.39	91.06 ^c ± 1.45	93.12 ^{bc} ± 1.87
N-balance : N-intake [PPV%]	63.55 ^a ± 2.53	60.66 ^a ± 2.43	44.82 ^b ± 2.47	59.73 ^a ± 4.68
N-retention ³⁾ : N-intake [NPU%]	75.44 ^{ab} ± 2.37	72.73 ^b ± 2.34	50.88 ^c ± 2.41	81.30 ^a ± 4.47
Biological value [BV%] ⁴⁾	79.56 ^b ± 2.58	74.21 ^b ± 1.87	55.89 ^c ± 2.84	87.31 ^a ± 4.56
Model parameter b-value	901 ^a ± 56	840 ^a ± 50	837 ^a ± 168	776 ^a ± 56

* $p \leq 0.001$

1) Data related to $BW_{kg}^{0.67}$ (metabolic BW)

2) Daily basal endogenous N-losses of the gut = $58.1 \text{ mg}/BW_{kg}^{0.67}$

3) $N\text{-retention} = N\text{-balance} + NMR^5)$ ($NMR = 127.6 \text{ mg}/BW_{kg}^{0.67}$)

4) $N\text{-retention} : \text{true digested } N\text{-intake} (\%)$

5) $NMR = N\text{-maintenance requirement}$ (sum of basal endogenous losses via faeces and urine)

The results demonstrate that the initial BW of rats in diet 4 was significantly lower due to the pre-feeding period with the low protein diet 4. This observation was continued up to the end of the collection period. According to the graded dietary supply of Isolexx, the daily N-intake per metabolic BW differed significantly.

000056

Accordingly, the faecal N-excretion was highest due to diet 3 with 30% Isolexx. Between diets 1 and 2, no significant difference of N-intake and faecal N-excretion was observed. A tendency for lower faecal N-output following the Vitalexx diet 2 was stated ($p \leq 0.001$). However, at $p \leq 0.05$, the faecal N-excretion with diet 2 was significantly declined. Accordingly, apparent N-digestibility provided by the Vitalexx diet 2 was improved ($p \leq 0.001$). True N-digestibility responded in same manner, indicating significant effects between diet 1 and 2 only at $p \leq 0.05$. Observed digestibility effects due to Isolexx diets 3 and 4 with quite different protein supply were as expected. N-excretion via urine was quite similar in diets 1 and 2, but tended to be higher in the Vitalexx diet 2. The observed difference was significant at $p \leq 0.05$. Actually it is not clear which is the main reason for this observation reflecting lower efficiency of the Vitalexx protein in the post-absorptive utilization process. Summarizing the efficiency of utilization on gut and metabolic level, several parameters were applied (PPV %; NPU %). Both of the parameters indicate that Vitalexx yielded a lower efficiency as compared to Isolexx, but not significantly at p-levels under study. However, the BV as reflection of post-absorptive protein utilization only was significantly lower in diet 2 at $p \leq 0.05$. The observed effects at different levels of Isolexx are in agreement with the expected changes following graded dietary protein supply.

According to PDCAAS calculation (A-Table 9a), lysine was the first limiting amino acid in both of the protein sources Isolexx and Vitalexx, respectively. The observed PDCAAS based on AA-analyses of our lab were very similar at 0.86 (Isolexx) or 0.87 (Vitalexx). More details and PDCAAS of other AAs are given in the annex. Due to the assumption that especially lysine could be underestimated by the AA analysis of our lab, additional AA analyses were conducted at LUFA Nord-West (Oldenburg, Germany). The new data of AA analyses and derived PDCAAS are added in annex tables 9b and 10b, respectively.

Based on the current AA-analyses, Isolexx (1.04) and Vitalexx (1.08) were very similar in PDCAAS, but superior to Dunasoy (0.94). Casein (1.47) was on highest level of PDCAAS. It has to be noted that the derived data for Canola proteins were based on lysine as the limiting AA, but Dunasoy and Casein were based on the sum of sulphur containing AA as limiting AA in the individual feed protein.

In conclusion of Exp.I it can be summarized that there is a strong evidence for any type of damage to one or more of the amino acids in Vitalexx which is not reflected on the digestibility level. This modification could be related to any type of lysine damage, as reported earlier for "Carpenter lysine". Accordingly, lysine was detected as the first limiting amino acid in the plant proteins under study. Consequently, any damage related to this amino acid will be reflected by decline of protein quality parameters as observed for PPV, NPU and BV, respectively.

Experiment II

Results of this study are summarized in table 3.

Table 3: Summary of N-balance studies with Isolexx and Vitalexx as single feed protein sources for rats in comparison with Dunasoy and Casein

	DIET 1	DIET 2	DIET 3	DIET 4
	15% Isolexx	17.48% Vitalexx	14.59% Dunasoy	14.23% Casein
Initial BW (g)	100.2 ^a ± 4.6*	98.9 ^a ± 4.5	98.1 ^a ± 3.4	102.3 ^a ± 4.4
Final BW (g)	123.2 ^a ± 4.5	122.1 ^a ± 3.9	120.9 ^a ± 3.4	128.6 ^a ± 4.8
DM-intake (g/d)	10.41 ^a ± 0.19	10.33 ^a ± 0.14	10.59 ^a ± 0.04	10.44 ^b ± 0.05
N-intake ¹⁾ (mg/d)	1052 ^{ab} ± 22	1016 ^b ± 26	1073 ^a ± 23	1001 ^b ± 26
N-excretion faeces ¹⁾ (mg/d)	144 ^a ± 14	93 ^c ± 7	113 ^b ± 5	84 ^c ± 3
N-excretion urine ¹⁾ (mg/d)	244 ^{ab} ± 19	305 ^a ± 29	259 ^a ± 18	194 ^b ± 42
N-balance ¹⁾ (mg/d)	664 ^{ab} ± 22	618 ^b ± 41	701 ^a ± 30	723 ^a ± 46
N-digestibility, apparent (%)	86.35 ^c ± 1.25	90.85 ^{ab} ± 0.56	89.43 ^b ± 0.34	91.56 ^a ± 0.28
N-digestibility, true ²⁾ (%)	91.87 ^c ± 1.26	96.57 ^a ± 0.64	94.85 ^b ± 0.37	97.37 ^a ± 0.27
N-balance : N-intake [PPV%]	63.16 ^b ± 1.56	60.80 ^b ± 3.22	65.32 ^b ± 2.01	72.18 ^a ± 4.05
N-retention ³⁾ : N-intake [NPU%]	75.29 ^b ± 1.57	73.37 ^b ± 3.10	77.21 ^b ± 1.93	84.93 ^a ± 4.06
Biological value [BV%] ⁴⁾	81.96 ^{ab} ± 1.83	75.98 ^b ± 3.18	81.40 ^{ab} ± 1.87	87.23 ^a ± 4.16
Model parameter b-value	884 ^b ± 31	832 ^b ± 59	936 ^{ab} ± 46	1052 ^a ± 92

* p ≤ 0.001

1) Data related to $BW_{kg}^{0.67}$ (metabolic BW)

2) Daily basal endogenous N-losses of the gut = 58.1 mg/ $BW_{kg}^{0.67}$

3) N-retention = N-balance + NMR⁵⁾ (NMR = 127.6 mg/ $BW_{kg}^{0.67}$)

4) N-retention : true digested N-intake (%)

5) NMR = N-maintenance requirement, according to the sum of daily basal endogenous losses via faeces and urine

Similar dietary protein supply between experimental diets in Exp.II yielded no significant effect on initial and final BW, respectively. Due to restricted feed supply, the dry matter intake between diets was also very similar. According to Exp.I, the Vitalexx diet 2 provided significant lower faecal N-output than Isolexx diet 1. Faecal N-excretion of the Vitalexx diet 2 was very similar to the animal protein diet 4 with Casein. Soyprotein source Dunasoy yielded significant lower faecal N-output when compared to the Isolexx diet 1. Consequently, the Isolexx diet 1 achieved both the lowest apparent and true N-digestibility, significantly different from the other protein sources. However, PPV and NPU

were not significantly different between the plant protein diets on $p \leq 0.001$ level. At $p \leq 0.05$, the Dunasoy diet 3 was superior to the Vitalex diet 2. The N-digestibility of Casein diet 4 was numerically higher than plant based diets, but not significantly in general (see table 3). Total protein utilization (PPV, NPU) of the animal protein diet 4 was superior to the plant based diets 1-3. Post absorptive N-utilization (BV) was also highest in diet 4, but, due to the observed standard deviation with this diet, only in part with statistical significance. The lower BV of the Vitalex diet 2, as observed in Exp.I, was confirmed by the results in Exp.II.

According to the calculated PDCAAS (A-Table 10a), the sulfur-containing AAs methionine plus cysteine were identified as the first limiting AAs in the protein fraction of Dunasoy. The observed PDCAAS (0.83) was below the data of Isolex and Vitalex, respectively. However, the yielded protein utilization *in vivo* tended to be higher when compared to the Canola proteins. The PDCAAS of the animal protein source Casein was superior to all of the plant protein sources under study, further details are given in the annex.

As already stated with Exp.I, an additional AA analysis was conducted and the new PDCAAS data are summarized in A-Table 10b.

In conclusion of Exp.II it can be summarized that the observed discrepancy between Isolex and Vitalex as single protein source in Exp.I was confirmed in Exp.II. Accordingly, it can be speculated that any type of lysine modification is achieved by processing of the protein source Vitalex. As related to Dunasoy, Vitalex yielded lower protein utilization both on the total (PPV, NPU) and on the post-absorptive level (BV) in spite of the highest level of detected protein digestibility. The protein quality of Casein for the laboratory rat was not achieved by any of the plant protein sources under study.

General conclusion

Both of the conducted experiments led to the conclusion that the Canola protein sources Isolex and Vitalex are highly digestible proteins for the rat, as indicated by true protein digestibility above 90%. However, a distinction between the proteins was observed which indicates increased amino acid absorption from the hydrolysed protein source (Vitalex). Furthermore, improved absorption did not yield improved total protein utilization with Vitalex as protein source, indicating any loss of bioavailability of lysine which was the dietary amino acid in limiting position. This observation should be taken into account when examining the treatment steps of Canola protein hydrolysis and drying procedures as well. In addition, the protein quality of Dunasoy as a reference protein was not completely achieved by the Canola protein sources. However, for Isolex the observed difference in dietary protein quality as related to Dunasoy was marginal. Both the *in vivo*

protein quality and PDCAAS of Casein was higher in comparison to all of the plant proteins under study. However, based on repeated external AA-analyses the PDCAAS of the Canola proteins under study were superior to Dunasoy as derived from the PDCAAS of lysine (Canola proteins) or methionine+cysteine (Dunasoy).

Finally, it has to be noted that the described parameters of protein quality were derived from rat studies which utilized the individual feed proteins as single protein sources without any supplementation of crystalline amino acids or making use of combination with other feed proteins to overcome individual amino acid deficiencies or imbalances. However, this would be the typical situation for application of the proteins under study in diet composition under feeding conditions. From this point of view, the yielded protein quality data can be improved by adding the detected AA in limiting position.

References

Gaßmann, B., 2006: Aminosäuren und Proteine. Teil 2: Proteine. Ernährungs-Umschau **53** (5): 176-181.

Horszczaruk, F. und Bock, H.-D., 1963: Eine Modifikation des von K. Schiller vorgeschlagenen Stoffwechselläufigs für Ratten. Zeitschrift Versuchstierkunde **2**: 126-131.

Liebert, F., 2008: Modelling of protein metabolism yields amino acid requirements dependent on dietary amino acid efficiency, growth response, genotype and age of growing chicken. Avian Biology Research **1** (3): 101-110.

Naumann, K. and Bassler, R., 1976-1997. Die chemische Untersuchung von Futtermitteln. Methodenbuch, Bd. III., Verlag Neumann-Neudam.

Samadi and Liebert, F., 2008: Modelling the optimal lysine to threonine ratio in growing chickens depending on age and efficiency of dietary amino acid utilisation. British Poultry Science **49** (1): 45-54.

Wecke, C. and Liebert, F., 2009: Lysine requirement studies in modern genotype barrows dependent on age, protein deposition and dietary lysine efficiency. Journal Animal Physiology and Animal Nutrition **93** (3): 295-304.

000060

Annex - Tables

A-Table 1a: Results of analysed feed proteins

AA	ISOLEXX		VITALEXX		DUNASOY 90	CASEIN
	gAA/100 g CP		gAA/100 g CP		gAA/100 g CP	
	analysed	certified	analysed	certified	analysed	
Cys ox	2.13	2.10	2.25	2.33	0.97	0.29
Met ox	1.96	2.08	2.12	2.29	1.21	2.86
Asp	8.37	8.65	6.54	7.30	11.63	7.26
Thr	4.11	3.90	4.80	4.29	3.76	4.25
Ser	4.14	4.67	4.59	5.21	5.08	5.56
Glu	19.80	20.02	19.88	20.14	18.28	20.62
Pro	6.30	5.78	6.33	6.21	5.06	10.45
Gly	4.99	5.17	5.31	5.53	3.91	1.75
Ala	4.53	4.69	5.01	5.31	4.12	2.97
Val	4.43	5.17	4.92	5.93	4.21	5.79
Ileu	3.82	4.23	4.39	4.85	4.46	4.96
Leu	7.23	7.61	7.59	7.92	7.55	9.15
Tyr	2.59	3.18	1.66	2.18	3.79	5.65
Phe	4.22	4.50	4.15	4.47	5.20	5.10
His	2.54	2.77	3.15	3.49	2.24	2.60
Lys	4.61	5.95	4.54	5.48	5.18	6.97
Arg	6.56	8.05	4.06	5.52	6.78	3.44

A-Table 1b: Results of analysed feed proteins according to LUFA 9/2010

AA	ISOLEXX		VITALEXX		DUNASOY 90	CASEIN
	gAA/100 g CP		gAA/100 g CP		gAA/100 g CP	
	analysed	certified	analysed	certified	analysed	
Cys ox	2.30	2.10	2.65	2.33	1.09	0.55
Met ox	2.11	2.08	2.37	2.29	1.39	3.23
Thr	4.07	3.90	4.95	4.29	3.88	5.75
Lys	5.57	5.95	5.62	5.48	6.61	9.26
Try	1.43	-	1.39	-	1.27	1.25

A-Table 2: Experimental diets (Exp.I) as mixed

	Diet1	
	%	g/1.5kg final feed
Isolexx	15	225
Wheat starch	55,7	835.5
Soybean oil	6	90
Sucrose	10	150
Cellulose powder	5	75
Mineral premix Altromin	6	90
Vitamin premix Altromin	2	30
Titanium dioxide	0,3	4.5
	100	1500

	Diet 2	
	%	g/1,5kg final feed
Vitalexx	17.48	262.2
Wheat starch	53.22	798.3
Soybean oil	6	90
Sucrose	10	150
Cellulose powder	5	75
Mineral premix Altromin	6	90
Vitamin premix Altromin	2	30
Titanium dioxide	0.3	4.5
	100	1500

	Diet 3	
	%	g/1.5kg final feed
Isolexx	30	450
Wheat starch	40.7	610.5
Soybean oil	6	90
Sucrose	10	150
Cellulose powder	5	75
Mineral premix Altromin	6	90
Vitamin premix Altromin	2	30
Titanium dioxide	0.3	4.5
	100	1500

	Diet 4	
	%	g/1.5kg final diet
Isolexx	7.5	112.5
Wheat starch	63.2	948
Soybean oil	6	90
Sucrose	10	150
Cellulose powder	5	75
Mineral premix Altromin	6	90
Vitamin premix Altromin	2	30
Titanium dioxide	0.3	4.5
	100	1500

A-Table 3: Composition of the mineral premix (according to provider)

Altromin™ Mineral and Micro Nutrients Nr. 201014 (6%)

Ingredient	Unit	Content
Crude Ash	mg/kg	839799.490
Calcium	mg/kg	148070.343
Phosphorus	mg/kg	97355.040
Digestible Phosphorus	mg/kg	97355.040
Magnesium	mg/kg	8784.265
Sodium	mg/kg	39229.405
Potassium	mg/kg	116496.447
Sulfur	mg/kg	10535.808
Chlorine	mg/kg	63510.382
Iron	mg/kg	4664.018
Manganese	mg/kg	1733.858
Zinc	mg/kg	387.541
Copper	mg/kg	85.209
Iodine	mg/kg	7.504
Molybdenum	mg/kg	3.314
Fluorine	mg/kg	70.076
Selenium	mg/kg	3.835
Cobalt	mg/kg	2.080
Aluminium	mg/kg	0.070

A-Table 4: Composition of the vitamin premix (according to provider)

Altromin™ Vitamin Mixture Nr. 201005 (2%)		
Ingredient	Unit	Content
Vitamin A	I.E./kg	750000.000
Vitamin D3	I.E./kg	25000.000
Vitamin E	mg/kg	7500.000
Vitamin K3 as Menadione	mg/kg	500.000
Vitamin B1	mg/kg	1000.000
Vitamin B2	mg/kg	1000.000
Vitamin B6	mg/kg	750.000
Vitamin B12	mg/kg	1.500
Nicotinic Acid	mg/kg	2500.000
Pantothenic Acid	mg/kg	2500.000
Folic Acid	mg/kg	500.00000
Biotin	mg/kg	10.000
Choline Chloride	mg/kg	50000.000
Benzoic Acid	mg/kg	5000.000
Inositol	mg/kg	5000.000
Vitamin C	mg/kg	975.000

A-Table 5: Individual data Exp.I-1

Rat number	Diet	Initial BW (g)	Final BW (g)	Average BW (g)	DM-intake (g/d)	N-intake (mg/LMkg ^{0.67} /d)	N-excretion faeces (mg/LMkg ^{0.67} /d)	N-excretion urine (mg/LMkg ^{0.67} /d)	N - balance (mg/LMkg ^{0.67} /d)
1	1*	96.5	119.5	108	10.3016	1075.1423	99.3159	320.5564	655.2700
2	1	93	119.8	106.4	10.2680	1082.4147	101.4267	281.5858	699.4022
3	1	95	120.5	107.75	10.2939	1076.0167	101.0012	295.3328	679.6827
4	1	90.5	115.7	103.1	10.1736	1095.3371	128.7092	243.5179	723.1100
5	1	99.5	123.5	111.5	10.1507	1037.0038	126.5926	273.6283	636.7828
6	1	91.5	115.9	103.7	10.2391	1098.1124	117.3172	264.5610	716.2342
7	1	93	117.7	105.35	10.1142	1073.3027	107.7937	247.5325	717.9764
8	1	99.3	123.7	111.5	10.2802	1050.2328	126.1436	292.9143	631.1750
9	2**	94	118.4	106.2	10.2642	1056.9479	114.2458	343.0299	599.6723
10	2	92	118.1	105.05	10.2383	1062.0061	70.1229	340.5086	651.3746
11	2	95	119.2	107.1	10.2398	1048.4986	68.6189	319.3242	660.5556
12	2	80.9	107.1	94	10.2611	1146.6549	81.2294	344.7304	720.6950
13	2	99	123.9	111.45	10.2991	1026.8098	77.7126	327.3065	621.7908
14	2	99.4	119.7	109.55	10.3174	1040.5473	75.7072	305.1820	659.6580
15	2	100	126.4	113.2	10.2900	1015.2473	71.5402	348.0642	595.6429
16	2	91.5	118.6	105.05	10.2991	1068.3125	74.9402	365.8258	627.5465
17	3***	103	129.3	116.15	10.3444	2043.1614	284.1621	850.4086	908.5906
18	3	92	126.3	109.15	10.3996	2141.4162	241.1838	814.8088	1085.4237
19	3	97.5	124.9	111.2	10.3720	2109.2721	224.3171	980.0006	904.9543
20	3	102	130.5	116.25	10.3996	2052.8807	203.3499	964.2015	885.3292
21	3	98	126.6	112.3	10.3352	2087.9720	259.3668	907.9236	920.6816
22	3	94	121.6	107.8	10.3720	2153.6159	262.7288	936.4679	954.4192
23	3	85	116.1	100.55	10.3904	2260.4589	293.5969	949.2261	1017.6360
24	3	103	136.8	119.9	10.3720	2005.4598	205.9750	918.6308	880.8540
25	4****	87	100.9	93.95	10.2386	589.1272	99.1045	157.4558	332.5669
26	4	85.5	98.3	91.9	10.2386	597.9000	93.7886	108.3373	395.7741
27	4	80	92.5	86.25	10.2189	622.6645	106.9955	107.2731	408.3958
28	4	95	106.6	100.8	10.2341	561.7438	86.7224	177.5687	297.4527
29	4	91.5	106.1	98.8	10.1324	563.6837	86.9941	129.9874	346.7021
30	4	92	112.7	102.35	10.2113	554.7935	103.0256	136.9577	314.8103
31	4	69.5	82.8	76.15	10.0172	663.4900	127.5914	156.3973	379.5013
32	4	83.5	95.9	89.7	10.0111	594.1815	90.4468	140.0600	363.6747

* Diet 1:15% Isolexx ** Diet 2: 17.48% Vitalexx *** Diet 3: 30% Isolexx **** Diet 4: 7.5% Isolexx

00064

A-Table 6: Individual data Exp.I-2

Rat number	Diet	N-digestibility. apparent (%)	N-digestibility. true (%)	PPV (%)	NPU (%)	BV (%)	b-value
1	1*	90.7625	96.1665	60.9473	72.8155	75.7182	848.3566
2	1	90.6296	95.9972	64.6150	76.4035	79.5892	923.6847
3	1	90.6134	96.0130	63.1666	75.0251	78.1406	891.8701
4	1	88.2494	93.5537	66.0171	77.6665	83.0181	958.8894
5	1	87.7925	93.3951	61.4060	73.7107	78.9235	846.2200
6	1	89.3165	94.6074	65.2241	76.8441	81.2242	942.8884
7	1	89.9568	95.3700	66.8941	78.7827	82.6074	968.1839
8	1	87.9890	93.5211	60.0986	72.2483	77.2534	825.7965
9	2**	89.1910	94.6879	56.7362	68.8087	72.6689	767.8227
10	2	93.3971	98.8679	61.3344	73.3494	74.1892	851.8969
11	2	93.4555	98.9968	63.0001	75.1699	75.9317	879.5546
12	2	92.9160	97.9829	62.8520	73.9800	75.5030	911.3859
13	2	92.4316	98.0899	60.5556	72.9824	74.4036	828.1478
14	2	92.7243	98.3079	63.3953	75.6581	76.9603	884.6192
15	2	92.9534	98.6762	58.6697	71.2381	72.1938	792.5551
16	2	92.9852	98.4237	58.7418	70.6859	71.8180	805.6623
17	3***	86.0920	88.9357	44.4698	50.7151	57.0244	768.3503
18	3	88.7372	91.4503	50.6872	56.6459	61.9417	1222.7012
19	3	89.3652	92.1197	42.9036	48.9531	53.1408	737.9777
20	3	90.0944	92.9246	43.1262	49.3418	53.0988	724.7755
21	3	87.5781	90.3607	44.0945	50.2057	55.5615	773.6172
22	3	87.8006	90.4984	44.3171	50.2420	55.5170	814.5300
23	3	87.0116	89.5819	45.0190	50.6639	56.5559	920.8633
24	3	89.7293	92.6264	43.9228	50.2854	54.2885	734.4189
25	4****	83.1777	93.0398	56.4508	78.1099	83.9533	735.6424
26	4	84.3137	94.0310	66.1940	87.5354	93.0920	854.3598
27	4	82.8165	92.1474	65.5884	86.0810	93.4167	846.4102
28	4	84.5619	94.9047	52.9517	75.6666	79.7291	699.2975
29	4	84.5669	94.8741	61.5065	84.1433	88.6895	798.6587
30	4	81.4299	91.9023	56.7437	79.7432	86.7696	743.8271
31	4	80.7697	89.5264	57.1977	76.4294	85.3708	738.9798
32	4	84.7779	94.5561	61.2060	82.6809	87.4411	792.2632

* Diet 1:15% Isolexx ** Diet 2: 17.48% Vitalexx *** Diet 3: 30% Isolexx **** Diet 4: 7.5% Isolexx

A-Table 7: Individual data Exp.II-1

Rat number	Diet	Initial BW (g)	Final BW (g)	Average BW (g)	DM- intake (g/d)	N-intake (mg/LMkg ^{0.67} /d)	N-excretion faeces (mg/LMkg ^{0.67} /d)	N-excretion urine (mg/LMkg ^{0.67} /d)	N - balance (mg/LMkg ^{0.67} /d)
1	1*	102	123.5	112.75	10.4582	1049.9877	167.8619	387.2116	662.7760
2	1	106	130	118	10.5843	1030.7406	131.8278	405.3617	625.3788
3	1	99	122.5	110.75	10.4828	1065.1557	149.5801	396.2191	668.9366
4	1	94	119.5	106.75	10.5336	1097.0222	145.4405	413.2465	683.7757
5	1	101	124	112.5	10.2966	1035.3061	141.6742	373.7019	661.6041
6	1	97	117	107	10.0720	1047.3067	153.9742	388.2336	659.0731
7	1	106.5	129	117.75	10.6028	1034.0069	130.3962	379.7184	654.2885
8	1	96	120	108	10.2135	1055.4273	128.0702	356.4560	698.9714
9	2**	104	126	115	10.4422	997.4584	92.7733	451.0533	546.4050
10	2	99	123	111	10.2869	1006.2115	93.3800	394.2886	611.9229
11	2	99.5	124	111.75	10.4453	1017.1016	85.2675	390.8661	626.2355
12 outlier	2	-	-	-	-	-	-	-	-
13	2	102	124.5	113.25	10.1670	981.2001	88.8214	396.1489	585.0512
14	2	95	118	106.5	10.4852	1054.4471	103.5882	418.1694	636.2777
15	2	91	115.5	103.25	10.1639	1043.5793	101.4719	373.4859	670.0935
16	2	101.5	124	112.75	10.4314	1009.7094	85.7645	360.6462	649.0633
17	3***	101.5	125	113.25	10.6287	1052.6063	115.9390	384.8967	667.7097
18	3	96	121	108.5	10.5735	1077.6334	110.4944	353.1101	724.5233
19	3	97	120	108.5	10.5765	1077.9461	108.9293	335.7224	742.2238
20	3	93	117	105	10.6287	1107.3241	122.0012	378.8446	728.4795
21	3	101.5	122	111.75	10.5182	1051.0144	111.9498	388.8622	662.1523
22	3	98.5	120.5	109.5	10.6287	1076.6242	112.5337	369.2092	707.4150
23	3	102	125.5	113.75	10.5735	1044.0506	108.1342	368.0898	675.9607
24	3	95	116	105.5	10.5735	1098.0695	117.3661	397.7802	700.2893
25	4****	104.5	129.5	117	10.5099	998.2331	85.5875	326.0082	672.2250
26	4	104.5	129.5	117	10.4075	988.5120	89.1744	267.0500	721.4619
27	4	97	123.5	110.25	10.3800	1025.9445	85.6781	269.6092	756.3354
28	4	105	132	118.5	10.4044	979.8231	81.1608	257.8202	722.0028
29	4	99.5	124.5	112	10.4533	1022.3475	84.4776	357.6058	664.7417
30	4	96	122.5	109.25	10.4671	1040.8857	86.4644	231.3551	809.5306
31	4	103.5	130.5	117	10.3892	986.7709	80.2410	263.2761	723.4948
32	4	108.5	136.5	122.5	10.5114	968.1183	82.7768	255.3716	712.7468

* Diet 1: 15% Isolexx ** Diet 2: 17.48% Vitalexx *** Diet 3: 14.59% Dunasoy **** Diet 4: 14.23% Casein

990000

A-Table 8: Individual data Exp.II-2

Rat number	Diet	N-digestibility. apparent (%)	N-digestibility. true (%)	PPV (%)	NPU (%)	BV (%)	b-value
1	1*	84.0130	89.5464	63.1223	75.2748	84.0624	882.3805
2	1	87.2104	92.8471	60.6728	73.0522	78.6801	831.2364
3	1	85.9570	91.4116	62.8018	74.7812	81.8072	881.0462
4	1	86.7423	92.0384	62.3302	73.9617	80.3596	882.2682
5	1	86.3157	91.9276	63.9042	76.2291	82.9230	892.7110
6	1	85.2981	90.8456	62.9303	75.1139	82.6830	877.8387
7	1	87.3892	93.0082	63.2770	75.6173	81.3018	880.3020
8	1	87.8656	93.3704	66.2264	78.3163	83.8769	946.4559
9	2**	90.6990	96.5238	54.7797	67.5722	70.0058	725.6981
10	2	90.7196	96.4938	60.8145	73.4958	76.1663	827.7119
11	2	91.6166	97.3289	61.5706	74.1160	76.1501	843.9005
12 outlier	2	-	-	-	-	-	-
13	2	90.9477	96.8690	59.6261	72.6306	74.9781	801.7752
14	2	90.1761	95.6861	60.3423	72.4434	75.7095	831.3412
15	2	90.2765	95.8439	64.2111	76.4382	79.7528	901.4301
16	2	91.5060	97.2601	64.2822	76.9195	79.0863	891.7059
17	3***	88.9855	94.5052	63.4339	75.5562	79.9493	889.2759
18	3	89.7466	95.1380	67.2328	79.0736	83.1146	977.5119
19	3	89.8947	95.2846	68.8554	80.6927	84.6860	1013.9295
20	3	88.9823	94.2292	65.7874	77.3107	82.0453	959.1655
21	3	89.3484	94.8764	63.0013	75.1419	79.1998	880.3736
22	3	89.5475	94.9440	65.7068	77.5586	81.6888	944.2416
23	3	89.6428	95.2077	64.7441	76.9657	80.8398	912.0898
24	3	89.3116	94.6027	63.7746	75.3950	79.6964	912.1956
25	4****	91.4261	97.2464	67.3415	80.1241	82.3928	946.5683
26	4	90.9789	96.8564	72.9846	85.8929	88.6807	1058.8775
27	4	91.6489	97.3119	73.7209	86.1582	88.5382	1097.1969
28	4	91.7168	97.6464	73.6871	86.7098	88.7998	1069.4698
29	4	91.7369	97.4199	65.0211	77.5022	79.5548	909.9548
30	4	91.6932	97.2750	77.7732	90.0320	92.5542	1210.0747
31	4	91.8683	97.7562	73.3194	86.2505	88.2302	1065.2403
32	4	91.4497	97.4511	73.6219	86.8021	89.0725	1061.7703

* Diet 1:15% Isolexx ** Diet 2: 17.48% Vitalexx *** Diet 3: 14.59% Dunasoy

**** Diet 4: 14.23% Casein

A-Table 9a: PDCAAS of Canola proteins according to AA-analysis of our lab

Reference protein humans								
AA	His	Ile	Leu	Lys	Met+Cys	Phe+Tyr	Thr	Val
mg AA/g protein	18	25	55	51	25	47	27	32
Test protein (Isolexx)								
AA	His	Ile	Leu	Lys	Met+Cys	Phe+Tyr	Thr	Val
mg AA/g protein	25.4	38.2	72.3	46.1	40.9	68.1	41.4	44.3
AAS	1.41	1.53	1.31	0.90	1.64	1.45	1.53	1.38
TPD (%)*	94.83							
PDCAAS	1.34	1.45	1.25	0.86	1.55	1.37	1.45	1.31
Test protein (Vitalex)								
AA	His	Ile	Leu	Lys	Met+Cys	Phe+Tyr	Thr	Val
mg AA/g protein	31.5	43.9	75.9	45.4	43.7	58.1	48	49.2
AAS	1.75	1.76	1.38	0.89	1.75	1.24	1.78	1.54
TPD (%)*	98.00							
PDCAAS	1.72	1.72	1.35	0.87	1.71	1.21	1.74	1.51

*) true protein digestibility

A-Table 9b: PDCAAS of Canola proteins according to AA-analysis of LUFA 9/2010

Reference protein humans								
AA	His	Ile	Leu	Lys	Met+Cys	Phe+Tyr	Thr	Trp
mg AA/g protein	-	-	-	51	25	-	27	7
Test protein (Isolexx)								
AA	His	Ile	Leu	Lys	Met+Cys	Phe+Tyr	Thr	Trp
mg AA/g protein	-	-	-	55.75	23.00	-	40.65	14.29
AAS	-	-	-	1.09	1.77	-	1.51	2.04
TPD (%)*	94.83							
PDCAAS	-	-	-	1.04	1.68	-	1.43	1.93
Test protein (Vitalex)								
AA	His	Ile	Leu	Lys	Met+Cys	Phe+Tyr	Thr	Trp
mg AA/g protein	-	-	-	56.21	50.21	-	49.51	13.95
AAS	-	-	-	1.10	2.01	-	1.83	1.99
TPD (%)*	98.00							
PDCAAS	-	-	-	1.08	1.97	-	1.79	1.95

*) true protein digestibility

A-Table 10a: PDCAAS of Dunasoy and Casein according to AA-analysis of our lab

Reference protein humans								
AA	His	Ile	Leu	Lys	Met+Cys	Phe+Tyr	Thr	Val
mg AA/g protein	18	25	55	51	25	47	27	32
Test protein (Dunasoy 90)								
AA	His	Ile	Leu	Lys	Met+Cys	Phe+Tyr	Thr	Val
mg AA/g protein	22.4	44.6	75.5	51.8	21.8	91.9	37.6	42.1
AAS	1.24	1.78	1.37	1.02	0.87	1.96	1.39	1.32
TPD (%)	94.85							
PDCAAS	1.18	1.69	1.30	0.96	0.83	1.85	1.32	1.25
Test protein (Casein)								
AA	His	Ile	Leu	Lys	Met+Cys	Phe+Tyr	Thr	Val
mg AA/g protein	32.2	44.1	81.5	62.1	28.1	95.8	37.9	51.6
AAS	1.29	1.77	1.48	1.22	1.12	2.04	1.40	1.61
TPD (%)	97.37							
PDCAAS	1.25	1.72	1.44	1.19	1.09	1.99	1.37	1.57

*) true protein digestibility

A-Table 10b: PDCAAS of Dunasoy and Casein according to AA-analysis of LUFA 9/2010

Reference protein humans								
AA	His	Ile	Leu	Lys	Met+Cys	Phe+Tyr	Thr	Trp
mg AA/g protein	-	-	-	51	25	-	27	7
Test protein (Dunasoy 90)								
AA	His	Ile	Leu	Lys	Met+Cys	Phe+Tyr	Thr	Trp
mg AA/g protein	-	-	-	66.1	24.8	-	3.88	1.27
AAS	-	-	-	1.30	0.99	-	1.44	1.82
TPD (%)	94.85							
PDCAAS	-	-	-	1.23	0.94	-	1.37	1.73
Test protein (Casein)								
AA	His	Ile	Leu	Lys	Met+Cys	Phe+Tyr	Thr	Trp
mg AA/g protein	-	-	-	92.57	37.75	-	57.49	12.54
AAS	-	-	-	1.82	1.51	-	2.13	1.79
TPD (%)	97.37							
PDCAAS	-	-	-	1.77	1.47	-	2.07	1.74

*) true protein digestibility

000068

APPENDIX 3
Feeding Studies on Canola or B. Juncea
Meal Pressed Cake in Field Animals

000070

Appendix 3 Feeding Studies on Canola or B. Juncea Meal Pressed Cake in Field Animals

Reference	Study Design/ Objective	Species & Number/Sex	Test Substance	Dose	NOAEL/ LOAEL*	Duration	Results
Bell, et al. 1972	Histopathological analysis of tissues of rats and mice fed diets containing <i>B. juncea</i> diet	<ul style="list-style-type: none"> ▪ Male weanling mice and rats 	<i>B. juncea</i> and other mustard diets	20% crude protein	Adequate data in the paper for calculating NOAEL/LOAEL	4 and 6 weeks for mice and rats respectively	<ul style="list-style-type: none"> ▪ Thyroid enlargement of the rapeseed meal –fed rats was apparent microscopically ▪ When compared to other meals, rats consuming <i>B. juncea</i> meal fared better in body and organ weight gain ▪ Histopathological assays along with the performance data showed a greater response by rats than by mice to the presence of glucosinolates in the diet
Marangos and Hill, 1976	Use of rapeseed meal and mustard seed meal as protein source in diets for laying pullets	<ul style="list-style-type: none"> ▪ Shaver Starcross 288 layer type pullets ▪ Aged 17 weeks 	<i>B. juncea</i> , <i>B. comprestis</i> , <i>B. napus</i>	15% crude protein	Adequate data in the paper for calculating NOAEL/LOAEL	6 weeks	<ul style="list-style-type: none"> ▪ Thyroid of birds fed on rapeseed meal diets during the laying period was significantly heavier than those of birds fed on diets containing mustard seed or soybean ▪ Meals comprising <i>B. juncea</i> did not have any significant effect on egg production or thyroid enlargement

000071

Appendix 3 Feeding Studies on Canola or B. Juncea Meal Pressed Cake in Field Animals

Reference	Study Design/ Objective	Species & Number/Sex	Test Substance	Dose	NOAEL/ LOAEL*	Duration	Results
Cilly et al. 1977	Mustard cake (<i>B. juncea</i>) as a substitute for groundnut cake in chick diets	<ul style="list-style-type: none"> ▪ Male White Leghorn chicks ▪ 1 week old 	Three types of <i>Brassica</i> seeds, including <i>B. juncea</i>	Mustard cake consists of 37.2% crude protein, 27.5% true protein, 12.6% available carbohydrate and 2.09% tannins	<p>1600 mg/kg.bw/day</p> <p>Daily feed consumption is 543 g</p> <p><i>NOAEL is based on following assumptions:</i></p> <p>Average weight of a chick is ~ 125g. This is based on follow:</p> <p>Approximate weight of chick at start of experiment - ~50g and end of experiment was ~200 g</p> <p>Or $50+200=250g$ Or $250/2=125g$ is the average weight of a chick.</p>	6 weeks	<ul style="list-style-type: none"> ▪ <i>B. juncea</i> cake had no effect on the growth rate of the chicks or either breed although some thyroid enlargement was seen ▪ <i>B. juncea</i> cake did not affect body composition of the chicks

000072

Appendix 3 Feeding Studies on Canola or B. Juncea Meal Pressed Cake in Field Animals

Reference	Study Design/ Objective	Species & Number/Sex	Test Substance	Dose	NOAEL/ LOAEL*	Duration	Results
Cilly, et al. 1978	Nutritive value of <i>B. juncea</i> and other mustard seeds	<ul style="list-style-type: none"> ▪ Broiler and White Leghorn chicks ▪ 1 week old 	<i>B. Juncea</i> seed cake after extraction of oil	Crude protein content 25 %	<p>888 mg/kg.bw/day</p> <p>Daily feed consumption is 445 g</p> <p><i>NOAEL is based on following assumptions:</i></p> <p>Average weight of a chick is ~ 125g. This is based on follow:</p> <p>Approximate weight of chick at start of experiment - ~50g and end of experiment was 200 g based on weight gain in 4 weeks.</p> <p>Or $50+200=250g$ Or $250/2=125g$ is the average weight of a chick.</p>	4-weeks	<ul style="list-style-type: none"> ▪ The broiler chicks fed <i>B. juncea</i> in their diet gained significantly more weight than those fed with Taramira or groundnut diets ▪ The chicks of either breed utilized dietary protein containing <i>B. juncea</i> and other varieties with the same efficiency as that of the groundnut diet ▪ The protein digestibility and metabolizable energy content of Brassica seed cake were higher for broilers than for egg-type chicks

000073

Appendix 3 Feeding Studies on Canola or B. Juncea Meal Pressed Cake in Field Animals

Reference	Study Design/ Objective	Species & Number/Sex	Test Substance	Dose	NOAEL/ LOAEL*	Duration	Results
Bell, et al. 1981	Effect of alkali treatment and amino acid supplementation on the nutritive value of yellow and oriental mustard meal for swine	<ul style="list-style-type: none"> ▪ Crossbred pigs ▪ Average weight 27.3 kg 	<i>B. juncea</i> and <i>B hirta</i> "Sabre" meal	16-17 % crude protein	<p><u>~8225 mg/kg.bw/day</u></p> <p>Daily feed of pigs in the <i>B. juncea</i> group ~ 1.5 kg</p> <p><i>NOAEL is based on following assumptions:</i></p> <p>Average weight of pig ~ 31 kg. This is based on follow:</p> <p>Weight of pigs at start of experiment- 27 kg</p> <p>Daily weight gain of pigs-410g. Therefore, total weight gain in 12 weeks is 34 kg.</p> <p>Or 27+34=61kg Or 61/2=30.5kg is the average weight of pig</p>	12 weeks	<ul style="list-style-type: none"> ▪ Heat treatment resulted in significant reductions in glucosinolate content in <i>B. juncea</i> diet ▪ Growth responses of swine showed oriental mustard meal to be inferior to Sabre meal and both inferior to soy bean meal ▪ Digestibility of Sabre and oriental protein was 64% and 87%, respectively ▪ The nutritional differences between the mustard meals was attributed to glucosinolates affecting palatability or thyroid function, since available energy, protein or lysine content were not sufficiently limiting in the diets used in the study ▪ Nitrogen to protein conversion factors was somewhat similar in both the mustard diets and soy bean diets

000074

Appendix 3 Feeding Studies on Canola or B. Juncea Meal Pressed Cake in Field Animals

Reference	Study Design/ Objective	Species & Number/Sex	Test Substance	Dose	NOAEL/ LOAEL*	Duration	Results
Bell, et al. 1984	Amino acid supplementation of ammoniated mustard (<i>B. juncea</i>) meal for use in swine feeds	<ul style="list-style-type: none"> ▪ Pigs (cross bred barrows) ▪ 40 in number ▪ 25-52 kg 	Expelled <i>B. juncea</i> , canola and soy bean meal as a protein supplement in six barley:wheat (2:1) diets	<p>Feeding trial: 45% crude protein</p> <p>Finisher diet: 14% crude protein</p> <p>Average crude protein:</p> <p>~30%</p>	<p>11285 mg/kg.bw/day</p> <p>Daily feed of pigs in the <i>B. juncea</i> group 2.37 kg</p> <p><i>NOAEL is based on following assumptions:</i></p> <p>Average weight of pig ~ 63 kg. This is based on follow:</p> <p>Weight of pigs at start of experiment - 25 kg and end of experiment is 100 kg</p> <p>Or $25+100=125\text{kg}$ Or $125/2=62.5\text{ kg}$ is the average weight of pig.</p>	Time duration between the weight of the pig reached from 24-52 kg	<ul style="list-style-type: none"> ▪ Compared to the unsupplemented mustard diet, supplemented lysine significantly improved the feed gain ratio, while adding both lysine and isoleusine improved the daily weight gain. ▪ The performance of the pigs over the entire experiment showed no significant difference among protein supplements in terms of growth and efficiency of feed utilization.

000075

Appendix 3 Feeding Studies on Canola or B. Juncea Meal Pressed Cake in Field Animals

Reference	Study Design/ Objective	Species & Number/Sex	Test Substance	Dose	NOAEL/ LOAEL*	Duration	Results
Blair, 1984	Nutritional evaluation of ammoniated mustard meal	Male day-old broiler chicks	<i>B. juncea</i> meal	Crude protein ~25%	<p>717 mg/kg.bw/day</p> <p>Daily feed consumption is 1247 g</p> <p><i>NOAEL is based on following assumptions:</i></p> <p>Average weight of a chick is ~ 434g. This is based on follow:</p> <p>Approximate weight of chick at start of experiment - ~25g and end of experiment was 843.</p> <p>Or $25+843=868g$ Or $868/2=434g$ is the average weight of a chick.</p>	4 weeks	<ul style="list-style-type: none"> ▪ The first experiment showed that up to 10% ammoniated mustard meal could be included in the diets of chicks successfully although thyroid enlargement was observed in chicks ▪ The second experiment showed that up to 20% ammoniated mustard meal could successfully be included in the diets of chicks provided lysine was supplemented

920006

Appendix 3 Feeding Studies on Canola or B. Juncea Meal Pressed Cake in Field Animals

Reference	Study Design/ Objective	Species & Number/Sex	Test Substance	Dose	NOAEL/ LOAEL*	Duration	Results
Khan, et al. 1995	Hematological and histological studies after curry leaf (<i>Murraya koenigii</i>) and mustard (<i>B. juncea</i>) feeding in rats	<ul style="list-style-type: none"> ▪ Male SD rats ▪ 80-85g 	Curry leaf (<i>Murraya koenigii</i>) and mustard (<i>B. juncea</i>) seeds	10% <i>B. juncea</i> seeds	Not determined since amount of crude protein in seeds not provided	60 days	<ul style="list-style-type: none"> ▪ Whole curry leaf and powdered mustard seeds fed to rats at doses equal to normal human intake did not cause any adverse effect on food efficiency ratio, red and white blood cell count, total count, differential counts, or on the levels of blood constituents, like serum electrolytes, blood urea, hemoglobin, total serum protein, albumin-globulin ratio, fibrin level, glycosylated hemoglobin and the activity of serum enzymes (GOT and GPT) ▪ No histopathological changes were observed in the liver of rats administered powdered mustard seeds

000077

Appendix 3 Feeding Studies on Canola or B. Juncea Meal Pressed Cake in Field Animals

Reference	Study Design/ Objective	Species & Number/Sex	Test Substance	Dose	NOAEL/ LOAEL*	Duration	Results
Khan, et al. 1996	Effect of <i>Murraya koenigii</i> and <i>B. juncea</i> on lipid profile in 1-2 dimethyl hydrazine induced colon carcinogenesis in rats	<ul style="list-style-type: none"> ▪ SD rats ▪ 8 weeks old ▪ 80-100g weight 	<i>Murraya koenigii</i> and <i>B. juncea</i> seed	Not mentioned	Not determined since amount of crude protein in seeds not provided	15 weeks	<ul style="list-style-type: none"> ▪ The level of cholesterol and phospholipids decreased in the group administered curry leaves and mustard seeds (experimental group) when compared to the control group ▪ The cholesterol phospholipid ratio showed an elevated level in the 1, 2-dimethyl hydrazine (1,2 DMH) group when compared to mustard group ▪ Bile acids and neutral sterols showed a sharp increase in the mustard group in liver and feces when compared to the control group ▪ Morphological and histological studies revealed that the mean number of neoplasms in the colon and intestine were significantly low in the mustard fed group

820000

Appendix 3 Feeding Studies on Canola or B. Juncea Meal Pressed Cake in Field Animals

Reference	Study Design/ Objective	Species & Number/Sex	Test Substance	Dose	NOAEL/ LOAEL*	Duration	Results
Newkirk, et al. 1997	Nutritional evaluation of <i>B. juncea</i> seed and other <i>Brassica</i> samples in broiler diets	<ul style="list-style-type: none"> ▪ Broiler chickens 	<i>B. juncea</i> seed and other <i>Brassica</i> varieties	Crude protein range from 45%-47.2%	Adequate data not available for NOAEL/LOAEL calculation	21 days	<ul style="list-style-type: none"> ▪ Meals derived from <i>B. juncea</i> contained more crude protein and less total dietary fiber than <i>B. napus</i> or <i>B. rapa</i> varieties ▪ <i>B. juncea</i> meals contained more glucosinolates than <i>B. napus</i> and <i>B. rapa</i>, respectively ▪ <i>B. juncea</i> meals were equal or superior to <i>B. napus</i> and <i>B. rapa</i> meals for nutrient retention and apparent ileal protein digestibility ▪ Broilers fed <i>B. juncea</i> meals grew as quickly and converted feed to body weight gain as efficiently to 21 days of age as those birds fed <i>B. napus</i> and <i>B. rapa</i> meals ▪ It was concluded that the nutritional values of meal from <i>B. juncea</i> was equal or superior to that of canola meal samples derived from <i>B. napus</i> and <i>B. napa</i> cultivars

Appendix 3 Feeding Studies on Canola or B. Juncea Meal Pressed Cake in Field Animals

Reference	Study Design/ Objective	Species & Number/Sex	Test Substance	Dose	NOAEL/ LOAEL*	Duration	Results
Begum, et al. 1998	Hematobiochemic al studies on the toxic effects of expeller variety of mustard cake (<i>B. juncea</i>) in broiler chickens	<ul style="list-style-type: none"> ▪ Broiler chicks ▪ 100 in number ▪ 2 weeks old 	<i>B. juncea</i> diet	<i>B. juncea</i> diet replacing 20, 30, 50, and 100% ground nut cake diet	Not determined since amount of crude protein in seeds not provided	6 weeks	<ul style="list-style-type: none"> ▪ Decreasing trend of total erythrocyte, leukocyte count, Packed Cell Volume (PCV) and Hemoglobin in all the treated group of chicks. However, the values were still under the normal range up to 30% of replacement meal ▪ It was concluded that the groundnut cake meal when substituted up to 30% with <i>B. juncea</i> in broiler ration does not influence any change in the hematobiochemical variables in chicks

Appendix 3 Feeding Studies on Canola or B. Juncea Meal Pressed Cake in Field Animals

Reference	Study Design/ Objective	Species & Number/Sex	Test Substance	Dose	NOAEL/ LOAEL*	Duration	Results
Tripathi, et al. 1998	Effect of high glucosinolate diet on growth, carcass quality, and hematology	<ul style="list-style-type: none"> ▪ Aviv Astra lambs ▪ 9±0.56 kg body weight ▪ 25±3 days of age 	Defatted mustard meal (<i>B. juncea</i>)	<ul style="list-style-type: none"> ▪ 3-4 % total glucosinolate content ▪ 35-40% protein content 	<p><u>10975 mg/kg.bw/day</u></p> <p>Daily feed of lambs in the <i>B. juncea</i> group 0.44 kg</p> <p><i>NOAEL is based on following assumptions:</i></p> <p>Average weight of pig ~ 16 kg. This is based on follow:</p> <p>Weight of lambs at start of experiment - 8.5 kg and end of experiment was 23 kg</p> <p>Or $8.5+23=31.5\text{kg}$ Or $31.5/2=15.75\text{ kg}$ is the average weight of lamb.</p>	180 days	<ul style="list-style-type: none"> ▪ Total weight gain and body weight gain was significantly higher in groundnut meal basal diet than in mustard meal based diet ▪ Feed intake was similar in both the groups ▪ Total protein, globulin and glucose contents were similar in both the groups, whereas albumin and thiocyanate levels were significantly higher in mustard meal diet

000081

Appendix 3 Feeding Studies on Canola or B. Juncea Meal Pressed Cake in Field Animals

Reference	Study Design/ Objective	Species & Number/Sex	Test Substance	Dose	NOAEL/ LOAEL*	Duration	Results
Tripathi, et al. 1999	Feeding value of mustard oil cake	<ul style="list-style-type: none"> ▪ Ewes ▪ 18 in number ▪ In advanced gestational stage 	Mustard oil cake	~20% crude protein in dry meal	Adequate data not available for calculating NOAEL/LOAEL values	120 days	<ul style="list-style-type: none"> ▪ The dry matter intake per unit metabolic body size in all the groups (groundnut cake, mustard oil cake and 12-hour water soaked and sundried mustard oil cake) was similar ▪ Milk fat was similar in all 3 groups ▪ Lactose and solids-not fat (SNF) were higher in mustard oil cake fed ewes as compared to groundnut cake fed animals ▪ Other milk constituents such as protein, total solids, and ash were similar and within the range ▪ The serum proteins, albumin , and globulin were higher in groundnut cake fed group as compared to mustard cake fed group ▪ The blood biochemical variables were within the normal ranges ▪ It was found that the water soaking of mustard cake did not improve digestive efficiency, but decreased serum and milk thiocyanate content

000082

Appendix 3 Feeding Studies on Canola or B. Juncea Meal Pressed Cake in Field Animals

Reference	Study Design/ Objective	Species & Number/Sex	Test Substance	Dose	NOAEL/ LOAEL*	Duration	Results
Giri, et al. 2000	Feed intake, digestibility, plane of nutrition, and weight gain by growing bulls fed on grain less diets (including mustard cake) containing different nitrogen sources	<ul style="list-style-type: none"> ▪ Cross bred bulls ▪ 25 in number ▪ Weight approximately 305 Kg 	Grain less diets (including mustard cake)	20% of crude protein	<p><u>2450 mg/kg.bw/day</u></p> <p>Daily feed of bulls in the mustard meal group ~4.3kg</p> <p><i>NOAEL is based on following assumptions:</i></p> <p>Average weight of bull ~ 351kg. This is based on follow:</p> <p>Weight of bulls at start of experiment - 309 kg and end of experiment is 392 kg</p> <p>Or $309+392=701\text{kg}$ Or $701/2=350.5\text{ kg}$ is the average weight of a bull.</p>	196 days	<ul style="list-style-type: none"> ▪ The dry matter intake and digestibility of the nutrients except crude protein were similar in all groups ▪ A positive nitrogen, calcium and phosphorous balance was observed in all groups ▪ Average daily weight gains were similar in animals fed control diet (barley 30%) and mustard oil cake

Appendix 3 Feeding Studies on Canola or B. Juncea Meal Pressed Cake in Field Animals

Reference	Study Design/ Objective	Species & Number/Sex	Test Substance	Dose	NOAEL/ LOAEL*	Duration	Results
Tripathi, et al. 2001a	Performance of crossbred calves on acid processed or copper and iodine supplemented high glucosinolate mustard meal incorporated diets	<ul style="list-style-type: none"> ▪ Male cross bred calves ▪ 230±15.4 days age ▪ 86.6±2.7 body weight 	Treated and untreated <i>B. juncea</i> meal	89.8% dry matter, 28.3% crude protein	<p><u>5557 mg/kg.bw/day</u></p> <p>Daily feed of a calf in the untreated <i>B. juncea</i> group 2.06 kg (2.96 roughage +1.17 concentrate=4.13/2=.06)</p> <p><i>NOAEL is based on following assumptions:</i></p> <p>Average weight of a calf ~ 104kg. This is based on follow:</p> <p>Weight of calves at start of experiment - ~87kg and end of experiment is 120 kg (Average daily gain was 194g times 168 days=32.59kg)</p> <p>Or 87+120=207kg Or 207/2=103.5 kg is the average weight of a calf.</p>	24 weeks	<ul style="list-style-type: none"> ▪ The calves fed HCl-treated mustard meal diet gained more weight as compared to control diet ▪ Body composition of calves in all the groups was similar ▪ It was concluded that mustard meal after HCl treatment can be utilized as suitable substitute for soy bean meal in the diet of growing crossbred calves

Appendix 3 Feeding Studies on Canola or B. Juncea Meal Pressed Cake in Field Animals

Reference	Study Design/ Objective	Species & Number/Sex	Test Substance	Dose	NOAEL/ LOAEL*	Duration	Results
Tripathi, et al. 2001b	Effect of untreated mustard (Brassica juncea) HCl-treated or copper or iodine supplemented meal on nutrient utilization, liver enzymes, thyroid hormones and growth	<ul style="list-style-type: none"> ▪ Male crossbred calves ▪ 24 in number ▪ 230±15.4 days age ▪ 86.6±2.73 body weight 	Treated or untreated <i>B. juncea</i> and soy bean meal	282.8g crude protein/kg of meal	<p>8952 mg/kg.bw/day</p> <p>Daily feed of a calf in the untreated <i>B. juncea</i> group is 4.03 kg</p> <p><i>NOAEL is based on following assumptions:</i></p> <p>Average weight of calf ~126 kg. This is based on follow:</p>	25 weeks	<ul style="list-style-type: none"> ▪ Average daily gain of calves fed with HCl-treated mustard meal, mustard meal with copper sulfate and potassium iodide, and soy bean meal diets was similar but higher than in calves fed untreated mustard meal. ▪ Treatment with mustard meal with HCl and supplemented with Cu and I increased crude protein and metabolized energy intake, as well as digestibility of nutrients, versus calves fed with untreated mustard meal ▪ Inclusion of CuSO₄ and KI to the mustard meal improved calf growth ▪ Treatment with mustard meal with HCl improved metabolized energy intake, higher digestibility of nutrients, higher serum protein levels, lower levels of liver enzymes, and higher levels of thyroid hormones, as well as higher growth rate with negligible effects on calf performance ▪ It was concluded that mustard meal treated with HCl or untreated mustard meal with Copper and KI can replace effectively soybean diets of calves

580000

Appendix 3 Feeding Studies on Canola or B. Juncea Meal Pressed Cake in Field Animals

Reference	Study Design/ Objective	Species & Number/Sex	Test Substance	Dose	NOAEL/ LOAEL*	Duration	Results
Tripathi, et al. 2001c	Effect of soy bean meal with mustard meal on intake, digestibility, growth performance and body composition	<ul style="list-style-type: none"> ▪ Male crossbred calves ▪ 24 in number ▪ 240±15.4 days age ▪ 87±2.52 body weight 	Treated or untreated <i>B. juncea</i> meal and soy bean meal	459 g crude protein/kg dry matter	<p>18260 mg/kg.bw/day</p> <p>Daily feed of a calf in the untreated <i>B. juncea</i> group is 4.13 kg</p> <p><i>NOAEL is based on following assumptions:</i></p> <p>Average weight of a calf ~ 104kg. This is based on follow:</p> <p>Weight of calves at start of experiment - ~87kg and end of experiment is 120 kg (Average daily gain was 194g times 168 days=32.59kg)</p> <p>Or 87+120=207kg Or 207/2=103.5 kg is the average weight of a calf.</p>	16 weeks	<ul style="list-style-type: none"> ▪ Differences among treatment groups in dry matter intake of oat hay and in total dry matter intake as a percent of body weight favored the soy bean meal diet, whereas acid-treated mustard meal and untreated mustard meal-fed calves had similar dry matter intake ▪ The fed conversion ratio was lowest and growth rate was highest in calves fed treated mustard meal diets ▪ Serum albumin was lowest in calves fed the untreated mustard meal diet ▪ It was concluded that acid treatment mustard meal can effectively replace soy bean meal as a protein source without substantive detrimental effects on overall calf performance and has beneficial effects on performance of growing calves compared to untreated mustard meal

Appendix 3 Feeding Studies on Canola or B. Juncea Meal Pressed Cake in Field Animals

Reference	Study Design/ Objective	Species & Number/Sex	Test Substance	Dose	NOAEL/ LOAEL*	Duration	Results
Grover, et al. 2002	Hypoglycemic and antihyperglycemic effect of <i>B. juncea</i> diet and their effect on hepatic glycogen content and the key enzymes of carbohydrate metabolism	<ul style="list-style-type: none"> ▪ Male and female Albino rats ▪ 190-220 g weight 	<i>B. juncea</i> diet	5, 10 and 15%	Not determined since amount of crude protein in seeds not provided	7 days and 5 weeks	<ul style="list-style-type: none"> ▪ <i>B. juncea</i> diet (10 and 15%) showed significant antihyperglycemic effect in alloxan but not in streptozotocin-induced diabetes Albino rats ▪ <i>B. juncea</i> diet failed to modulate the hepatic glycogen content and enzyme activities ▪ It was concluded that <i>B. juncea</i> diet can be of use in the management of pre-diabetic state or moderate diabetes. However, for control of severe diabetes it is not of much use.

000087

Appendix 3 Feeding Studies on Canola or B. Juncea Meal Pressed Cake in Field Animals

Reference	Study Design/ Objective	Species & Number/Sex	Test Substance	Dose	NOAEL/ LOAEL*	Duration	Results
Kumar, et al. 2002	Mustard cake as a source of dietary protein for growing lambs	<ul style="list-style-type: none"> ▪ Cross bred male lambs ▪ 18 in number ▪ 6-7 months of age ▪ Body weight 12.8±0.48 kg 	Mustard cake meal replaced at 50 and 100% control meal	18% crude protein	<p><u>8848 mg/kg.bw/day</u></p> <p>Daily feed of lambs in the <i>B. juncea</i> group 934 g</p> <p><i>NOAEL is based on following assumptions:</i></p> <p>Average weight of pig ~ 19 kg. This is based on follow:</p> <p>Weight of lambs at start of experiment - 12.8 kg and end of experiment was 24.3 kg</p> <p>Or $12.8+24.3=37.1\text{kg}$ Or $37.1/2=18.5\text{ kg}$ is the average weight of lamb.</p>	120 days	<ul style="list-style-type: none"> ▪ The total dry matter did not differ among the experimental and control groups ▪ No significant body weight gain was observed, the animals fed on 50% mustard meal gained more weight as compared to 100% and control group ▪ It was concluded that peanut cake may completely be replaced with mustard cake without effecting feed intake, feed efficiency, nitrogen balance, mineral balance and growth performance of growing lambs

880000

Appendix 3 Feeding Studies on Canola or B. Juncea Meal Pressed Cake in Field Animals

Reference	Study Design/ Objective	Species & Number/Sex	Test Substance	Dose	NOAEL/ LOAEL*	Duration	Results
Grover, et al. 2003	Oral feeding study on kidney function and glucose levels	<ul style="list-style-type: none"> ▪ Streptozotocin Diabetic mice ▪ 30-50g ▪ Both sexes 	<i>B. juncea</i> (BJ) seed powder and <i>Murraya koeingii</i> (MK) leaves	<ul style="list-style-type: none"> ▪ 10% of <i>B. juncea</i> seed powder ▪ 15% of powdered leaves of <i>Murraya koeingii</i> 	Not determined since amount of crude protein in seeds not provided	60 days	<ul style="list-style-type: none"> ▪ Urine volume per day and urinary albumin was significantly higher in diabetic control group as compared to normal control group ▪ Feeding of the BJ/MK showed a trend towards improvement in most of the clinical and biochemical variables, results were not statistically different from the diabetic control group except for the serum creatinine values in BJ-fed rats on day 70 ▪ The diet is suggested as a preferable food adjuvant for diabetic patients

680000

Appendix 3 Feeding Studies on Canola or B. Juncea Meal Pressed Cake in Field Animals

Reference	Study Design/ Objective	Species & Number/Sex	Test Substance	Dose	NOAEL/ LOAEL*	Duration	Results
Sen and Bhattacharyya , 2003	Nutritional effects of <i>B. juncea</i> seed protein on growing rats	<ul style="list-style-type: none"> ▪ Male albino rats ▪ 60±2 g 	<i>B. juncea</i> protein rich fraction and casein	Mustard seed-26% protein; mustard seed protein rich fraction- 78% of the diet	Adequate data not available for calculating NOAEL/LOAEL values	28 days	<ul style="list-style-type: none"> ▪ Mustard seed extracted enzymatically with cellulose in presence of hexane gave a product with reduced levels of undesirable factors which could prove beneficial from nutritional point of view ▪ Liver lipid concentration was lower in the protein rich fraction-fed rats than that of the casein diet group ▪ Growth, protein efficiency ratio, serum lipid and protein concentration and organ weight between the two groups fed casein and mustard seeds protein fraction were comparable suggesting that mustard seed protein fraction was comparable with casein

060000

Appendix 3 Feeding Studies on Canola or B. Juncea Meal Pressed Cake in Field Animals

Reference	Study Design/ Objective	Species & Number/Sex	Test Substance	Dose	NOAEL/ LOAEL*	Duration	Results
Das and Singhal, 2005	Effects of feeding chemically treated mustard cake on growth, thyroid and liver functions and carcass characteristics in kids	<ul style="list-style-type: none"> ▪ Cross bred male kids ▪ 4.5 months ▪ 11.7 kg body weight 	Treated and untreated mustard cake	19.6 % of crude protein	<p><u>5488 mg/kg.bw/day</u></p> <p>Daily feed of kids in the untreated <i>B. juncea</i> group 0.42 kg (Total DM intake 47.4kg/week or 0.42/day)</p> <p><i>NOAEL is based on following assumptions:</i></p> <p>Average weight of kid ~ 15 kg. This is based on follow:</p> <p>Weight of kids at start of experiment - 11.6 kg and end of experiment was 17.3 kg</p> <p>Or $11.6+17.3=28.9\text{kg}$ Or $28.9/2=14.5\text{ kg}$ is the average weight of a kid.</p>	13 week	<ul style="list-style-type: none"> ▪ Palatability of treated mustard cake based concentrate mixture was higher than the untreated mustard cake ▪ Total in take of concentrate mixture did not vary among both groups ▪ The average body weight gain was similar among both groups ▪ No evidence of inflammation, hemorrhage or malignant pathology was observed in tissues of both groups ▪ The clinical and biochemical variables remained similar in both groups ▪ It was concluded that despite the reduction in glucosinolate content by chemical treatment of mustard cake, its feeding as sole protein source did not improve performance over untreated mustard cake

Appendix 3 Feeding Studies on Canola or B. Juncea Meal Pressed Cake in Field Animals

Reference	Study Design/ Objective	Species & Number/Sex	Test Substance	Dose	NOAEL/ LOAEL*	Duration	Results
Tripathi, et al. 2008	Effect on caecal fermentation characteristics, blood composition and growth by administration of mustard meal	<ul style="list-style-type: none"> ▪ Soviet Chinchilla and White Giant breed weaning rabbits ▪ 40 in number 	Mustard meal (<i>B. juncea</i>) and soy bean meal diet	376 g/kg crude protein; 0, 80 160 and 245 g/kg mustard meal	<p><u>21147 mg/kg.bw/day</u></p> <p>Daily feed of rabbits in the highest <i>B. juncea</i> group 128 g</p> <p><i>NOAEL is based on following assumptions:</i></p> <p>Average weight of kid ~ 2326g. This is based on follow assumptions:</p> <p>Weight of rabbit at start of experiment - 314g and end of experiment will be 2326 based on average daily weight gain of 27g. The average weight gain in 56 days will be 1512. Or 314+1512=4652 Or 4652/2=2326g</p>	8 weeks	<ul style="list-style-type: none"> ▪ Mustard meal –incorporated diets had higher digestibility and linearly, higher metabolizable energy content than control group having soy bean diet ▪ Average daily gain (ADG) reduced linearly with increasing mustard meal level in diet, still 80 and 160 g mustard meal diets had similar ADG compared to that of soy bean meal ▪ Caecum weight reduced linearly with increasing mustard meal levels in diet ▪ Blood hemoglobin, packed cell volume and lymphocytes were higher on 245 mustard meal diets, whereas white blood cell count reduced linearly ▪ Serum liver enzymes increased linearly while other biochemical variables were not influenced by mustard meal diet ▪ It was concluded that rabbits can replace up to 66% soy bean protein in rabbit feeding, whereas complete replacement of soy bean diet with mustard diet reduced feed intake and ADG by 23% and 13%, respectively

Appendix 3 Feeding Studies on Canola or B. Juncea Meal Pressed Cake in Field Animals

Reference	Study Design/ Objective	Species & Number/Sex	Test Substance	Dose	NOAEL/ LOAEL*	Duration	Results
Ravichandran, et al. 2008	Comparative assessment of soybean meal with high and low glucosinolate rapeseed mustard cake as protein supplement on performance of growing crossbred calves	<ul style="list-style-type: none"> ▪ Male calves ▪ Weight 62.9±3.8 kg 	Soy bean, low glucosinolat e <i>B. napus</i> and high glucosinolat e <i>B. juncea</i> diets	20% crude protein	<p><u>4880 mg/kg.bw/day</u></p> <p>Daily feed of a calf in the high 1.83 kg glucosinolate group is kg</p> <p><i>NOAEL is based on following assumptions:</i></p> <p>Average weight of a calf ~ 75 kg. This is based on follow:</p> <p>Weight of calves at start of experiment - ~64kg and end of experiment is 86kg</p> <p>Or 64+86=150kg Or 150/2=75 kg is the average weight of a calf.</p>	120 days	<ul style="list-style-type: none"> ▪ Despite genetic variability in the gluconinolates contents of various diets it had no adverse effect on nutrient utilization in growing calves ▪ Nutrient digestibility and density and balances of nitrogen, calcium and phosphorous by calves did not differ significantly among groups ▪ Average daily gain was significantly lower in calves fed high gluconinolate supplement as compared to other groups ▪ All the other biochemical variables remained within the normal levels in all the three groups

APPENDIX 4
Allergenicity Assessment of Canola
Protein Isolates and Concentrates

000095

REPORT: Analysis of the potential allergenicity of canola protein isolates and concentrates using *Brassica juncea* and *Brassica napus* as source materials.

Report prepared by Joe Baumert, Food Allergy Research & Resource Program (FARRP), December, 2009.

I. Purpose of the Report

Food allergies affect an estimated 3.5-4.0% of the total population in the United States (Sicherer et al., 2004). The symptoms associated with food allergies can range from relatively mild and transitory reactions to severe and life-threatening anaphylactic reactions (Sampson, 2005). The prevalence of food allergies, especially among children under the age of 18, has increased in the United States over the past 5-10 years (Sicherer et al., 2002; Sicherer et al., 2003; Branum et al., 2008). As a result of the apparent increase in the prevalence of food allergies and greater awareness of food allergies, novel foods and food ingredients are often subjected to pre-market allergenicity assessment (Poulsen et al., 2004; Goodman et al., 2007). Appropriate labeling strategies should be used if there is any indication that a newly introduced food ingredient might pose a risk to a sensitive group of consumers (Hefle and Taylor, 2004).

The purpose of this report is to assess the potential allergenicity of three food ingredients (Advantaxx 70™: Canola Protein Concentrate; Isolexx™: Canola Protein Isolate; Vitalexx™: Hydrolyzed Protein Isolate) derived from canola (*Brassica juncea* and *Brassica napus*). This assessment will be included as part of the self-affirmation process to establish the food ingredients as generally recognized as safe (GRAS) ingredients as outlined by the United States Food and Drug Administration (21 CFR 170.30(b)). The focus of this assessment is two fold: (1) to evaluate whether proteins contained in the isolates and concentrates could potentially cross-react with known allergens from other sources due to the similarities of sequence/structure, and (2) to evaluate whether the presence of more concentrated products containing mustard-like allergens (such as BioExx protein isolates and concentrates in food) would increase the prevalence of mustard allergy in the population.

II. Classification of *Brassica* Species

The family of Brassicaceae (formally known as Cruciferae) contains approximately 300 genera and 3000 species that grow in temperate and tropical regions throughout the world (Weiss, 2002). The genus *Brassica* contains over 160 species including some valuable edible crops and oilseeds

(radish, rutabaga, cabbage, cauliflower, broccoli, brussel sprouts, turnip, watercress, horseradish, mustard, and rapeseed) (Rance, 2003; Figueroa et al., 2005).

There are three main types of mustard seeds produced worldwide: pale yellow or white mustard (*Sinapis alba*, formally classified as *Brassica alba*), black mustard (*Brassica nigra*), and brown or oriental mustard (*Brassica juncea*). The *Brassica* and *Sinapis* genera share close botanical lineage but have subtle physiological differences observed in the leaves, petals, and fruit bristles. It should also be noted that *Brassica juncea* is referred to as oilseed rape in some regions of the world; however, it is one of the predominant mustard varieties, along with yellow mustard, used in the United States, Canada, and Europe for edible mustard products. Oilseed rape (or rapeseed) includes the species of *Brassica napus* and *Brassica rapa* (also known as *Brassica campestris*). Canola is an oilseed rape variant that was conventionally bred to contain low levels of erucic acid and glucosinolates in the seeds.

III. Known Food Allergens of *Brassica* Species

Much of the allergen assessment for this report is focused on *Brassica juncea* and *Brassica napus* as these are the two source materials used to make the canola protein isolate and concentrate products. Additional classified allergens from the *Brassicaceae* family will be briefly noted.

Aero and contact allergens from turnip (*Brassica rapa*; Bra r 2) have been characterized as well as a lipid transfer protein from cabbage (*Brassica oleracea*; Bra o 3). Bra o 3 has been shown to inhibit IgE binding in CAP inhibition assays to mugwort pollen, broccoli, and peach indicating that there is potential for cross-reactivity. Clinical cross-reactivity between most *Brassicaceae* species has not been documented, with the exception of mustard and rapeseed (Rance, 2003).

Allergenic food proteins in mustard have been identified and characterized. These include *Sin a 1* and *Bra j 1* which are 2S albumin seed storage proteins from *Sinapis alba* and *Brassica juncea*, respectively (Menedez-Arias et al., 1988; Monsalve et al., 1993). These 2S albumin proteins share 80% sequence identity and have been shown to share a homologous IgE-binding epitope indicating that individuals that are known to be sensitive to one species of mustard are likely to show sensitivity to other species as indicated in IgE immunoblotting experiments (Monsalve et al., 1993). *Sin a 1* is a basic, low molecular weight protein (14 kDa) composed of two polypeptide chains of 39 and 88 amino acids. The heavy chain and light chain are linked by two disulfide bonds (Menedez-Arias et al., 1988). *Bra j 1* (14 kDa in size) is also composed of two polypeptide chains of 37 and 92 amino acids linked by two disulfide bonds (Monsalve et al.,

1993). Proteins of this class have been shown to be highly resistant to proteolytic and thermal denaturation which may allow the 2S albumins to resist digestion and interact with the immune system for longer periods of time (Astwood et al., 1996; Moreno et al., 2008). An allergenic 2S albumin (napin protein) has also been characterized from *Brassica napus* (Bra n 1) and in vitro cross-reactivity between Bra n 1 and Sin a 1 has been described in the literature (Monsalve et al., 1991; Asero et al., 2002).

Palomares et al. (2005) identified an additional novel allergen from *Sinapis alba* (Sin a 2) which is an 11S globulin of approximately 51 kDa in size under non-reducing conditions. Upon reduction, two subunits of 36 and 23 kDa can be found. Sera from nine of the thirteen (69%) mustard allergic individuals showed significant IgE reactivity to Sin a 2 (Palomares et al., 2005; Palomare et al., 2007). Allergenic 11S globulins have not been identified and characterized from *Brassica juncea* or *Brassica napus* to date.

IV. Protein Characterization of Canola Protein Isolate and Concentrate Samples

BioExx conducted a sedimentation velocity analysis of the three protein samples derived from *B. juncea* --Advantaxx 70™: Canola Protein Concentrate; Isolexx™: Canola Protein Isolate; and Vitalexx™: Hydrolyzed Protein Concentrate. (Reported by John Philo, Report # POS102609). Since the BioExx proteins analyzed are from a *B. juncea* cultivar we expect that these isolates and concentrates will contain the identical or very similar allergenic protein sequences found in other *B. juncea* cultivars. However, sedimentation analysis cannot reveal these precise sequences because this technique is not as precise as other techniques and it is likely that several proteins in the extract will have similar sedimentation velocities. Sedimentation velocity analysis does, however, reveal the relative amounts of different sedimentation groups (i.e., 2S, 7S, and 11S) present in the concentrate and isolate products. These studies show that the 2S, 7S, and 11S proteins are the major proteins present in the (unhydrolyzed) BioExx *B. juncea* products.

Rapeseed protein meal (*Brassica napus*) has been shown to contain two predominant classes of seed storage proteins: 11S/12S globulin (cruciferin) which represents 25-65% of the protein content and 2S albumin (napin) (Berot et al., 2005). Cruciferin (a member of the 11S globulin family) is composed of hexamers with a mean molecular mass of 300 kDa. Each of the six subunits dissociates at extreme pH into six subunits composed of two polypeptide chains of approximately 30 and 20 kDa linked by a disulfide bond (Berot et al., 2005). The napin proteins belong to the 2S albumin storage proteins and have an estimated molecular mass of 12-14 kDa. Similar to the 2S albumins in mustard, the napins from rapeseed are comprised of two polypeptide chains (4.5 and 10 kDa in size), held together by two disulfide bonds.

The canola protein isolates and concentrates contain proteins from both *Brassica napus* and *Brassica juncea*. The canola protein concentrate (Advantaxx 70™) contained approximately 52% 2S proteins, 37% 11S proteins, and 1% 7S proteins according to the sedimentation velocity analysis. The canola protein isolate (Isolexx™) contained approximately 28% 2S proteins, 57% 11S proteins, and 3% 7S proteins.

The Vitalex product is derived from enzyme hydrolysis of the source proteins. Enzyme hydrolysis can be less predictable than acid hydrolysis due to environmental factors that can affect the enzyme activity rates so there can be peptide fragments of larger sizes that could maintain their allergenicity. This may confound, in this case, the recognized paradigm that if a protein is hydrolyzed it is unlikely to be allergenic. We commonly refer to the Food Chemical Codex (5th edition) specification for acid hydrolysis of protein when conducting a risk assessment of the potential allergenicity of hydrolyzed protein ingredients from commonly allergenic sources such as soy, wheat, and milk. Acid hydrolysates with a degree of hydrolysis of >62% (alpha-amino/total nitrogen ration) have generally not been shown to be allergenic to consumers with allergies to the source material. This guideline has been used by infant formula manufacturers for years that are making hypoallergenic formulas from casein and these formulas have been shown to be safe for the vast majority of infants.

V. Protein Bioinformatics/Sequence Search

We conducted a bioinformatics search to compare the known sequences of the *Brassica juncea* 2S albumin protein, and *Brassica napus* 2S and 11S proteins. Previously published sequences of these proteins were compared to known and putative allergenic proteins (1386 sequence entries) listed in AllergenOnline.com version 9.0 database. A FASTA (version 35.04) search using the default search and scoring criteria of Pearson (2000) was performed. The default scoring matrix is BLOSUM 50 (Henikoff and Henikoff, 1996). Statistical values are calculated for each search, compared to expected values, and sequence alignments are indicated in the output. Expectation values (E values) indicate the probable evolutionary homology and structural similarity. Distantly related sequences that do not indicate significant similarity but may be considered related evolutionarily (share a common ancestor or share a common three-dimensional structure) will generally have E values of less than 0.02. An E value of 0.02 does not mean that the overall structures are sufficiently similar that IgE antibodies from individuals allergic to one protein will recognize the other protein. Highly similar sequences that probably represent close homology generally have E values of 1e-7 or less. Proteins with E values greater than 1 e-7 may share some degree of amino acid sequence homology but are not likely to share immunologic or allergenic cross-reactivity whereas E values less than 1 e-30 are much more likely to be cross-

reactive in at least some individuals (Hileman et al., 2002). If the E value indicates that close homology exists, the percent identity over the length of the intact protein is evaluated. Since E values depend to a great degree on the scoring matrix, the size of the database used for the search comparison and many other factors, E values are not commonly used as the only value for the evaluation and interpretation of immunological significance. A more common comparison is the percent identity. A query (search) protein sharing greater than 70% identity over its length relative to a known allergen is likely to be cross-reactive or share IgE binding. Those that have less than 50% identity are not likely to be cross-reactive (Aalberse, 2000).

A BLAST search (NCBI Entrez) was used to compare each query protein sequence against the entire Entrez Protein database, with a limit option selected to query entries for “*allergen*” so that comparisons were made only to proteins identified as allergens. This was done to ensure that the query proteins were compared against newly discovered allergens that have not been entered into the AllergenOnline database. The same criteria as outlined for determining potential cross-reactivity to a known allergen were used in this search also.

VI. Bioinformatics Search Results

The FASTA search of AllergenOnline version 9.0 was used to compare the potential sequential and inferred structural similarity of the Bra j 1 (from *B. juncea*) and Bra n 1 (from *B. napus*) to all known and putative allergenic proteins (1386 sequence entries) in the AllergenOnline database. Bra j 1 (GI: 32363444) was found to share significant identity (>70% identity) to Bra n 1 (GI: 75107016) and Sin a 1 (GI: 51338758) with 89 and 80%, respectively (Appendix A). Additionally, Bra n 1 (GI: 75107016) shared 83% identity to Sin a 1 (GI: 51338758) (Appendix B). A number of Sin a 1 isotopes showed similar sequence identity to both Bra j 1 and Bra n 1 with approximate identity of 80% (data not shown). No other known allergens were shown to share significant identity to these 2S albumins.

The 509 amino acid cruciferin CRU1 protein (11/12S protein) of *Brassica napus* (GI: 461840) was used as the query sequence in the FASTA search of the AllergenOnline database. This protein is likely a major component of the canola protein isolate and concentrate products as indicated in previous work conducted by Berot et al. (2005). Although this protein has not been reported to be a known food allergen, it is important to identify any significant sequence homology that it may have with known food allergens. The *Brassica napus* cruciferin CRU1 protein shares approximately 92% identity with the known 11S globulin allergen of *Sinapis alba* (Appendix C). This indicates likely cross-reactivity or IgE binding between these two proteins. Clinical cross-reactivity between these two proteins has not been reported to date. Serum testing

and probably clinical testing would be required to verify actual clinically significant cross-reactivity.

In summary, there is a very strong sequence homology between all of the tested 2S proteins in *B. juncea*, *B. napus* and *S. alba*. There is also a strong sequence homology between the cruciferin 11/12S protein in *B. napus* and the known 11S protein in *S. alba*. These findings imply that sufficient amino acid sequence homology exists between these proteins and they are likely to share immunologic or allergic cross reactivity with one another.

VII. Cross-Reactivity with Other Major Food Allergens

The *Brassica* proteins Bra j 1, Bra n 1, and the 11S cruciferin protein of *B. napus* do not share significant sequence identity to known allergens of the top eight major allergens (cow's milk, egg, peanut, soybean, wheat, tree nuts, fish, and crustacean shellfish). Appendix D outlines the sequence identity of the *Brassica* proteins to seed storage proteins of major food allergens using the FASTA search of AllergenOnline. Minor sequence identity was observed to known seed storage proteins/allergens from walnut, Brazil nut, cashew, wheat, peanut, and soybean; however, in all cases the sequence identity was less than 50%, indicating that these proteins are not likely to be cross-reactive with the *Brassica* proteins. Additionally, no clinical cross-reactivity between proteins of the *Brassica* family and other protein families of the Big 8 allergens has been reported to date. As expected, no sequence identity to milk, egg, fish, or shellfish was observed as proteins from animal sources do not typically share sequence identity to seed storage proteins.

VIII. Mustard Allergy and Prevalence Rates

Several clinical case reports of mustard allergy have been described in the literature. A majority of these reports have originated in European countries (Dannake and White, 1987; Jorro et al., 1995; Kanny et al., 1995; Kavli and Moseng, 1987; Malet et al., 1993; Meding, 1985; Monreal et al., 1992; Panconesi et al., 1980; Vidal et al., 1991; Valescchi et al., 2000; Windström and Johansson, 1986). Only two clinical reports of allergy to mustard have been reported in North America, making mustard allergy quite rare in Canada and the United States. Yip and Zimmerman (1999) reported 5 cases of mustard allergy in children who experience moderate to severe reactions, 2 of the subjects reported laryngeal edema or anaphylaxis that required emergency medical attention. Connors et al. (2006) reported a single case involving a 50 year-old woman with a history of anaphylactic reactions upon exposure to mustard. Reactions associated with allergy to mustard have been reported to range from mild and transitory symptoms to severe and life-threatening anaphylaxis. Two blinded oral food challenge studies

have been conducted by clinical allergists in France and Spain. Figureroa et al. (2005) conducted a double-blind, placebo-controlled food challenge (DBPCFC) using *Sinapis alba* mustard. Thirty-eight mustard allergic subjects with a clinical history of allergic reactions upon consumption of mustard and positive skin prick tests to mustard were recruited for this study. Fourteen of these 38 subjects did not undergo the DBPCFC because of either a history of severe symptoms (n = 4) or because of denial of consent. Twenty-four subjects were challenged. Fourteen of the 24 challenged subjects (58%) showed a positive reaction upon oral challenge with mustard. Ten of these subjects experienced mild, subjective symptoms such as oral allergy syndrome (OAS). Two subjects had moderate to severe reactions with one subject showing symptoms of bronchial asthma and another developed systemic anaphylaxis. The lowest eliciting dose in the most severe case was 156 mg mustard sauce. The most sensitive subject reacted with mild, subjective symptoms at a dose of 44 mg of mustard sauce while the mean cumulative dose for the group was 891±855 mg of mustard sauce (equivalent to 125 mg±119 mg of mustard seeds). Morisset et al. (2003) also conducted a DBPCFC with 24 subjects being challenge with *Brassica juncea* mustard. All of these subjects were selected to be included in the DBPCFC on the basis of a positive skin prick test to mustard (*B. juncea*). Seven out of the 24 subjects had a positive reaction upon oral challenge with mustard. Symptoms in this study were mild to moderate including eczema, urticaria, rhinitis, abdominal pain, diarrhea, and wheezing. The lowest eliciting doses were noted at 40 mg and 440 mg of mustard seasoning. These subjects experienced mild reactions such as urticaria and rhinitis.

The prevalence of mustard allergen is not known for many countries in the world. Most of the prevalence estimates have come from researchers in France where it is estimated that mustard allergy is the third or fourth most common food allergy and the most common spice allergy (Rance et al., 2001). The European Union mandates the declaration of mustard on the labels of pre-packaged foods regardless of the amount (Commission Directive 2007/68/EC, November 27, 2007). The presence of mustard in formulated products can be difficult to identify when it appears labeled as “spices” or “flavorings.” This may be one of the contributing factors for the decision by Health Canada which is also finalizing legislation to require the declaration of mustard on packaged food products. Health Canada has defined mustard to include white or yellow mustard (*Sinapis alba*), brown mustard (*Brassica juncea*), and black mustard (*Brassic nigra*).

X. Discussion

The canola protein isolates and concentrates (Advantaxx 70™: Canola Protein Concentrate; Isolexx™: Canola Protein Isolate; Vitalexx™: Hydrolyzed Protein Isolate) are derived from *Brassica napus* (rapeseed) and *Brassica juncea* (mustard). Both of these contain known 2S

albumin seed storage proteins that are considered to be food allergens (Bra n 1 and Bra j 1). These allergens show considerable amino acid sequence identity to the 2S albumin allergen of *Sinapis alba* (Sin a 1) of mustard. These proteins are not novel food proteins in the human diet however. *Sinapis alba* and *Brassica juncea* are the two most common sources of mustard seed used in cooking and food processing in Canada and the United States where the prevalence of mustard allergy remains quite rare.

The prevalence rates of both soybean allergy and milk allergy have not been shown to increase dramatically as a result of the use of concentrate and isolate products from these sources in finished food products. The proposed levels (0.5 – 10%) to be used in finished products and the intended uses for the canola protein concentrates and isolates are quite similar to the levels and uses of whey protein isolates and dairy product solids which obtained GRAS notification by the FDA in 2000 (GRN No. 37).

Since this product contains known allergens from *Brassica napus* and *Brassica juncea* which show considerable sequence homology to known allergens from *Sinapis alba*, it is likely in my expert opinion that individuals who are allergic to mustard will also be react upon consumption of products containing rapeseed/canola protein. Source labeling of products containing these isolates and concentrates should be considered (i.e. contains canola and mustard protein isolates or concentrates) to alert mustard allergic individuals. These isolates and concentrates should not pose any additional allergenic risk as cross-reactivity between mustard/rapeseed and other foods is not known to occur and has not been documented.

XI. Conclusion

Mustard protein is a proven food allergen. However, it is not one of the major allergens in the U.S. and it is apparent that the prevalence of mustard allergy is low in the U.S. although it is significantly higher in Europe. One purpose of this report was to try to determine whether an increase in the exposure to the proteins from the BioExx products would increase the prevalence rate of mustard type allergy. The second purpose was to determine whether there would be any potential for significant cross-reactivity between the allergens in the BioExx proteins and the top eight major food allergens in the U.S.

Potential increased prevalence

It is anticipated that BioExx products will be used as protein sources in processed food and that this use will expose some consumers to higher levels of the *B. juncea* allergens (2S proteins) than they would otherwise be exposed to through consumption of mustard as a condiment or

levels typically used in flavoring or spice blends used in some processed products. There is a concern that this increased exposure might induce mustard-like allergy in people who tolerate, without affect, the lower exposures to common 2S mustard (*B. juncea*) protein allergens. As noted above, previous experience with the introduction of protein isolate and concentrate products into the human diet from known allergenic sources such as soy protein concentrates and isolates, whey protein concentrates and isolates, and milk protein did not increase the sensitization of humans. Although it is impossible to prove that an increase in sensitization will not occur in this case, since increased prevalence did not occur in the cited similar instances we consider it unlikely that the additional exposure to mustard protein will increase the low incidence of mustard allergy.

Possible cross-reactivity with the major food allergens

As discussed above, the Brassica proteins Bra j 1, Bra n 1, and the 11S cruciferin protein of *B. napus* do not share significant sequence identity to the top eight major food allergens in the U.S. (milk, egg, peanut, soybean, wheat, tree nuts, fish, and crustacean shellfish). As expected, no sequence identity to milk, egg, fish or shellfish was observed as proteins from animal sources do not typically share sequence identity to seed storage proteins. The absence of sequence homology between mustard protein and the top eight allergens in the U.S. makes it unlikely that there will be any cross-reactivity between the eight major allergens and the BioExx protein products.

Appendix D. Sequence identity of *Brassica juncea* and *Brassica napus* proteins to major allergens (Big 8).

Brassica juncea 2S Albumin Allergen (Bra j 1):

Species	Common Name	Allergen	% Sequence Identity to Bra j 1	Expectation Value (E Value)
<i>Juglans regia</i>	English Walnut	Jug r 1	50.00%	1.3 e-008
<i>Juglans nigra</i>	Black Walnut	Jug n 1	32.40%	e 0.0063
<i>Bertholletia excelsa</i>	Brazil Nut	Ber e 1	28.70%	e 0.012
<i>Anacardium occidentale</i>	Cashew	Ana o 3	32.50%	e 0.16
<i>Triticum aestivum</i>	Wheat	gliadin	32.40%	0.34
<i>Arachis hypogaea</i>	Peanut	Ara h 2.02	NR*	e 1.7
<i>Arachis hypogaea</i>	Peanut	Ara h 6	NR	e 2.5
<i>Glycine max</i>	Soybean	β -conglycinin	NR	e 5.8

Brassica napus 2S Albumin Allergen (Bra n 1):

Species	Common Name	Allergen	% Sequence Identity to Bra n 1	Expectation Value (E Value)
<i>Juglans regia</i>	English Walnut	Jug r 1	30.60%	0.00037
<i>Juglans nigra</i>	Black Walnut	Jug n 1	32.40%	e 0.00022
<i>Bertholletia excelsa</i>	Brazil Nut	Ber e 1	30.60%	e 0.0033
<i>Anacardium occidentale</i>	Cashew	Ana o 3	34.80%	e 0.0093
<i>Triticum aestivum</i>	Wheat	gliadin	31.50%	0.24
<i>Arachis hypogaea</i>	Peanut	Ara h 6	25%	e 0.27
<i>Arachis hypogaea</i>	Peanut	Ara h 2.02	NR*	e 2.2

Brassica napus cruciferin (11S globulin):

Species	Common Name	Allergen	% Sequence Identity to <i>Brassica napus</i> cruciferin	Expectation Value (E Value)
<i>Juglans regia</i>	English Walnut	Jug r 4	44.50%	3 e -039
<i>Bertholletia excelsa</i>	Brazil Nut	Ber e 2	39.70%	1.5 e 036
<i>Anacardium occidentale</i>	Cashew	Ana o 2	46.20%	6.9 e -039
<i>Corylus avellana</i>	Hazelnut	Cor a 9	45.60%	4.2 e -038
<i>Glycine max</i>	Soybean	Glycinin G1	37.10%	1.1 e -028
<i>Glycine max</i>	Soybean	Glycinin G2	35.40%	4.1 e -018
<i>Glycine max</i>	Soybean	Glycinin G3	36.00%	4.2 e -024
<i>Glycine max</i>	Soybean	Glycinin G4	31.40%	6.0 e -018
<i>Arachis hypogaea</i>	Peanut	Ara h 3/4	34%	1.9 e -016

*NR - Percent identity was not reported in the FASTA full length search of AllergenOnline

**Proteins sharing >70% identity with a known allergen are likely to be cross-reactive or share IgE binding.

Proteins sharing < 50% identity with a known allergen are not likely to be cross-reactive.

References:

- Aalberse, R.C. 2000. Structural biology of allergens. *J. Allergy Clin. Immunol.* 106:228-38.
- Asero, R., Mistrello, G., Roncarbolo, D., Amoto, S. 2002. Allergenic similarities of 2S albumins. *Allergy.* 57:62-3.
- Astwood, J. D., Leach, J.N., Fuchs, R.L. 1996. Stability of food allergens to digestion in vitro. *Nat. Biotechnol.* 14:1269-73.
- Berot, S., Compoin, J.P., Larre, C., Malabat, C., Gueguen, M.J. 2005. Large scale purification of rapeseed proteins (*Brassica napus* L.). *J. Chromot. B.* 818:35-42.
- Branum, A.M., Lukacs, S.L., 2008. Food allergy among U.S. children: Trends in prevalence and hospitalizations. NCHS data brief, no 10. Hyattsville, MD: National Center for Health Statistics.
- Connors, L.A., Yang, W.H., Lacuesta, G.A. 2006. Case reports of seed anaphylaxis: mustard, flax, and sunflower seed. *J. Allergy Clin. Immunol.* 117:S52.
- Dannake, C. J., I. R. White. 1987. Cutaneous allergy to mustard in a salad maker. *Contact Derm.* 6: 212-214.
- Figuroa, J., C. Blanco, A. G. Dumpiérrez, L. Almeida, N. Ortega, R. Castillo, et. al. 2005. Mustard allergy confirmed by double-blind placebo-controlled food challenges: clinical features and cross-reactivity with mugwort pollen and plant-derived foods. *Allergy* 60:48-55.
- Goodman, R.E., Taylor, S.L., Yamamura, J., Kobayashi, T., Kawakami, H., Kruger, C.L., Thompson, G.P. 2007. Assessment of the potential allergenicity of milk basic protein fraction. *Food Chem Toxicol.* 45:1787-94.
- Hefle, S.L., Taylor, S.L. 2004. Food allergy and the food industry. *Curr. Allergy Asthma Rep.* 4:55-9.
- Henikoff, J.G., Henikoff, S. 1996. Blocks database and its applications. *Methods Enzymol.* 266:88-105.
- Hileman, R.E., Silvanovich, A., Goodman, R.E., Rice, E.A., Holleschak, G., Astwood, J.D., Hefle, S.L. 2002. Bioinformatic methods for allergenicity assessment using a comprehensive allergen database. *Int. Arch. Allergy Immunol.* 128:280-91.
- Jorro, G., Morales, C., Brasó, V.J., Peláez, A. 1995. Mustard allergy: three cases of systemic reaction to ingestion of mustard sauce. *J. Invest. Allergol. Clin. Immunol.* 5: 54-56.
- Kanny, G., Fremont, S., Talhouarne, G., Nicolas, J.P., Moneret-Vautrin, D.A. 1995. Anaphylaxis to mustard as a masked allergen in "chicken dips". *Ann. Allergy Asthma Immunol.* 75: 340-342.

- Kavli, G., Moseng, D. 1987. Contact urticaria from mustard in fish-stick production. *Contact Derm.* 17: 153-155.
- Malet, A., Valero, A., Lluch, M., Bescos, M., Amat, P., Serra, E. 1993. Hypersensitivity to mustard seed. *Allergy* 48: 62-63.
- Meding, B. 1985. Immediate hypersensitivity to mustard and rape. *Contact Derm.* 13: 121-122.
- Menendez-Aria, L., Moneo, I., Dominguez J., Rodrigues, R. 1988. Primary structure of the major allergen of yellow mustard (*Sinapis alba* L.) seed, Sin a I. *Eur J Biochem.* 177:159-66.
- Monreal, P., Botey, J., Pena, M., Marin, A., Eseverri, J.L. 1992. Mustard allergy: two anaphylactic reactions to ingestion of mustard sauce. *Ann. Allergy.* 69: 317-320.
- Monsalve, R.I., Villalba, M., Lopez-Otin, C., Rodriguez, R. 1991. Structural analysis of the small chain of the 2S, napin nIII, from rapeseed. Chemical and spectroscopic evidence of an intramolecular bond formation. *Biochem. Biophys. Acta.* 1078:265-72.
- Monsalve, R.I., Gonzalez de la Pena, M.A., Menendez-Arias, L., Lopez-Otin, C., Villalba, M., Rodriguez R. 1993. Characterization of a new oriental-mustard (*Brassica juncea*) allergen, Bra j IE: detection of an allergenic epitope. *Biochem. J.* 293:625-32.
- Moreno, F.J., Clemente, A. 2008. 2S albumin storage proteins: What makes them food allergens? *Open Biochem. J.* 2:16-28.
- Morisset, M., Moneret-Vautrin, D.A., Maadi, F., Fremont, S., Guenard, L., Croizier, A., Kanny, G. 2003. Prospective study of mustard allergy: first study with double-blind placebo-control food challenge trials. *Allergy* 58:295-299.
- Palomares, O., J. Cuesta-Herranz, A. Vereda, S. Sirvent, M. Villalba, and R. Rodríguez. 2005. Isolation and identification of an 11S globulin as a new major allergen in mustard seeds. *Ann. Allergy Asthma Immunol.* 94: 586-592.
- Palomares, O., Vereda, A., Cuesta-Herranz, J., Villalba, M., and Rodrigues, R. 2007. Cloning, sequencing, and recombinant production of Sin a 2, an allergenic 11S globulin from yellow mustard seeds. *J Allergy Clin. Immunol.* 119:1189-1196.
- Panconesi, E., Sertoli, A., Fabbri, P., Giorgini, S., Spallanzi, P. 1980. Anaphylactic shock from mustard after ingestion of pizza. *Contact Derm.* 6: 294-295.
- Pearson, W.R. 2000. Flexible sequence similarity searching with the FASTA3 program package. *Methods Mol. Biol.* 132:185-219.
- Poulsen, L.K. 2004. Allergy assessment of food or ingredients derived from biotechnology, gene-modified organisms, or novel foods. *Mol. Nutr. Food Res.* 48:413-23.

- Rance, R. Abbal, M., Dutau, G. 2001. Mustard allergy in children. *Pediat. Pulmon.* 32:44-45.
- Rance, F. 2003. Mustard as a new food allergy. *Allergy.* 58:287-88.
- Sampson, H.A. 2005. Food allergy – accurately identifying clinical reactivity. *Allergy.* 60:19-24.
- Sicherer, S.H. 2002. Food allergy. *Lancet.* 360:701-10.
- Sicherer, S.H., Munoz-Furlong, A., Sampson, H.A. 2003. Prevalence of peanut and tree nut allergy in the United States determined by means of random digit dial telephone survey: a 5-year follow-up study. *J Allergy Clin. Immunol.* 112:1203-7.
- Sicherer, S.H., Munoz-Furlong, A., Sampson, H.A. 2004. Prevalence of seafood allergy in the United States determined by a random telephone survey. *J. Allergy Clin. Immunol.* 114:159-65.
- Valescchi, R., Lechissa, P., Cortinovia, R., Cologni, L. 2002. Contact urticaria syndrome from mustard in anchovy fillet sauce. *Contact Derm.* 42: 114.
- Vidal, C., Díaz, C., Sáez, A., Rodriguez, M., Iglesias, A. 1991. Anaphylaxis to mustard. *Postgrad. Med. J.* 67: 401.
- Weiss, E. A. 2002. *Spice crops.* CABI Publishing, Wallingford, UK, pp. 23-44.
- Windström, L., Johansson, S.G.O. 1986. IgE-mediated anaphylaxis to mustard. *Acta Derm. Venereol.* 66: 70-71.
- Yip, L.Y., Zimmerman, B. 1999. Mustard allergy: Uncommon allergy with a common spice. *Canadian J. Allergy Clin. Immunol.* 4:76-78.

APPENDIX 5
GRAS Expert Panel

000113

EXPERT PANEL OPINION

On the Generally Recognized as Safe (GRAS) Status of BioExx Canola Proteins (Isolexx™ and Vitalexx™) for use as food ingredients in foods where protein is used for functional or nutritional purposes

May 16, 2011

The undersigned, an independent panel of experts, qualified by their scientific training and national and international experience to evaluate the safety of food and food ingredients (the “Expert Panel”), was specially convened by Keller and Heckman LLP, on behalf of their client BioExx Specialty Proteins, Ltd. (BioExx), and asked to evaluate the safety and “Generally Recognized As Safe” (“GRAS”) status of the proposed uses of canola proteins derived from *Brassica juncea* (*B. juncea*) and *Brassica napus* (*B. napus*) in multiple food applications. The safety evaluation focused on two canola protein products: Canola protein isolate (Isolexx™), and Hydrolyzed canola protein isolate (Vitalexx™). These canola protein products are intended for use as food ingredients in foods where protein is used for functional or nutritional purposes, for example bakery products, snack foods, beverages (including nutritional beverages), soups, dairy products, dry instant milkshake mixes and protein drinks, instant powdered nutritional beverages, processed meat products, vegetarian food products/meat analogues, and meal replacement/nutritional bars.

The Expert Panel reviewed a draft GRAS Notification Document prepared by Keller and Heckman LLP that summarized the manufacturing processes for Isolexx™ and Vitalexx™ including the safety of the substances used in manufacturing, the results of analyses of the finished products for toxigenic substances and microbial pathogens of potential concern, specifications for the products, estimated consumption of these canola protein products from the proposed food uses, nutritional considerations, and information that is available in the peer-reviewed scientific literature relating to the safety of Isolexx™ and Vitalexx™ including animal feeding trials with canola proteins, production animal feeding experience with canola meal, and allergenic potential in humans.

Following this review, the Expert Panel convened via telephone conference call, and independently, jointly, and unanimously concluded that the canola protein isolate (Isolexx™) and hydrolyzed canola protein isolate (Vitalexx™) products manufactured by BioExx Specialty Proteins, Ltd., consistent with current good manufacturing practice (cGMP) and meeting appropriate food-grade specifications, are safe for their intended use as food ingredients in foods where protein is used for functional or nutritional purposes, for example bakery products, snack

000114

foods, beverages (including nutritional beverages), soups, dairy products, dry instant milkshake mixes and protein drinks, instant powdered nutritional beverages, processed meat products, vegetarian food products/meat analogues, and meal replacement/nutritional bars. The Expert Panel further concluded that these intended uses are Generally Regarded As Safe (GRAS) based on scientific procedures. It is also the opinion of this Expert Panel that other qualified experts would concur with its conclusions.

(b) (6)

Michael W. Pariza
Professor Emeritus, Food Science
University of Wisconsin-Madison
Member, Michael W. Pariza Consulting LLC

(b) (6)

Walter H. Glinsmann, M.D.
Glinsmann Inc.
Arlington, VA

17 May 2011

(b) (6)

Joe L. Baumert, Ph.D.
Assistant Professor
Food Allergy Research and Resource Program
University of Nebraska-Lincoln

Ramos-Valle, Moraima

From: Drozen, Melvin S. [Drozen@khlaw.com]
Sent: Wednesday, June 15, 2011 11:34 PM
To: Ramos-Valle, Moraima
Cc: Pelonis, Evangelia C.
Subject: BioExx Specialty Proteins Ltd./Canola Protein GRAS Notification

Dear Ms. Ramos-Valle:

I received your voice message and understand you have spoken with Ms. Pelonis. The purpose of this email is to provide authorization for you or anyone at FDA to speak with Eve Pelonis(or other Keller and Heckman personnel) with regard to this pending GRAS notice.

Let me know if you have any questions.

Best regards.

Mel Drozen.

Melvin S. Drozen
tel: 202.434.4222 | fax 202.434.4646 | drozen@khlaw.com
1001 G Street, N.W., Suite 500 West | Washington, D.C. 20001

Keller and Heckman LLP
Serving Business through Law and Science®

Washington, D.C. | Brussels | San Francisco | Shanghai

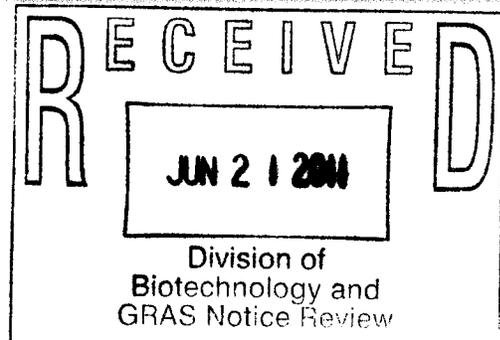
Visit our websites at www.khlaw.com or www.packaginglaw.com for additional information on Keller and Heckman.

Please consider the environment before printing this email.

This message and any attachments may be confidential and/or subject to the attorney/client privilege, IRS Circular 230 Disclosure or otherwise protected from disclosure. If you are not a designated addressee (or an authorized agent), you have received this e-mail in error, and any further use by you, including review, dissemination, distribution, copying, or disclosure, is strictly prohibited. If you are not a designated addressee (or an authorized agent), we request that you immediately notify us of this error by reply e-mail and then delete it from your system.

000116

1001 G Street, N.W.
Suite 500 West
Washington, D.C. 20001
tel. 202.434.4100
fax 202.434.4646



Writer's Direct Access
Melvin S. Drozen
(202) 434-4222
drozen@khlaw.com

June 17, 2011

Via Overnight Mail

Office of Food Additive Safety (HFS-225)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Pkwy
College Park, MD 20740

Re: GRAS Notification for Canola Protein Isolate (Isolexx[™]) and
Hydrolyzed Canola Protein Isolate (Vitalex[™])

Dear Sir or Madam:

This letter provides further information for the GRAS Notification submitted on behalf of our client BioExx Specialty Proteins, Ltd. (BioExx) for Canola Protein Isolate (Isolexx[™]) and Hydrolyzed Canola Protein Isolate (Vitalex[™]) derived from *Brassica juncea* (*B. juncea*) and *Brassica napus* (*B. napus*) for use as food ingredients.

BioExx has determined that these canola proteins are generally recognized as safe (GRAS) based on scientific procedures in accordance with 21 C.F.R. § 170.30(b) and conforms to the guidance issued by the Food and Drug Administration (FDA) under *proposed* 21 C.F.R. § 170.36, 62 Fed. Reg. 18938 (Apr. 17, 1997). The GRAS determination has also been evaluated by experts qualified by scientific training and experience to assess the safety of the canola proteins under the conditions of their intended use in food.

The analytical data, published studies, and information that are the basis for this GRAS determination are available for FDA review and copying at reasonable times at Keller and Heckman LLP, 1001 G Street, NW, Suite 500W, Washington, DC 20001 or will be sent to FDA upon request.

Sincerely,

(b) (6)

Melvin S. Drozen

000117

SUBMISSION END

000118