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Analysis of Glabrous Canary Seeds by ELISA, Mass Spectrometry, and Western Blotting for the Absence of Cross-Reactivity with Major Plant Food Allergens

Joyce Irene Boye,*[,†] Allaoua Achouri,[†] Nancy Raymond,[†] Chantal Cleroux,[‡] Dorcas Weber,[‡] Terence B. Koerner,[‡] Pierre Hucl,[§] and Carol Ann Patterson[⊥]

[†]Food Research and Development Centre, Agriculture and Agri-Food Canada, 3600 Casavant Boulevard West, Saint-Hyacinthe, Quebec, Canada

[‡]Food Research Division, Bureau of Chemical Safety, Health Canada, Ottawa, Canada

[§]Crop Development Centre, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5A8, Canada

[⊥]The Pathfinders Research and Management Limited, Saskatoon, Saskatchewan S7N 0S5, Canada

ABSTRACT: Glabrous (hairless) canary seed belongs to the Poaceae (Gramineae) family and could serve as an alternative source of gluten-free cereal grain. In this study, allergenic cross-reactivities between hairless, dehulled canary seeds (*Phalaris canariensis*) and major allergenic proteins from gluten, soy, peanuts, tree nuts, sesame, and mustard were studied using commercial enzyme-linked immune sorbent assay (ELISA) kits specific for these target allergens. Mass spectrometry (MS) and immunoblotting were further used to assess for the presence of gluten-specific protein fragments. MS results revealed the likely presence of proteins homologous with rice, oat, corn, carrot, tomato, radish, beet, and chickpea. However, no presence of celiac-related gluten fragments from wheat, rye, barley, or their derivatives was found. Immunoblotting studies yielded negative results, further confirming the absence of gluten in the canary seed samples tested. No cross-reactivities were detected between canary seeds and almond, hazelnut, mustard, peanut, sesame, soy, walnut, and gluten using ELISA.

KEYWORDS: *glabrous canary seed, gluten-free, gluten-sensitive enteropathy, celiac disease*

INTRODUCTION

Gluten-sensitive (GS) enteropathy or celiac disease (CD) is an immune-mediated food sensitivity that affects about 0.3−1% of the population.[1,2] CD is considered an autoimmune disease because of the presence of tTG-targeting autoantibodies in the serum and the intestinal mucosa of patients.[3] Symptoms of CD include anemia, gastrointestinal disorders, stunted growth, dermatitis, and endocrine disorders, among many others.[4] In practical terms the disease can be considered a spectrum disorder with some individuals showing no symptoms whereas others experience extreme debilitating symptoms. The offending proteins in CD are found in the gliadin fraction of gluten (i.e., prolamin in wheat, secalin in rye, and hordein in barley).[5−7]

To avoid symptoms and disease exacerbation, GS individuals must avoid all sources of gluten in the diet. Gluten avoidance can however be a major challenge, as cereals containing gluten (e.g., wheat, barley, rye, spelt, kamut, triticale, and their derivatives) are frequently used in food preparation (e.g., as flours in bakery products, sauces and seasonings, anticaking agents, spices, and icing sugar). This limits choice and further puts the nutritional status of CD patients at risk.

Growing awareness of CD and its impacts on health has garnered interest to identify non-gluten-containing cereal that could be safe for GS individuals. Non-gluten-containing cereals frequently used in product formulation include rice, teff, quinoa, millet, buckwheat, sorghum, maize, and amaranth. As the environmental conditions for growing these grains are variable, availability of regular supplies is not always assured.

An alternative grain that may potentially be considered for GS individuals is glabrous canary seed (*Phalaris canariensis* L.). Annual canary seed belongs to the Poaceae (Gramineae) family, the Pooideae subfamily, and the Agrostideae tribe. It is different from reed canarygrass (*Phalaris arundinacea* L.), which is a perennial forage grass of the same family and genus.[7] There are over 15 species in the *Phalaris* genus, and of the annual species of the genus, *P. canariensis* is the only one grown as a grain crop.[8] The glabrous (hairless) trait and yellow-colored grain trait were derived by mutagenesis of the certified pubescent (hairy) parent *P. canariensis,* cultivar Keet.[9]

The chemical composition of canary seed shows that it has potential as a food crop. Glabrous canary seed contains an average of 24% protein, 8% crude fat, 56% starch, and 7% total dietary fiber.[10] Phytate levels are about twice that of wheat, and levels of trypsin inhibitor and alpha-amylase inhibitor are similar to those found in other cereal grains.[11] There are also smaller amounts of soluble sugars and ash in this grain.[12] Indeed, canary seed is a richer source of most required minerals relative to wheat.[10] Baking tests show that bread made with 100% hairless canary seed flour is significantly lower in loaf volume and crust and crumb color than wheat bread. Bread

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Table 1. Enzyme-Linked Immuno Sorbent Assay (ELISA) Commercial Test Kits Used for the Assessment of Allergen Cross-Reactivity between Glabrous Canary Seed and Selected Priority Allergens

allergen	company	test kit	result expression	limit of detection/quantification (LOD)/(LOQ), ppm	range of quantification, ppm	extraction method	antibody
almonds	Gen-Probe/Tepnel	Biokit	almond	0.1 (LOD)	1−20	1 to 10 in tris(hydroxymethyl) aminomethane/sodium chloride/gelatin	polyclonal antibody specifically detects almond proteins
	Neogen	Veratox	almond	2.5 (LOQ)	2.5−25	1 to 25 in PBS solution + additive	
	R-Biopharm	Ridascreen	almond	1.7 (LOD)	2.5−20	1 to 20 in buffer solution	anti-almond antibodies
gluten	Gen-Probe/Tepnel	Biokit	gluten	1.0 (LOD)	3−50	1 to 10 in buffer solution with gluten extraction additive and 40% ethanol	monoclonal (401/21) reactive to glutenin and gliadin
	Neogen	Veratox	gliadin/gluten	5.0 (LOQ)	5−50 gliadin	1:10 in 40% ethanol, then 1:40 in PBS + additive	
	Neogen	Veratox	gliadin R5	2.5 (LOQ)	2.5−40	gliadin renaturing cocktail solution	
	R-Biopharm	Ridascreen	gliadin/gluten	3.0 (gluten) (LOD)	5−80 ppb gliadin	1 to 10 in 60% ethanol	monoclonal R5 to gliadin fractions
hazelnut	Gen-Probe/Tepnel	Biokit	hazelnut	0.1 (LOD)	1.6−32	1 to 10 in tris(hydroxymethyl) aminomethane/sodium chloride/gelatin	polyclonal antibody specifically detects hazelnut proteins
	Neogen	Veratox	hazelnut	2.5 (LOQ)	2.5−25	1 to 25 in PBS solution + additive	
	R-Biopharm	Ridascreen	hazelnut	1.5 (LOD)	2.5−20	1 to 20 in buffer solution + 1 g milk powder	
mustard	ELISA System	mustard seed protein residue	mustard seed protein	1.0 (LOQ)	1−10	1 to 10 in extraction buffer solution	anti-mustard seed protein antibodies
	Neogen	Veratox	mustard	2.5 (LOQ)	2.5−25	1 to 25 in PBS solution + additive	
	Sedium R&D	ELISA kit	mustard	0.06 (LOD)	0.5−15	1 to 10 in extraction buffer solution	
peanut	Gen-Probe/Tepnel	Biokit	peanut	0.1 (LOD)	1−20	1 to 10 in tris(hydroxymethyl) aminomethane/sodium chloride/gelatin	polyclonal antibody specifically detects conarachin (Ara h 1)
	Neogen	Veratox	peanut	2.5 (LOQ)	2.5−25	1 to 25 in PBS solution + additive	
	R-Biopharm	Ridascreen	peanut	1.5 (LOD)	2.5−20	1 to 20 in extraction buffer solution	polyclonal antibody specifically detects peanut protein, including the peanut allergen Ara h 1
sesame	ELISA System	sesame residue detection	sesame seed	0.5 (LOQ)	0.5−5	1 to 10 in extraction buffer solution	specific anti-sesame seed 2S-albumin antibodies
	Gen-Probe/Tepnel	Biokit	sesame	0.1 (LOD)	6.125−100	1 to 10 in tris(hydroxymethyl) aminomethane/sodium chloride/gelatin	sesame antibodies
soy	ELISA System	soy protein residue detection	soy flour protein	2.5 (LOQ)	2.5−25	1 to 10 in extraction buffer solution	specific anti-soy trypsin inhibitor and other soy protein antibodies
	Neogen	Veratox	soy flour protein	2.5 (LOQ)	2.5	1 to 25 in PBS solution + additive	
walnut	Gen-Probe/Tepnel	Biokit	walnut	0.25 (LOD)	2.4−120	1 to 10 in tris(hydroxymethyl) aminomethane/sodium chloride/gelatin	polyclonal antibody specifically detects walnut proteins

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Table 2. Website Links for Detailed Information on the ELISA Commercial Kits Used for Determining Allergen Cross-Reactivity

allergen	company	test kit	detailed method (Web site link)
almonds	Gen-Probe/Tepnel	Biokit	http://www.tepnel.com/
	Neogen	Veratox	http://www.neogen.com/foodsafety/V_Product_List.asp?Test_Kit_Cat=203
	R-Biopharm	Ridascreen	http://www.r-biopharm.com/main.php?
gluten	Gen-Probe/Tepnel	Biokit	http://www.tepnel.com/
	Neogen	Veratox	http://www.neogen.com/foodsafety/V_Product_List.asp?Test_Kit_Cat=203
		Gliadin R5	
	R-Biopharm	Ridascreen	http://www.r-biopharm.com/main.php?
hazelnut	Gen-Probe/Tepnel	Biokit	http://www.tepnel.com/
	Neogen	Veratox	http://www.neogen.com/foodsafety/V_Product_List.asp?Test_Kit_Cat=203
	R-Biopharm	Ridascreen	http://www.r-biopharm.com/main.php?
mustard	ELISA System	Mustard Seed P.	http://www.elisas.com.au/
	Neogen	Veratox	http://www.neogen.com/foodsafety/V_Product_List.asp?Test_Kit_Cat=203
	Sedium R&D	ELISA kit	http://www.sedium-rd.cz/en/biotech/products/allergens-in-food/mustard-elisa-kit
peanut	Gen-Probe/Tepnel	Biokit	http://www.tepnel.com/
	Neogen	Veratox	http://www.neogen.com/foodsafety/V_Product_List.asp?Test_Kit_Cat=203
	R-Biopharm	Ridascreen	http://www.r-biopharm.com/main.php?
sesame	ELISA System	sesame residue	http://www.elisas.com.au/
	Gen-Probe/Tepnel	Biokit	http://www.tepnel.com/
soy	ELISA System	soy protein	http://www.elisas.com.au/
	Neogen	Veratox	http://www.neogen.com/foodsafety/V_Product_List.asp?Test_Kit_Cat=203
walnut	Gen-Probe/Tepnel	Biokit	http://www.tepnel.com/

with loaf volume, specific volume, and crust color comparable to those of the wheat control was achieved by using up to 25% of hairless canary seed or 15% of roasted canary seed flour, thus demonstrating its potential to replace a percentage of wheat flour in products or 100% in products such as cookies, cakes, and flat breads for GS individuals.

Other more recent studies also show that hairless canary seed has significantly higher levels of phosphorus, sulfur, magnesium, calcium, iron, manganese, and zinc than wheat and oat. Thus, from a nutritional perspective, glabrous canary seed could be a good source of macronutrients such as protein and fiber as well as a source of some important micronutrients including phenolic compounds and carotenoids.[13−15]

Reports of the pollen of perennial forage canarygrass (e.g., *Phalaris aquatica*, *P. arundinacea*) as a major environmental allergen and incidents of allergic reactions to canaryseed on inhalation during handling have been cited in the literature. Using IgE antibodies from sera of 24 grass-pollen-allergic subjects, 17 allergenic fractions of canarygrass (*P. aquatica*) pollen were identified ranging in molecular mass from 14 to 100 kDa. A 34 kDa protein fraction was found to have the highest frequency of IgE binding (77%) and was tentatively designated as Pha a I.[16] Another study reported immediate rhinoconjunctivitis, asthma, and contact urticaria while handling bird food caused by black seed (*Guizotia abyssinica*) used as an ingredient in canary food.[17]

In order to establish the safety of canary seeds for human consumption from a food allergy perspective and further allow the mitigation of potential risks that could arise with their consumption, studies are required to confirm the absence of cross-reactivity with major allergenic proteins. The specific objective of this study, therefore, was to assess the safety of glabrous canary seed from an allergy perspective by analyzing for the presence of allergenic cross-reactivities between glabrous canary seed and allergenic proteins in the following foods: gluten, soy, peanuts, tree nuts, sesame, and mustard. Mass spectrometry (MS), bioinformatics, and immunoblotting were used to assess the homology of glabrous canary seed proteins with those from known gluten sources (wheat, barley, and rye). Additionally, commercially available ELISA kits were used for the detection of cross-reactivities with each target food allergen as indicated in Tables 1 and 2.

MATERIALS AND METHODS

Materials and Chemicals. Hairless canary seed (*P. canariensis* L.) cultivars, CDC Maria (brown-colored grain), and two yellow-colored grain cultivars, C05041 and C05091, were used in this study. They were grown in three-replicate randomized complete block experiments in three locations in Saskatchewan, Canada, over a two-year period (2007 and 2008). Harvested samples from each of the test plots were dehulled and hand-cleaned to remove any broken seeds or foreign material. Each sample was a blend of seeds from the same variety grown on different plots. Further cleaning was done by spraying the surface of the seeds with 70% ethanol, gentle cleaning using paper towels, followed by air-drying at room temperature in a fume hood. The samples were ground in a Waring commercial blender 7011 (model 31BL92, Waring Products Division, New Hartford, CT, USA) and stored at −20 °C until analyzed. Oat, millet, quinoa, teff, sorghum, and buckwheat (marketed as gluten-free) and Canadian western red spring wheat were also obtained through local suppliers, cleaned, milled with a coffee grinder, and similarly stored for Western blotting analysis.

Serum samples of 10 patients with wheat allergy were purchased from PlasmaLab International (Everett, WA, USA). The sera were

Table 3. Protein Hits Identified in the MASCOT Mass Spectrometric Database with a MOWSE Score of 38 or Higher for the CDC Maria, C05041, and C05091 Canary Seed Sample

mass spectrometry protein hits (common name in parentheses)
CDC Maria (*sample as is*)
AVE3_AVESA Avenin-3/*Avena sativa* (oats)
SSG1_AVESA 12S seed storage globulin 1/*Avena sativa* (oats)
AVEE_AVESA Avenin-E/*Avena sativa* (oats)
SSG2_AVESA 12S seed storage globulin 2/*Avena sativa* (oats)
GLUA2_ORYSJ Glutelin type-A 2/*Oryza sativa* subsp. *japonica* (rice)
G3PC_ANTMA Glyceraldehyde-3-phosphate dehydrogenase, cytosolic/*Antirrhinum majus* (herbaceous perennial plant; common name snapdragon)
ALF_CICAR Fructose-bisphosphate aldolase, cytoplasmic isozyme/*Cicer arietinum* (chickpea)
EF1A1_DAUCA Elongation factor 1-alpha/*Daucus carota* (wild carrot)
OLEO2_ORYSI Oleosin 18 kDa/*Oryza sativa* subsp. *indica* (rice)
PDI_WHEAT Protein disulfide-isomerase/*Triticum aestivum* (wheat)[a]
H4_ARATH Histone H4/*Arabidopsis thaliana* (annual flowering plant)
HSP70_MAIZE Heat shock 70 kDa protein/*Zea mays* (corn)
CDC Maria (*cleaned sample*)
AVE3_AVESA Avenin-3/*Avena sativa* (oats)
AVEE_AVESA Avenin-E/*Avena sativa* (oats)
SSG1_AVESA 12S seed storage globulin 1/*Avena sativa* (oats)
SSG2_AVESA 12S seed storage globulin 2/*Avena sativa* (oats)
GLUA2_ORYSJ Glutelin type-A 2/*Oryza sativa* subsp. *japonica* (rice)
H4_ARATH Histone H4/*Arabidopsis thaliana* (annual flowering plant)
H2B10_ORYSI Histone H2B.10/*Oryza sativa* subsp. *indica* (rice)
G3PE_MAIZE Glyceraldehyde-3-phosphate dehydrogenase, cytosolic 3/*Zea mays* (corn)
G3PX_HORVU Glyceraldehyde-3-phosphate dehydrogenase, cytosolic/*Hordeum vulgare* (barley)[a]
EM4_WHEAT Em protein H5/*Triticum aestivum* (wheat)[a]
SEEP_RAPSA Late seed maturation protein P8B6/*Raphanus sativus* (radish)
EMB1_DAUCA EMB-1 protein/*Daucus carota* (wild carrot)
EMP1_ORYSJ Embryonic abundant protein 1/*Oryza sativa* subsp. *japonica* (rice)
C05041 (*sample as is*)
SSG1_AVESA 12S seed storage globulin 1/*Avena sativa* (oats)
AVE3_AVESA Avenin-3/*Avena sativa* (oats)
G3PE_MAIZE Glyceraldehyde-3-phosphate dehydrogenase, cytosolic 3/*Zea mays* (corn)
GLUA2_ORYSJ Glutelin type-A 2/*Oryza sativa* subsp. *japonica* (rice)
AVEE_AVESA Avenin-E/*Avena sativa* (oats)
G3PX_HORVU Glyceraldehyde-3-phosphate dehydrogenase, cytosolic/*Hordeum vulgare* (barley)[a]
HSP70_MAIZE Heat shock 70 kDa protein/*Zea mays* (corn)
H2B10_ORYSI Histone H2B.10/*Oryza sativa* subsp. *indica* (rice)
GLUB4_ORYSJ Glutelin type-B 4/*Oryza sativa* subsp. *japonica* (rice)
GLUB1_ORYSJ Glutelin type-B 1/*Oryza sativa* subsp. *japonica* (rice)
H4_ARATH Histone H4/*Arabidopsis thaliana* (annual flowering plant)
ATPBM_HEVBR ATP synthase subunit beta, mitochondrial/*Hevea brasiliensis* (rubber tree)
OLEO2_ORYSI Oleosin 18 kDa/*Oryza sativa* subsp. *indica* (rice)
ATP9_MAIZE ATP synthase subunit 9, mitochondrial/*Zea mays* (corn)
EF1A1_DAUCA Elongation factor 1-alpha/*Daucus carota* (wild carrot)
ALF_CICAR Fructose-bisphosphate aldolase, cytoplasmic isozyme/*Cicer arietinum* (chickpea)

mass spectrometry protein hits (common name in parentheses)
C05041 (*cleaned sample*)
AVE3_AVESA Avenin-3/*Avena sativa* (oats)
AVEE_AVESA Avenin-E/*Avena sativa* (oats)
SSG2_AVESA 12S seed storage globulin 2/*Avena sativa* (oats)
H32_CICIN Histone H3.2/*Cichorium intybus* (chicory)
H32_MEDSA Histone H3.2/*Medicago sativa* (alfafa)
GLUA2_ORYSJ Glutelin type-A 2/*Oryza sativa* subsp. *japonica* (rice)
G3PE_MAIZE Glyceraldehyde-3-phosphate dehydrogenase, cytosolic 3/*Zea mays* (corn)
UP01_VITRO Unknown protein 1 (Fragment)/*Vitis rotundifolia* (grapevine species)
G3PX_HORVU Glyceraldehyde-3-phosphate dehydrogenase, cytosolic/*Hordeum vulgare* (barley)[a]
EF2_BETVU Elongation factor 2/*Beta vulgaris* (beet)
H2B10_ORYSI Histone H2B.10/*Oryza sativa* subsp. *indica* (rice)
SEEP_RAPSA Late seed maturation protein P8B6/*Raphanus sativus* (radish)
EMB1_DAUCA EMB-1 protein/*Daucus carota* (wild carrot)
H4_ARATH Histone H4/*Arabidopsis thaliana* (annual flowering plant)
SUSY_ALNGL Sucrose synthase/*Alnus glutinosa* (alder)
SUS1_ORYSJ Sucrose synthase 1/*Oryza sativa* subsp. *japonica* (rice)
GRDH_DAUCA Glucose and ribitol dehydrogenase/*Daucus carota* (wild carrot)
GRDH_ORYSJ Glucose and ribitol dehydrogenase homologue/*Oryza sativa* subsp. *japonica* (rice)
EMP1_ORYSJ Embryonic abundant protein 1/*Oryza sativa* subsp. *japonica* (rice)
PROF2_SOLLC Profilin-2/*Solanum lycopersicum* (tomato)
REHYA_ORYSI 1-Cys peroxiredoxin A/*Oryza sativa* subsp. *indica* (rice)
EF1A1_DAUCA Elongation factor 1-alpha/*Daucus carota* (wild carrot)
C05091 (*sample as is*)
SSG1_AVESA 12S seed storage globulin 1/*Avena sativa* (oats)
AVE3_AVESA Avenin-3/*Avena sativa* (oats)
SSG2_AVESA 12S seed storage globulin 2/*Avena sativa* (oats)
AVEE_AVESA Avenin-E/*Avena sativa* (oats)
GLUA2_ORYSJ Glutelin type-A 2/*Oryza sativa* subsp. *japonica* (rice)
G3PC_ANTMA Glyceraldehyde-3-phosphate dehydrogenase, cytosolic/*Antirrhinum majus* (herbaceous perennial plant; common name snapdragon)
EF1A1_DAUCA Elongation factor 1-alpha/*Daucus carota* (wild carrot)
C05091 (*cleaned sample*)
AVE3_AVESA Avenin-3/*Avena sativa* (oats)
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SSG2_AVESA 12S seed storage globulin 2/*Avena sativa* (oats)
AVEE_AVESA Avenin-E/*Avena sativa* (oats)
GLUA2_ORYSJ Glutelin type-A 2/*Oryza sativa* subsp. *japonica* (rice)
H4_ARATH Histone H4/*Arabidopsis thaliana* (annual flowering plant)
EF1A1_DAUCA Elongation factor 1-alpha/*Daucus carota* (wild carrot)
OLEO2_ORYSI Oleosin 18 kDa/*Oryza sativa* subsp. *indica* (rice)
HSP70_MAIZE Heat shock 70 kDa protein/*Zea mays* (corn)
SUS1_ORYSJ Sucrose synthase 1/*Oryza sativa* subsp. *japonica* (rice)
SEEP_RAPSA Late seed maturation protein P8B6/*Raphanus sativus* (radish)
EMB1_DAUCA EMB-1 protein/*Daucus carota* (wild carrot)

[a]Wheat and/or barley.

portioned in aliquots and stored at −80 °C until use. Mouse anti-human IgE (monoclonal) was purchased from SouthernBiotech (Birmingham, AL, USA). Wheat gluten and β-lactoglobulin polyclonal antibodies and goat anti-rabbit secondary antibodies conjugated to horseradish peroxidase (HRP) were purchased from Cederlane (Burlington, Ontario, Canada) and Abcam Inc. (Cambridge, MA, USA), respectively. Rabbit polyclonal anti-sesame antibodies were donated by Health Canada Proteomic and Allergen Research Unit.

Dithiothreitol (DTT), iodoacetamide, [Glu1]-fibrinopeptide B, ammonium bicarbonate (NH_4HCO_3), acetonitrile (ACN), and formic acid (FA) were purchased from Sigma-Aldrich (Oakville, ON) and used without purification. Promega sequencing grade trypsin was purchased from Fisher Scientific (Nepean, ON), and ethanol was purchased from Commercial Alcohols (Brampton, ON). Water was purified with resistivity ≥18 MΩ using a Millipore Milli-Q Synthesis system from Fisher Scientific (Nepean, ON). The extraction solution used in

these experiments consisted of 60% ethanol and buffer (NH_4HCO_3, 100 mM, pH 8.5 with 1 M urea). Molecular mass calibration kit (10 to 250 kDa) from Bio-Rad (Bio-Rad Laboratories, Hercules, CA, USA) was used as molecular markers.

ELISA kits for selected priority allergens including standards were purchased from commercial suppliers as listed in Table 1. Information for each kit and the Web sites of each kit manufacturer for full protocols are provided in Tables 1 and 2. All other reagents and chemicals used were of analytical grade.

Sample Preparation for Mass Spectrometric Analysis. A sample of finely ground canary seed powder (5 g) was dispersed in the extraction solution (5 mL) by shaking on a vortex for 30 s. An aliquot of the supernatant (1 mL) was diluted, and the protein content determined using a BCA test from Fisher Scientific (Nepean, ON). Further dilutions were made to produce a standard concentration (25 μg/100 μL) for the digestion and subsequent mass spectrometry analysis. Dithiothreitol (10 mM in 100 mM ammonium bicarbonate) was added for reduction, while iodoacetamide (55 mM in 100 mM ammonium bicarbonate) was added for alkylation. Samples were then digested with trypsin, and finally, 1% formic acid was added to the mixture prior to MS analysis.

LC-MS/MS. Liquid chromatography (LC) and mass spectrometry (MS) were performed on a hybrid MALDI Q-TOF Premier (Waters, Milford, MA, USA) fitted with a nanolockspray source, which was coupled to a nanoAcquity UPLC system (Waters). The LC system was configured with a binary solvent manager to deliver the gradient system and an auxiliary solvent manager to deliver the reference mass standard solution for accurate mass corrections. The reference mass standard consisted of a solution of [Glu1]-fibrinopeptide B in 50% aqueous methanol, which was infused into the source at a flow rate of 0.200 μL/min and sampled every 10 s for 1 s. The separation system consisted of a trap column (Symmetry C_{18} 5 μm 180 μm × 20 mm, Waters) and an analytical column (BEH 130 C_{18} 1.7 μm 100 μm × 100 mm, Waters). Solvent A consisted of H_2O with 0.1% FA, and solvent B consisted of ACN with 0.1% FA. In a typical experiment digested canary seed protein samples were concentrated and desalted by injection (1 μL) onto the trap column for 2 min at a flow rate of 15.0 μL/min using 99% solvent A. The samples were then diverted to the analytical column and eluted at 400 nL/min. The elution program started with 99% A for 1 min followed by a gradient from 1% to 50% B in 50 min. The mass spectrometer was operated in positive ion mode (resolution >10000 fwhh), and the acquired data were collected using MassLynx v4.1. Data were collected in both MS survey scan mode and an automated data directed analysis (DDA) mode. MS survey scan data were acquired in continuum mode from m/z 400 to 1600 with a scan time of 0.60 s with an interscan delay of 0.02 s.

Data collected using DDA software were submitted directly to an in-house MASCOT server with software version 2.3.01. The tandem MS information was searched against the National Center for Biotechnology Information (NCBI) database (version 20100115) with up to two missed cleavages, a fixed carbamidomethylation modification at cysteine, and oxidation of methionine, and deamidation of asparagine and glutamine were specified as variable modifications. The peptide mass tolerance and the fragment mass tolerance were both set to 0.1 Da. The mass and the retention time data are collected for peptides that produced a significant identification or extensive homology (probability-based MOWSE score ≥38).

Identification of Protein Bands as Allergens by LC-MS/MS. The protein in-gel digestion and mass spectrometry experiments were performed by the Proteomics platform of the Eastern Quebec Genomics Center, Quebec, Canada. Detailed experimental parameters for the tryptic digestion, the mass spectrometry conditions, and data analysis were previously reported.[18] Scaffold (Scaffold_3_00_07, Proteome Software Inc., Portland, OR, USA) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm.[19] Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm.[20]

SDS-PAGE and Western Blot Analysis. SDS-PAGE and immunoblotting were used for the determination of allergenic proteins. SDS-PAGE was performed according to Laemmli[21] using 10−20% precast gel (Bio-Rad, St. Louis, MO, USA). Protein samples (2.5 mg/mL) were dispersed in Laemmli sample buffer (Bio-Rad) plus 5% β-mercaptoethanol and vortexed for 15 min. The dispersions were heated at 100 °C for 5 min and centrifuged before loading. A 10 μL amount of the supernatant of each sample was loaded in a well, and the gel was run at a constant voltage of 150 V for approximately 90 min at room temperature. After electrophoresis, the separated proteins were stained with Coomassie Brilliant Blue R-250 and scanned.

For immunoblotting, the separated proteins were subsequently transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, 0.45 mm) using a Criterion Blotter cell (Bio-Rad). The transfer was done for 1 h with a constant current of 100 V in 25 mM Tris-HCl (pH 8.3), 192 mM glycine, and 20% methanol, following the method of Towbin et al.[22] Blots were blocked in phosphate-buffered saline plus 0.1% Tween 20 containing 5% nonfat milk (NFM) powder or for 1 h at room temperature, and then the PVDF was washed three times with 0.1% Tween 20 in TBS and incubated overnight at 4 °C with pooled sera from wheat-allergic individuals (diluted 1:30 with 0.1% Tween 20 in TBS containing 1% BSA protease free) or with gluten-IgG (dilution 1:1000), β-lactoglobulin-IgG (dilution 1:10 000), or sesame-IgG (dilution 1:30 000) The membrane was washed and incubated for an additional 1 h with a goat anti-human IgE polyclonal (HRP) conjugate used as second antibody (diluted 1:5000) (KPL, Inc. Gaithersburg, MD, USA). Incubations were performed with gentle rocking. After washing, the blot was incubated in the Bio-Rad amplification reagent, washed, incubated in streptavidin-HRP, and washed again before incubation with the Opti-4CN substrate kit (Bio-Rad) to visualize the immunoreactive bands. To improve binding sensitivity and reduce nonspecific binding, a mouse anti-human IgE monoclonal antibody (dilution 1:2000) and a dilution of all antibodies in the blocker buffer (i.e., 3% nonfat dry milk) were also tested in comparison with the originally used goat anti-human IgE and 1% BSA diluting buffer.

Both the Coomassie-stained gels and immunostained membranes were scanned using an Epson Image Scanner III (Seiko Epson, Corporation, Nagano, Japan), and the generated files were analyzed with ImageQuant TL software (Amersham Biosciences) using the Kaleidoscope Precision Plus Protein Standards from Bio-Rad.

For the immunoblotting studies, the presence of a band in the immunoblot indicated the likely presence of an IgE antibody−antigen binding interaction.

Enzyme Linked Immunosorbent Assay (ELISA). ELISA analysis was carried out as per the instructions provided by the kit manufacturers (Tables 1 and 2). In general, glabrous canary seeds were ground into flour and extracted as per the kit instructions. Samples were dispersed in the extraction solutions provided with the ELISA kit and were continuously stirred while maintained at the temperature specified in the kit instructions and for the periods specified. The extracts were then filtered and tested according to the kit instructions. The protocol used varied slightly for the different test kits depending on whether the ELISA assay was direct, sandwich, or competitive. When cross-contamination was suspected, samples were cleaned with 70% ethanol and the extractions were repeated. To augment sensitivity for further confirmation, the proposed amounts indicated on the kit instructions were tripled and the extractions were repeated for some samples. Optical densities (OD) were measured at 450−650 nm depending on the kit instructions using a Bench Mark Plus microplate reader (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) controlled by Microplate Manager Software (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada). Due to reported variability in ELISA results from different test kits, two to three commercial test kits from different companies were used for each targeted allergen, extractions were done in triplicate for each kit, and each extract was analyzed in triplicate.

Quantitative determination of allergen content for ELISA was made using the standards provided in each kit and, when available, using the accompanying software provided by the kit manufacturer; otherwise the Excel software was used to calculate the linear/exponential

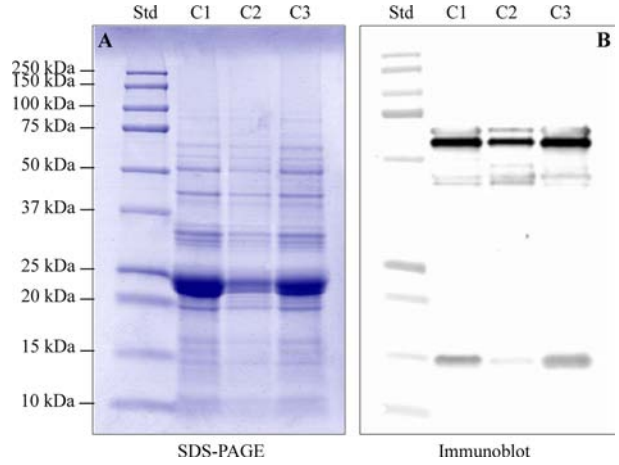

Figure 1. (A) SDS-PAGE patterns of canary seeds. (B) Immunoblot pattern of canary seeds using pooled sera of 10 wheat allergic individuals. Antibodies and streptavidin diluted in a nonfat dry milk buffer. Std, molecular weight standards; C1, CDC Maria; C2, C05041; and C3, C05091.

regression slopes of the standards and to determine the allergen content. Values below the limit of detection (LOD) or limit of quantitation (LOQ) were reported as such, indicating the absence of the target allergen. Values above the LOD or LOQ were reported as averages of all determinations. This indicated the likely presence of a target allergen. Data were statistically evaluated by one-way analysis of variance (ANOVA) using the PRISM software, version 3.02 (Graph Pad Software, Inc. San Diego, CA, USA). Significant differences between means were determined by Tukey's multiple comparison test procedure at the 5% significance level.

RESULTS AND DISCUSSION

Mass Spectrometry. The mass spectrometry MASCOT database search scores for protein hits were cut off at 38, which represents significant homology between the test sample and identified protein matches. Table 3 presents a list of the proteins identified from the database in the three canary seed samples, which were mostly homologous with rice, oats, corn, carrot, tomato, radish, beet, and chickpea proteins. The search showed no significant homology to celiac-related gluten fragments from wheat, rye, barley, or their derivatives in all three canary seed samples. For CDC Maria (Table 3), three hits were obtained indicating the likely presence of protein disulfide-isomerase (wheat), Em protein H5 (wheat), and cytosolic glyceraldehyde-3-phosphate dehydrogenase (barley) or proteins having similar homology. One hit suggesting the likely presence of cytosolic glyceraldehyde-3-phosphate dehydrogenase (barley) or a similar protein was found for C05041 (Table 3). Protein disulfide-isomerase, with a molecular mass of 56 533 Da, is an enzyme in the endoplasmic reticulum in eukaryotes that catalyzes the formation and breakage of disulfide bonds between cysteine residues within proteins as they fold.[23] Em protein H5 (molecular mass 10 060 Da) is a member of the small hydrophilic plant seed protein family. Cytosolic glyceraldehyde-3-phosphate dehydrogenase (molecular mass 33 236 Da) belongs to the glyceraldehyde-3-phosphate dehydrogenase family.

Gluten epitopes provoking celiac disease typically originate from the gliadin and glutenin fractions and contain high amounts of glutamine and proline amino acid residues and the signature "QP" amino acid sequence.[24] Examples of key epitopes reported in the literature include IIQPEQPAQ, FPQQPQQPYPQQP, FSQPQQQFPQPQ, LQPQQPFPQQPQQPYPQQPQ, and PQQPFPQPQQQFPQPQQPQQ.[25] The amino acid sequences of the three protein hits (i.e., protein disulfide-isomerase (wheat), Em protein H5 (wheat), and cytosolic glyceraldehyde-3-phosphate dehydrogenase (barley)), do not show any "QP" sequence, suggesting little likelihood of them containing a celiac-provoking epitope. Overall, the mass spectrometry results suggest either cross contact or homology between canary seed proteins and some proteins from rice, oats, corn, carrot, tomato, radish, beet, and chickpea proteins but not with gluten.

Immunoblotting. The SDS-PAGE protein profiles of the three canary seed samples are shown in Figure 1A. All three seeds showed similar electrophoretic profiles with the presence

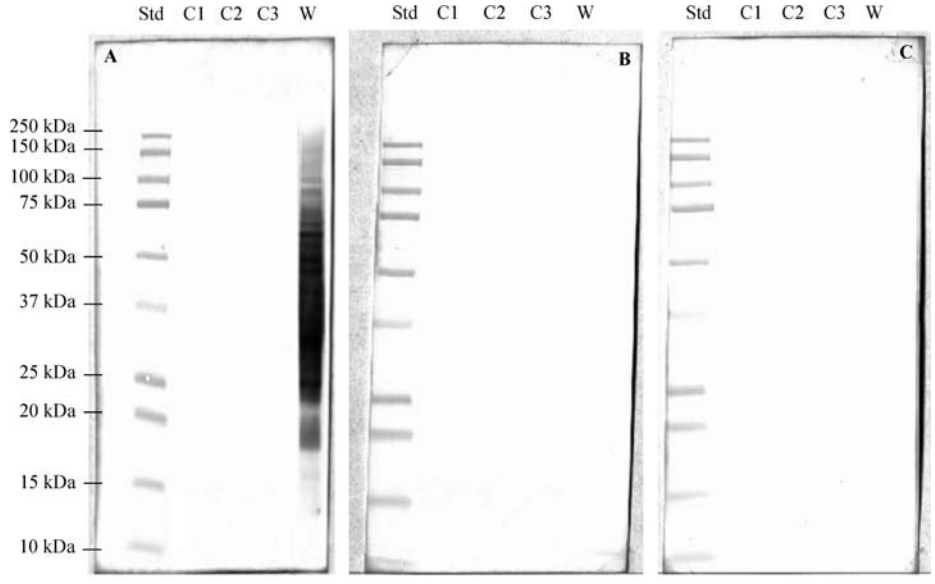

Figure 2. Immunoblots of canary seeds (C1, CDC Maria; C2, C05041; C3, C05091) and W-wheat (positive control) after incubation with (A) rabbit anti-gluten IgG antibody, (B) rabbit anti-β-lactoglobulin IgG antibody, and (C) rabbit anti-sesame IgG antibody. Antibodies and streptavidin were diluted in a nonfat dry milk buffer. The immunoblots were performed using the Opti-4CN colorimetric detection method.

of protein bands ranging in molecular mass from ~10 000 to 100 000 Da. The most prominent band had a molecular mass of ~20 000−25 000 Da.

Reactivities of proteins separated by SDS-PAGE were analyzed using pooled sera from 10 wheat allergenic individuals (Figure 1B). The amplified Opti-4CN detection kit used to perform colorimetric blotting requires the use of 1% BSA as diluent buffer for primary and secondary antibodies as well as the substrate reagents. The preliminary immunoblot of the three canary seed protein extracts using bovine serum albumin as antibody diluent buffer revealed strong binding with many of the canary seed protein bands. These results were intriguing, as the mass spectrometry results showed the absence of celiac-provoking gluten proteins. Binding, however, could sometimes result from nonspecific binding involving one of the detecting reagents used during the assay (e.g., BSA). Indeed, blotting of canary seed with only the secondary antibodies (no primary human sera used) resulted in the binding of some of the canary seed proteins, confirming the presence of nonspecific binding (data not shown).

Thus, the blotting was repeated this time using nonfat dry milk and Pierce protein-free blocking and superblock buffers as buffer diluent and blocking agents, respectively. Negative controls were also tested to reveal any nonspecific binding in the absence of human sera. The NFM blocking and diluent buffer showed good binding to all potential sites of nonspecific interaction, eliminating background altogether without altering or obscuring the epitope for antibody binding. Furthermore, as previously reported by other workers,[26] the use of a monoclonal secondary antibody (mouse anti-human IgE) gave better results with higher sensitivity than the polyclonal secondary antibody (goat anti-human IgE).

Optimal conditions for the immunoblot analysis of the canary seed with wheat human sera were thus obtained with the monoclonal secondary antibody and NFM as blocker (Figure 1B). As shown, the results revealed that some canary seed proteins were still recognized by the pooled sera of wheat allergic patients. Two strong antibody binding proteins with molecular mass (MM) ranging between 50 and 75 kDa were observed in addition to other polypeptides having MMs of 37−50 and 15 kDa. The three canary seed composites showed similar antibody-binding patterns.

Aalberse et al.[27] reported homologous allergens from phylogenetically related grasses. To determine if binding will be observed with gluten-specific antibodies, the work was repeated with polyclonal rabbit gluten IgG antibodies raised specifically against wheat gluten protein (immunogen). In addition, pooled sera of seven individuals allergic to sesame as well as anti-β-lactoglobulin antibody were tested as negative controls. As shown in Figure 2, despite the wheat sample used as positive control, no bands were observed in all three immune blots, suggesting the absence of gluten-specific proteins in the three canary seed samples.

Immunoblotting of Other Gluten-Free Cereals. To verify if binding will occur with other gluten-free cereals, the SDS-PAGE and blotting were performed on oat, millet, quinoa, teff, sorghum, and buckwheat as shown in Figure 3. Canadian western red spring wheat was used as positive control. The SDS-PAGE results (Figure 3A) showed major differences in the electrophoretic profiles of the cereals. This was expected, as the cereals belong to different plant families. As observed for canary seeds, when bovine serum albumin was used as blocking agent, the pooled wheat sera recognized practically all the different

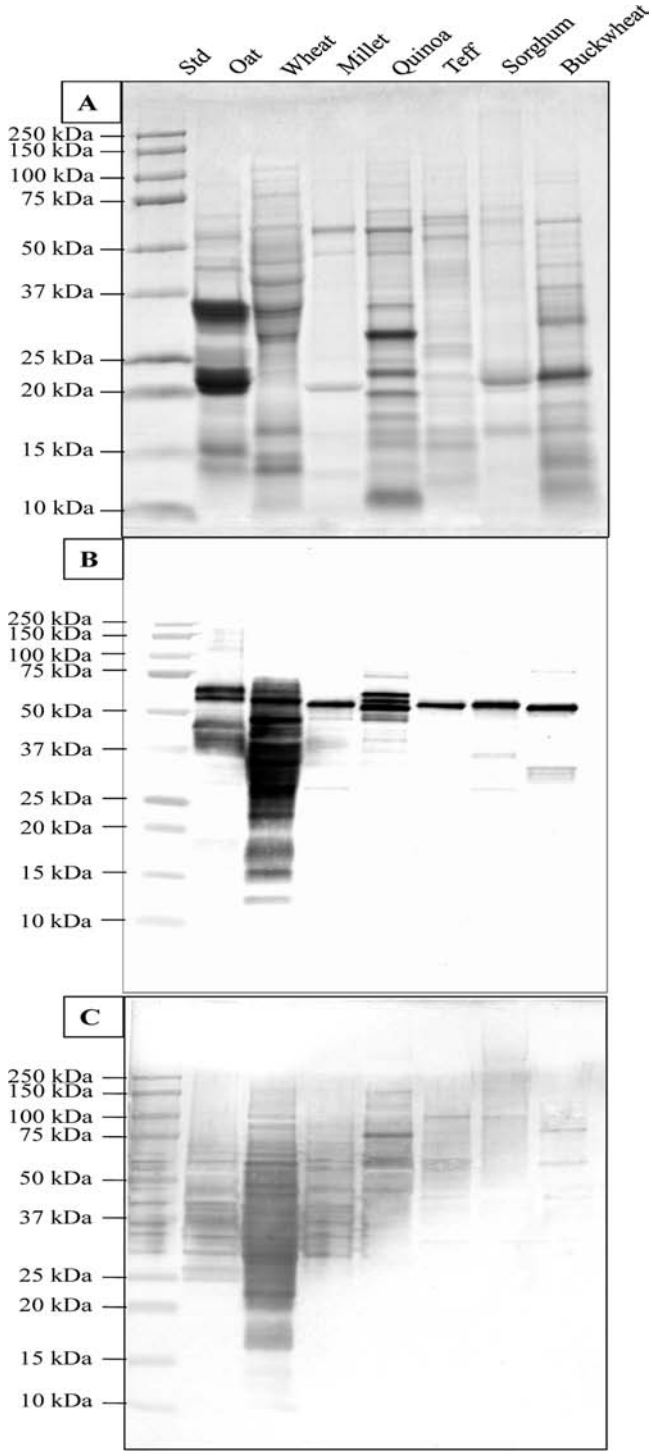

Figure 3. (A) SDS-PAGE patterns of gluten- and non-gluten-containing cereals. (B, C) Immunoblot patterns of gluten and non-gluten-containing cereals. (B) Bound IgE detected using pooled sera of 10 wheat allergic individuals. Antibodies and streptavidin were diluted in a nonfat dry milk buffer. (C) Immunoblot patterns bound using gluten-specific IgG antibodies (host, rabbit). Antibodies and streptavidin were diluted in nonfat dry milk. Std, molecular weight standards. The immunoblots were performed using the Opti-4CN colorimetric detection method.

polypeptide bands that were clearly visible in the SDS-PAGE profile of the other gluten-free cereals as well as some that were not previously evident. Blocking with nonfat dry milk instead of

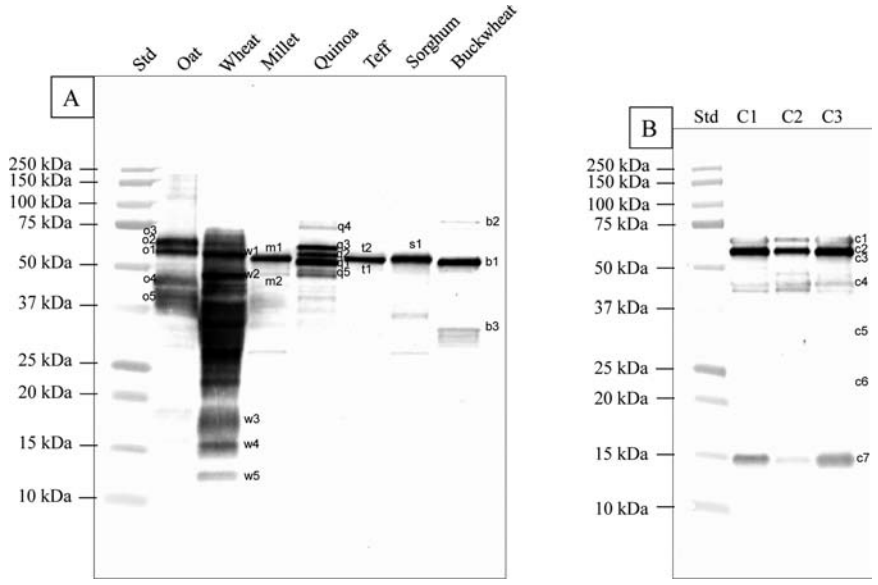

Figure 4. (A) Immunoblot patterns of gluten (wheat)- and non-gluten-containing cereals (oat, millet, quinoa, teff, sorghum, and buckwheat) and (B) immunoblots of canary seeds (C1, CDC Maria; C2, C05041; C3, C05091). Bound IgE was detected using pooled sera of 10 wheat allergic individuals.

bovine serum albumin revealed a different pattern with only a few bands recognized (Figure 3B). Prominent bands at approximately 50 to 75 kDa showed similar strong antibody-binding. Faint bands (low binding) were also observed for some proteins with MM above 37 kDa and at ~15 kDa. The Western blotting was repeated using rabbit polyclonal gluten antibodies with nonfat dry milk as blocking agent. The immunoblot (Figure 3C) revealed strong binding to many of the wheat proteins and some proteins in oat, millet, quinoa, teff, and to a lower extent with sorghum and buckwheat proteins, which could be due to either cross-reactivities or cross-contamination of the grains with gluten proteins.

In previous studies using radioallergosorbent testing (RAST) of sera from subjects sensitized to wheat and rye flour, a significant reaction was found with seed extracts of 12 cereals including wheat, durum wheat, triticale, cereal rye, barley, rye grass, oats, canarygrass, rice, maize, sorghum, and Johnson grass.[28]

Identification of Major Allergens (Excised Bands) by Mass Spectrometry. To confirm the identity of the predominant protein components recognized by antibodies in the wheat sera, electrophoresis of wheat, gluten-free cereals, and canary seeds was run again and the bands showing antibody–antigen binding during immunoblotting (Figure 4) were excised and further analyzed by LC/ESI-MS/MS. The list of proteins identified is presented in Table 4. Specifically, for the canary seeds, the allergenic protein bands (as shown in Figure 4B) were not identified by the MS technique as belonging to the *P. canariensis* family. To date, very few proteins from canary seeds have been sequenced. Howerver, using Mascot search, the tryptic peptides from the canary bands (C1–C7) were compared with peptides of known proteins listed in the National Center for Biotechnology Information (NCBI), and the results revealed significant sequence homology to several cereals in particular with rice (20% coverage), oat (16%), barley (14%), sorghum (10%), maize (5%), and granule-bound starch synthase 1 (GBSS1) from wheat (4%).

ELISA. ELISA results of the canary seeds for the different allergen kits tested and using the sample size indicated in the kit instructions are provided in Table 5. All the results were below the LOD/LOQ, except for two test kits (i.e., ELISA System kit for mustard and Neogen Veratox for gluten and mustard). The ELISA System mustard test kit gave significantly ($p < 0.05$) higher values for mustard, ranging between 0.7 and 13 ppm for the different canary seed composites. The LOQ for this kit was 1.0 ppm. The Neogen Veratox test kits also gave a value of 5.4 ppm (close to LOQ) for gluten and 36.5 ppm for mustard for CDC Maria, which was above the LOQ. A significant difference ($p < 0.05$) was observed between the mustard levels determined by the ELISA System kit and Neogen Veratox particularly for the CDC Maria variety. For the two other composites (C05041 and C05091) the Noegen Veratox results were below the LOQ of the kits (5 ppm for gluten and 2.5 ppm for mustard).

As the results for mustard and gluten using the sample size indicated in the kit instructions were above the LOQ (Table 5), the concentration requested by the kit instruction was tripled and the analysis was repeated. This was done intentionally in order to augment the sensitivity of the detection. Additionally, the canary seed groats were cleaned by washing with 70% ethanol, air-dried, and reanalyzed as per the kit instructions using one and three times the sample size recommended for extraction. The ELISA results obtained (Tables 6 and 7) were below the LOD/LOQ for all the kits retested, indicating the absence of cross-reactivity or cross-contamination with the targeted allergens. The results further suggest that the earlier positive detection was likely due to localized cross-contamination with some gluten- and mustard-containing material in the sample or a false positive.

A new Veratox kit for gluten that uses the Codex Alimentarius recommended "R5" gliadin antibody was used to test for the presence of gluten in the three different canary seed composites, and the results as shown in Table 7 confirmed the absence of cross-reactivity or gluten cross-contamination with the analyzed canary seed composites.

Table 4. Wheat, Nongluten Cereals, and Canary Seed Proteins Identified by LC/MS Analysis of Excised Bands from 1-DE, Selected by IgE Abs in Immunoblotting

band no.[a]	accession no.	protein identified	sequence coverage[b] (%)	MASCOT score[c]	theoretical mass (kDa)
W1	B7U6L4	wheat globulin 3	24	50	66
	A9YSK3	HMW glutenin subunit	14	52	70
W2	B2Y2R5	LMW glutenin subunit	12	52	45
W3	P17314	wheat α-amylase/trypsin inhibitor	20	52	18
W4	P01085 (Q19A44)	wheat α-amylase/trypsin inhibitor	95	52	13
W5	Q5UHH8	wheat α-amylase/trypsin inhibitor	90	52	13
O1	Q38780	AVESA 11S globulin (*Avena sativa* = oat)	38	53	59
O2	P14812	AVESA 12S globulin 2	33	53	59
O3	O49258	AVESA 12S globulin	25	53	58
O4	O49257	AVESA 12S globulin	14	51	53
O5	Q38780	AVESA 11S globulin	28	53	59
Q1−Q5	Q06AW2 (Q06AW1)	CHEQI 11S globulin A/B	13	52	54
M1−M2	DX3508	granule bound starch synthase I (GBSSI)	72	52	58
	DX3516				
T1−T2		not identified by Mascot			
S1	A5Y380	SORBI-GBSSI (from *Sorghum bicolor*)	55	54	66
B1	O23878	FAGES 13S globulin (*Fagopyrum esculentum*)	13	52	65
B2	O23878	FAGES 13S globulin 1 (*Fagopyrum esculentum*)	7.5	52	65
B3	A5H1X6	FAGES vicilin-like protein	24	51	16
	Q6QJL1	FAGES vicilin-like protein	17	52	16
C1	ND	significant homology with			
		wheat (GBSS1 = granule-bound starch synthase 1)	13	63.7	68
		maize	10	105.0	73
		rice	7.7	80.5	61
		oat	6.9	45.5	62
		sorghum	5.3	62.2	55
		barley	2.5	53.3	72
C2	ND	significant homology with			
		rice	20	49.9	66
		oat	13	45.5	53
		barley	7.6	53.3	61
		maize	4.2	53.0	68
		wheat (GBSS1)	2.8	54.1	64
C3	ND	significant homology with			
		barley	29	73.3	59
		maize	14	70.3	52
		oat	11	52.1	59
		wheat (GBSS1)	7.6	52.6	57
		rice	6.5	84.2	57
C4	ND	significant homology with			
		rice	16	57.0	45
		maize	11	70.0	43
		oat	10	75.0	58
		wheat (GBSS1)	6.8	78.6	57
		sorghum	5.7	58.2	46
C5	ND	significant homology with			
		oat	12	46	39
		barley	12	58.4	33
C6	ND	significant homology with			
		barley	17	59.6	27
		oat	16	39.7	25
		rice	16	57.0	17
C7	ND	significant homology with			
		rye	29	82.1	10
		maize	17	74	17
		oat	14	51.5	15

[a]Band numbers correspond to the gel in Figure 4. [b]Sequence coverage = total percentage of proteins amino acid sequence covered by peptides in MS/MS analysis. [c]MASCOT score > 40 indicates identification or extensive homology; HMW, high molecular weight; LMW, low molecular weight. ND: not determined (there are no canary seed protein sequences deposited in the NCBI dbEST).

Table 5. ELISA Results of Glabrous Canary Seed Analyzed As Is (Uncleaned) and Using Quantity Indicated in Kit Instructions[a]

			ELISA results		
allergen	company	test kit	CDC Maria, ppm	C05041, ppm	C05091, ppm
almond	Gen-Probe/Tepnel	Biokit	<LOD	<LOD	<LOD
	Neogen	Veratox	<LOQ	<LOQ	<LOQ
	R-Biopharm	Ridascreen	<LOD	<LOD	<LOD
gluten	Gen-Probe/Tepnel	Biokit	<LOD	<LOD	<LOD
	Neogen	Veratox	5.4 (≅LOQ)	<LOQ	<LOQ
	R-Biopharm	Ridascreen	<LOD	<LOD	<LOD
hazelnut	Gen-Probe/Tepnel	Biokit	<LOD	<LOD	<LOD
	Neogen	Veratox	<LOQ	<LOQ	<LOQ
	R-Biopharm	Ridascreen	<LOD	<LOD	<LOD
mustard	ELISA System	ELISA	13.0 a, A	0.7 b	6.9 c
	Sedium R&D	ELISA	<LOD	<LOD	<LOD
	Neogen	Veratox	36.5 B	<LOQ	<LOQ
peanut	Gen-Probe/Tepnel	Biokit	<LOD	<LOD	<LOD
	Neogen	Veratox	<LOQ	<LOQ	<LOQ
	R-Biopharm	Ridascreen	<LOD	<LOD	<LOD
sesame	ELISA System	ELISA	<LOQ	<LOQ	<LOQ
	Gen-Probe/Tepnel	Biokit	<LOD	<LOD	<LOD
soy	ELISA System	ELISA	<LOQ	<LOQ	<LOQ
	Neogen	Veratox	<LOQ	<LOQ	<LOQ
walnut	Gen-Probe/Tepnel	Biokit	<LOD	<LOD	<LOD

[a]LOD: limit of detection; LOQ: limit of quantification. Small letters (a, b, c) within a row indicate significant ($p < 0.05$) differences within the canary seed composites. Capital letters (A and B) within a column indicate significant ($p < 0.05$) differences between the ELISA kits for a particular variety.

Samples of the same groats were sent to the company for testing, and their results (not shown) further confirmed the absence of gluten.

In summary, glabrous canary seed could be a good alternative gluten-free cereal, and further in vitro experiments would confirm the immunogenic potential of digested canary seed proteins. CODEX[29] guidelines recommend that foods containing <20 ppm gluten may be labeled as gluten-free. All three techniques used in this study were negative for gluten; thus these methods could be used to support gluten-free labeling of products that contain glabrous canary seeds. As glabrous canary seed is likely to be grown in close proximity to other gluten-containing crops such as wheat, rye, and barley, the risk of cross-contamination during production, harvesting, and processing can be high if appropriate allergen management practices are not used. Food processors interested in using this grain in gluten-free foods would need to ensure that the grains are appropriately managed through production, transportation, and primary processing in order to ensure that they remain "gluten-free". Interestingly, the immunoblotting studies showed that non-gluten-containing cereals such as buckwheat, sorghum, teff, millet, quinoa, and oat contain proteins that are recognized by antibodies in wheat allergic sera and rabbit anti-gluten IgG raised against wheat gluten proteins. As IgE-mediated wheat allergy is different from GSE, the mechanisms underlying these binding interactions require further studies.

Table 6. ELISA Results of Glabrous Canary Seed Analyzed As Is (Uncleaned) and Using Three Times the Quantity Indicated in the Kit Instructions

			ELISA results		
allergen	company	test kit	CDC Maria	C05041	C05091
almond	Gen-Probe/Tepnel	Biokit	<LOD[a]	<LOD	<LOD
	Neogen	Veratox	<LOQ[b]	<LOQ	<LOQ
	R-Biopharm	Ridascreen	<LOD	<LOD	<LOD
gluten	Gen-Probe/Tepnel	Biokit	<LOD	<LOD	<LOD
	Neogen	Veratox	<LOQ	<LOQ	<LOQ
	R-Biopharm	Ridascreen	<LOD	<LOD	<LOD
hazelnut	Gen-Probe/Tepnel	Biokit	<LOD	<LOD	<LOD
	Neogen	Veratox	<LOQ	<LOQ	<LOQ
	R-Biopharm	Ridascreen	<LOD	<LOD	<LOD
nustard	ELISA System	ELISA	<LOQ	<LOQ	<LOQ
	Sedium R&D	ELISA	<LOD	<LOD	<LOD
	Neogen	Veratox	<LOQ	<LOQ	<LOQ
peanut	Gen-Probe/Tepnel	Biokit	<LOD	<LOD	<LOD
	Neogen	Veratox	<LOQ	<LOQ	<LOQ
	R-Biopharm	Ridascreen	<LOD	<LOD	<LOD
sesame	ELISA System	ELISA	<LOQ	<LOQ	<LOQ
	Gen-Probe/Tepnel	Biokit	<LOD	<LOD	<LOD
soy	ELISA System	ELISA	<LOQ	<LOQ	<LOQ
	Neogen	Veratox	<LOQ	<LOQ	<LOQ
walnut	Gen-Probe/Tepnel	Biokit	<LOD	<LOD	<LOD

[a]LOD: limit of detection. [b]LOQ: limit of quantification.

Table 7. ELISA Results for Glabrous Canary Seed Analyzed after Cleaning and Using One and Three Times the Quantity Indicated in the Kit Instructions

			ELISA results		
allergen	company	test kit	CDC Maria	C05041	C05091
A. Canary Seed Analyzed after Cleaning and Using Quantity Indicated in Kit Instructions					
gluten	Neogen	Veratox	<LOQ[a]	<LOQ	<LOQ
		Veratox R5	<LOQ	<LOQ	<LOQ
mustard	ELISA System	ELISA	<LOQ	<LOQ	<LOQ
	Neogen	Veratox	<LOQ	<LOQ	<LOQ
B. Canary Seed Analyzed after Cleaning and Using Three Times the Quantity Indicated in Kit Instructions					
gluten	Neogen	Veratox	<LOQ	<LOQ	<LOQ
mustard	ELISA System	ELISA	<LOQ	<LOQ	<LOQ
	Neogen	Veratox	<LOQ	<LOQ	<LOQ

[a]LOQ: Limit of quantification.

AUTHOR INFORMATION

Corresponding Author

*Tel: +1 450 768 3232. Fax: +1 450 773 8461 E-mail: joyce.boye@agr.gc.ca.

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Notes

The authors declare no competing financial interest.

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