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Detection and Quantification of Roundup Ready Soy in Foods by Conventional and Real-Time Polymerase Chain Reaction

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Transgenic soybean line GTS-40-3-2, marketed under the trade name Roundup Ready (RR) soy, was developed by Monsanto (USA) to allow for the use of glyphosate, the active ingredient of the herbicide Roundup, as a weed control agent. RR soy was first approved in Canada for environmental release and for feed products in 1995 and later for food products in 1996 and is widely grown in Canada. Consumer concern issues have resulted in proposed labeling regulations in Canada for foods derived from genetically engineered crops. One requirement for labeling is the ability to detect and accurately quantify the amount of transgenic material present in foods. Two assays were evaluated. A conventional qualitative Polymerase Chain Reaction (PCR) assay to detect the presence of soy and RR soy and a real-time PCR to quantify the amount of RR soy present in samples that tested positive in the first assay. PCR controls consisted of certified RR soy reference material, single transgenic soybeans, and a processed food sample containing a known amount of RR soy. To test real-world applicability, a number of common grocery store food items that contain soy-based products were tested. For some samples, significant differences in amplification efficiencies during the quantitative PCR assays were observed compared to the controls, resulting in potentially large errors in quantification. A correction factor was used to try to compensate for these differences.

KEYWORDS: Soybean; Roundup Ready; quantification; real-time PCR; processed foods: genetically modified

INTRODUCTION

The European Union (EU) has had regulations for the labeling of novel foods since 1997. As a consequence, research on the most efficient, reliable, and accurate methods of detecting and quantifying genetically modified (GM) ingredients has been intense (*1*−*12*). Current EU regulations stipulate that products containing an ingredient of which ≥0.9% originates from a GM product must be labeled. For example, if a pure soy product contains ≥1% soy derived from a GM soy variety, it must be labeled, but if it contains only 0.5%, it would not have to be labeled. If the product consisted of only 10% soy and 90% other, but 1% of the soy present was derived from a genetically GM soy variety, it would also require labeling, even though on a weight per weight basis it contains less GM soy than the pure soy product containing 0.5% GM soy that does not require labeling. No mandatory labeling legislation has been implemented in Canada to date. However, the Canadian Food and Drug Act allows for the voluntary labeling of biotechnology-derived foods. Currently, the Canadian General Standards Board (CGSB) in conjunction with the Canadian Council of Grocery Distributors has developed a National Standard for Canada for the voluntary labeling of foods and food ingredients obtained from biotechnology (*13*). The aim of the CGSB is to develop standards, based on recognized international protocols, that will provide a model for label declarations that are understandable and not misleading for Canadian consumers. Their goal is also to establish procedures to distinguish biotechnology-derived foods from conventionally produced foods from production to retail and to establish the use of testing and monitoring procedures. The CGSB does not legislate labeling requirements.

In Canada, there are over 40 plant products containing unique transgenic DNA events approved for food (*14*). Of these, one of the most widely grown in Canada, in 2002, is herbicide-tolerant (GTS-40-3-2) soybean (*15*), with over 100 varieties containing this event registered in Canada. GTS-40-3-2, also known as Roundup Ready (RR), was developed by Monsanto and confers tolerance to the glyphosate-based herbicide Roundup. The GTS-40-3-2 event consists of an enhanced cauliflower mosaic virus 35S promoter, a CTP4 leader sequence from *Petunia hybrida*, and the 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS) gene conferring the herbicide tolerance followed by an *Agrobacterium tumefaciens* nopaline synthase terminator (*15*, *16*). A number of methods have been developed for the detection of RR soy. These include protein-based methods for the detection of the EPSPS gene product in

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Table 1. Soy Foods Used in This Survey

food	degree of processing	form of soy ingredient	food	degree of processing	form of soy ingredient
miso	high	fermented beans	boiled soybean	low	whole bean
soy sauce	high	fermented beans	dried (organic) soybean	low	whole bean
natto	high	fermented beans	roasted soy nuts	low	whole bean
TVP[b]	high	tsp[c]	soy nut snack	low	whole bean
meat alternative 1	high	tsp/soy sauce	soy nut spread	low	whole bean
meat alternative 2	high	tsp	simulated bacon bits	low	flour/soy protein/hsp
cheesies	high	hsp[d]	protein bar 2	low	flour/lecithin/isp
cracker 1	high	hsp	biscuit	low	flour
gravy mix 1	high	oil/hsp	gravy mix 2	low	flour/hsp
frozen dessert	moderate	soy powder/isp	bread 1	low	flour/isp/lecithin
soup	moderate	soy milk	bread 2	low	defatted flour
soybean pâté	moderate	protein	soup mix	low	soy powder
protein bar 1	moderate	roasted soybean/isp[a]	nutritional bar	low	flour/isp/soybean/lecithin/soy butter
soy beverage	moderate	soy milk	cheese	low	flour/oil
meal replacement beverage	moderate	protein	crispbread	low	defatted flour
coffee whitener	moderate	whole bean	cracker 2	low	flour/lecithin
yogurt	moderate	soy milk	flour	low	flour
chocolate pudding	moderate	soy milk			
meat alternative 3	moderate	spc[e]/isp			
tofu	moderate	soybean curd			
infant formula 1	moderate	oil/isp/soy milk			
infant formula 2	moderate	oil/isp/soy milk			

[a] isp, isolated soy protein. [b] TVP, textured vegetable protein. [c] tsp, textured soy protein. [d] hsp, hydrolyzed soy protein. [e] spc, soy protein concentrate.

transgenic raw or unprocessed soy products (*15*, *17*−*19*) and PCR-based methods, both qualitative and quantitative, which can also be used for the more highly processed soy-based food products (*2*, *5*, *6*, *8*−*10*, *15*, *17*, *20*−*22*). Several of these methods have been officially approved or validated by other government agencies (*2*, *15*, *18*, *23*). In this study a variety of foods containing soy ingredients that are available in the local supermarkets were sampled using two different PCR protocols. Our goal was to first establish that soy DNA and Roundup Ready (RR) DNA could be detected in the foods by qualitative PCR, targeting the endogenous soy lectin gene and the CTP4−CTP EPSPS junction of the RR gene (*15*, *17*, *24*), and to determine from what type of foods it was possible to amplify soy DNA and to get an initial impression on the prevalence of genetically modified soy in common food products. Those products testing positive were then further analyzed by real-time quantitative PCR, targeting different regions but the same lectin and RR genes (*15*, *25*) to determine the level of Roundup Ready soy in these foods. Few studies to date have attempted to quantify the amount of transgenic material in processed foods to determine the practical feasibility of such testing.

MATERIALS AND METHODS

Soy Food Samples. A selection of 39 soy food products including vegetarian foods, dry foods, snack foods, condiments, desserts, soups, baby formulas, and beverages representing the different levels of processing (**Table 1**), were chosen from local supermarkets.

Soybean Reference Material. As reference material for qualitative and quantitative analysis, Roundup Ready soybean (variety S14M7) was obtained from a Canadian seed distributor. Because the purity of the seed lot was unknown, single-seed extractions were done to confirm that individual seeds were transgenic. One of these DNA extractions was then used as reference material. The single-seed extract was diluted to 40 ng/μL and serially diluted to 12 pg/μL. Six of these dilutions (5 μL per reaction) were chosen to represent the standard curve encompassing 200 ng−60 pg of DNA.

Quantitative PCR Control. Soybean powder certified reference material (CRM) IRRM-410S (Fluka) 5% Roundup Ready soy used for quantitative real-time PCR was purchased from Sigma (Canada). A processed soy food sample, meat pâté, from the Genetically Modified Material Analysis (GeMMA), Scheme, Report GeM18, with an assigned RR soy content of 8.5%, was also used as a control.

Extraction of Genomic DNA. Each soy food product was homogenized in a Waring electric blender and then ground using an ice-cold mortar and pestle. Approximately 200 mg or 1 g samples from the ground material were used in the subsequent DNA extraction procedure. The single S14M7 soybean seed reference material (~200 mg) was ground using a chilled mortar and pestle, whereas 50 mg of the CRM soybean powder was extracted directly.

Modified Wizard. Briefly, 860 μL of extraction buffer [10 mM Tris (pH 8.0), 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 1% (w/v) sodium dodecyl sulfate], 100 μL of (5 M) guanidine hydrochloride, and 40 μL of (20 mg/mL) proteinase K were added to 200 mg of each ground sample and vortexed to mix thoroughly. For 1 g samples, the extraction buffer, guanidine hydrocloride, and proteinase K were scaled up proportionally. The samples were incubated at 60 °C for 30−60 min followed by a further incubation at 75 °C for 20 min to inactivate the proteinase K. The supernatant generated after centrifugation at full speed for 20 min (to pellet debris) was incubated for 10 min at 60 °C with RNase A added to a final concentration of 1 mg/mL. Following a 3 min centrifugation at full speed, 1 mL of the supernatant was mixed with 1 mL of Wizard resin (Promega) and then applied to a Wizard mini column using a 3 or 5 mL capacity disposable Luer-Lok syringe. For samples that had a dark supernatant (roasted soy nuts, biscuit, natto, and simulated bacon bits), the column was washed with 1 mL of CQW wash buffer (Nucleospin Food Kit, Macherey-Nagel). All samples were washed with 2 mL of 80% ethanol and then centrifuged at full speed for 10 min followed by a 10 min incubation at 37 °C to evaporate residual ethanol. The nucleic acids were eluted in 100 μL of 70 °C 10 mM Tris, pH 8.0.

Qiagen Stool Kit. The soy sauce, chocolate pudding, and miso homogenized samples were dried at 37 °C for 24 h to remove excess moisture. To 1 g of dried sample, ASL buffer (supplied with the Stool Kit) was added until a consistency was reached such that the sample flowed freely in the tube (2−3 mL). The samples were vortexed until thoroughly homogenized. Following a 5 min incubation at 70 °C, the samples were centrifuged at full speed for 10 min to remove particulates. The supernatant was divided into multiple 2.0 mL microfuge tubes and 1 InhibitEX tablet added to each (1 tablet/1.2 mL supernatant). If the volume was <1.2 mL per tube, additional ASL buffer was added. The samples were vortexed to suspend the InhibitEX tablet and then incubated at room temperature for 1 min. The samples were centrifuged

Table 2. Primers Used for Qualitative and Quantitative PCR

primer/probe	sequence 5′ → 3′	product size (bp)	ref
lectin qualitative			
GMO3(forward)	gccctctactccacccccatcc	118	*15*
GMO4(reverse)	gcccatctgcaagccttttgtg		
RR qualitative			
RR04(forward)	ccccaagttcctaaatcttcaagt	180	*24*
RR05(reverse)	tgcgggccggctgcttgca		
lectin quantitative			
LecLC−F(forward)	cctctactccacccccatcca	114	*25*
LecLC-R(reverse)	ccatctgcaagccttttgtg		
LecLCFITC-1 (probe)	ttgccagcttcgccgcttc-FITC		
LecLCR705-1 (probe)	Red705-ttcaacttcaccttctatgcccctgac		
RR quantitative			
RRLC-F20(forward)	accgtcttcccgttaccttg	119	*25*
RRLC-R15(reverse)	gccgggcgtgttgag		
RRSoyaLCFITC-1 (probe)	gccgatggcctccgcaca-FITC		
RRSoyaLCR640-1 (probe)	Red640-gaagtccgccgtgctgctcg		

at full speed for 3 min to pellet the InhibitEX particles. The supernatants were pooled and then incubated for 10 min at 60 °C with 1 mg/mL final concentration of RNase A. Following centrifugation at full speed for 3 min, the pooled supernatants were treated with Qiagen Proteinase K solution and AL (supplied with the Stool Kit) buffer proportionally (for every 200 μL of supernatant, 15 and 200 μL of Proteinase K and AL buffer were added, respectively) according to the manufacturer's instructions. After incubation at 70 °C for 10 min, the amount of 95% ethanol was added according to the manufacturer's instructions. This entire mixture was then applied to the QIAamp spin column. The column was then washed, and the DNA was eluted with 100 μL of AE buffer according to the manufacturer's instructions.

Determination of Yield and Quality of Nucleic Acid Extracts. The nucleic acid extracts were measured using the Biochrom Ultrospec 2100 pro spectrophotometer in 200 mM NaOH (*15*). A conversion factor of 1 OD = 37 ng/μL was used to convert absorbance into concentration units. Sample purity was determined by measuring the A_{260}/A_{280} ratio. The extracts (100 ng−200 ng) were further analyzed by electrophoresis on an 0.8% agarose gel containing 0.1 μg/mL ethidium bromide.

Qualitative PCR Detection of Lectin and RR Sequences. PCR amplification was performed using 100−200 ng of DNA and 100−400 ng of DNA for the lectin and RR assays, respectively. Of the extracts that could not be accurately measured spectrophotometrically (cheesies and miso; $A_{260} < 0.1$), 10 μL of extract was used in the PCR reactions. A 25 μL reaction contained 1× PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), $MgCl_2$ (2.8 mM lectin; 1.8 mM RR; 2.0 mM chloroplast tRNA), 0.2 mM dNTP, 0.5 μM of each primer, and 1.25 units of Taq polymerase. Sequences of primers (Qiagen) used are shown in **Table 2**. The GeneAmp 9700 (Applied Biosystems) thermocycler was used with the following cycling program for lectin: 94 °C for 3 min, 35 cycles of 94 °C for 15 s, 60 °C for 15 s, 72 °C for 20 s, and a final extension at 72 °C for 5 min. The program for the RR PCR was similar except that 40 cycles were used. Products were electrophoresed on a 2% (w/v) agarose gel containing 0.1 μg/mL ethidium bromide.

The limit of detection (LOD) for both the lectin and RR qualitative PCR was determined from serial dilutions of S14M7 soybean seed DNA extracted using the modified Wizard method. Dilutions ranging from 50 ng to 10 pg (lectin) and from 80 to 1 pg (RR) were tested in triplicate. The LOD was defined as the lowest dilution for which the PCR product of expected size was visible by agarose gel electrophoresis in all three replicates. The conversion of mass to genome copy number was based on the haploid genome mass (1C value) for *Glycine max*, which is 1.13 pg, obtained from the Plant DNA C-values Database (*26*).

Real-Time Quantitative PCR of the Lectin and RR Genes. Fluorescence resonance energy transfer (FRET) hybridization probes were used to quantify in the LightCycler (Roche) real-time PCR system. The primers and probes (IT Biochem) used for the lectin and RR assays and the cycling conditions are described by Dahinden et al. (*15*, *25*). The donor probes for both targets were labeled with fluorescein-5-isothiocyanate (FITC), and the acceptor probes for lectin and RR genes were labeled with R705 and R640, respectively. Sequences of primers and probes used are shown in **Table 2**. PCR was performed in glass capillary tubes supplied by Roche. A total reaction volume of 20 μL contained 5 μL (15−200 ng) of DNA template, 50 mM Tris, pH 8.3, 0.4 μM of each probe, 0.25 mM of each dNTP, 0.5 μM of each primer, 250 μg/μL BSA, and 1 unit of Fast Start Taq polymerase (Roche). The lectin reactions contained 4.0 mM $MgCl_2$, and the RR reactions contained 2.5 mM $MgCl_2$. A serial dilution of $>3 \times 10^3$ (200 ng−60 pg per reaction) of total genomic DNA extracted from the S14M7 single seed, amplified in separate reaction tubes using either the lectin primers/probes or RR primers/probes, was used to generated the calibration curves for lectin and RR, respectively. The standard curves were the regression of crossing point (Cp) versus log of the nanograms of standard in each reaction, where the crossing point is the cycle at which the reaction fluorescence increases above a baseline level defined by the software (see **Table 3**). Each food sample was run in triplicate for each target. For the initial run, three different template concentrations for each sample were analyzed to determine which gave a Cp that best fit within the standard curve. In the subsequent run, this concentration was analyzed in duplicate, and the mean of all three Cp values was used in the analysis. Data were analyzed using LightCycler Data Analysis software version 3.5.5 and the "second derivative" algorithm.

Determination of Percent RR Soy Relative to Total Soy DNA in Food Samples. Two standard curves were generated from seven replicates of the lectin and RR assays. The equations from the linear regressions of these curves were used to calculate the nanograms of lectin or RR DNA in the food samples on the basis of the mean of the crossing points. The percent RR soy was determined from the ratio of nanograms of RR divided by the nanograms of lectin multiplied by 100. Some foods required more template in the RR reaction compared to the lectin reaction in order to bring the crossing points within the range of the standard curve (4 × template, meat alternatives 1 and 2, soup mix; 8 × template, protein bars 1 and 2;× 20 × template, tofu and 5% Fluka CRM), and therefore the RR values had to be divided by these factors before the ratio to lectin could be made.

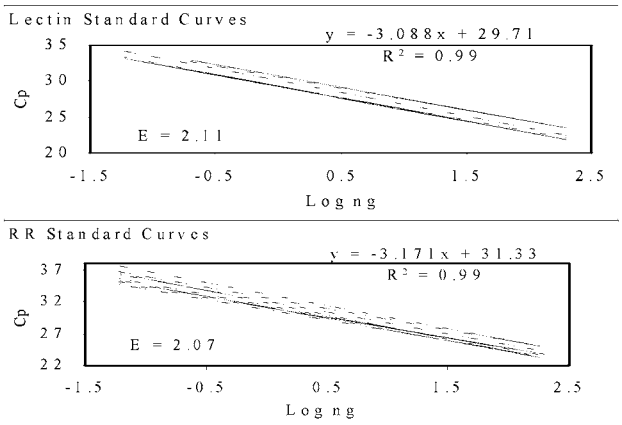
RESULTS

Classification of Soy-Containing Foods. The 39 foods chosen for this study had soy listed as an ingredient on the label and are representative of a wide range of soy-containing foods available in the Canadian marketplace. The soy ingredients included whole bean, flour, defatted flour, powder, soy protein, soy milk, and lecithin. The proportion of soy relative to other food ingredients varied from product to product and could not be experimentally determined. **Table 1** lists the soy foods and the degree of processing of the soy ingredients. Degree of processing was based on previously published descriptions of soybean products and their methods of preparation (*15*, *27*, *28*). Fermented, extruded (textured soy protein), pressed (lecithin and oil), and hydrolyzed soy protein soy ingredients were classified as highly processed. Mild alkaline extracted (isolated soy protein), water extracted (milk), precipitated (curd), and roasted ingredients were considered to be moderately processed. Whole beans and ground beans (flour) were considered to be the least processed. Foods with a number of different forms of soy in the ingredients were classified on the basis of the least processed form.

DNA Extraction. All samples were initially extracted using the modified Wizard method. Those samples that gave no or poor lectin amplification by qualitative PCR were re-extracted with the Qiagen Stool Kit (soy sauce, chocolate pudding, miso, gravy mix 1, natto, cracker 1, and cheesies). To determine the degree of degradation, and therefore the quality of the extracted DNA, ∼200 ng of each sample was visualized by agarose gel electrophoresis (**Figure 1**). Many of the food extracts contained

Table 3. Quantitative Results for the Soy Foods

Sample	Target	Standard (ng)	Cp[a]	SD[b]
Standard	Lectin	200.00	22.53	0.74
Standard	Lectin	100.00	23.61	0.77
Standard	Lectin	25.00	25.35	0.65
Standard	Lectin	6.25	27.17	0.58
Standard	Lectin	0.78	30.27	0.72
Standard	Lectin	0.06	33.36	0.32
Standard	RR	200.00	23.92	0.67
Standard	RR	100.00	25.06	0.62
Standard	RR	25.00	26.79	0.71
Standard	RR	6.25	28.82	0.83
Standard	RR	0.78	31.92	0.88
Standard	RR	0.06	35.02	0.39



Sample	Target	Cp[a]	SD[b]	ng Target	%RR/Soya	Corr. %RR/Soya
TVP	Lectin	25.58	0.10	21.7		
	RR	27.83	0.11	12.7	58	
Meat alternative 1	Lectin	24.77	0.06	39.8		
	RR[c]	33.14	0.08	0.067	0.17	<LOQ
Soup mix	Lectin	25.62	0.03	21.1		
	RR[c]	30.37	0.18	0.502	2.4	
Meat alternative 3	Lectin	26.53	0.08	10.7		
	RR[c]	33.80	0.29	0.042	0.39	5.0
Protein bar 1	Lectin	24.65	0.06	43.5		
	RR[d]	33.78	0.44	0.021	0.05	<LOQ
Protein bar 2	Lectin	25.16	0.11	29.7		
	RR[d]	34.61	0.33	0.012	0.04	<LOQ
Tofu	Lectin	25.39	0.09	25.1		
	RR[e]	33.87	0.38	0.008	0.03	0.43
Simulated bacon bits	Lectin	23.55	0.16	98.8		
	RR	25.85	0.03	53.5	54	
Frozen dessert	Lectin	26.44	0.20	11.5		
	RR	28.73	0.05	6.60	58	
Infant formula 1	Lectin	25.58	0.05	21.7		
	RR	27.73	0.13	13.7	63	
Bread 1	Lectin	28.30	0.37	2.86		
	RR	30.27	0.21	2.16	75	
Bread 2	Lectin	28.83	0.12	1.93		
	RR	30.81	0.18	1.46	76	2477
Biscuit	Lectin	26.41	0.10	11.7		
	RR	29.30	0.05	4.37	37	
Gravy mix 2	Lectin	27.60	0.11	4.82		
	RR	29.36	0.12	4.18	87	
5% Fluka	Lectin	25.30	0.05	26.8		
	RR[e]	26.92	0.06	1.23	4.6	
GeMMA Meat Pâté	Lectin	28.54	0.07	2.39		
	RR	33.85	0.18	0.16	6.7	

[a] Cp, crossing point (mean of nine replicates for standard curves and mean of three replicate for the food samples). [b] SD, standard deviation. [c] Four times more template used in the RR reaction compared to the lectin reaction. [d] Eight times more template used in the RR reaction compared to the lectin reaction. [e] Twenty times more template used in the RR reaction compared to the lectin reaction.

degraded DNA with an average fragment size of <564 bp. Yields from the various foods ranged from 0.15 μg/100 mg (soy sauce) to 38 μg/100 mg textured vegetable protein (TVP). The cheesies and miso samples extracted with the Qiagen Stool Kit both had absorbances at 260 nm of <0.1 and therefore could not be measured accurately. All of the samples had A_{260}/A_{280} ratios between 1.7 and 1.9 with the exception of soy sauce (2.3), cheesies (2.0), and coffee whitener (2.0).

Qualitative PCR. To determine if there was amplifiable soy DNA in the food extracts, PCR was performed using the lectin specific primers GMO3 and GMO4 (*15*). The LOD for the lectin assay was 50 pg (44 genome copies) of total soy DNA (data

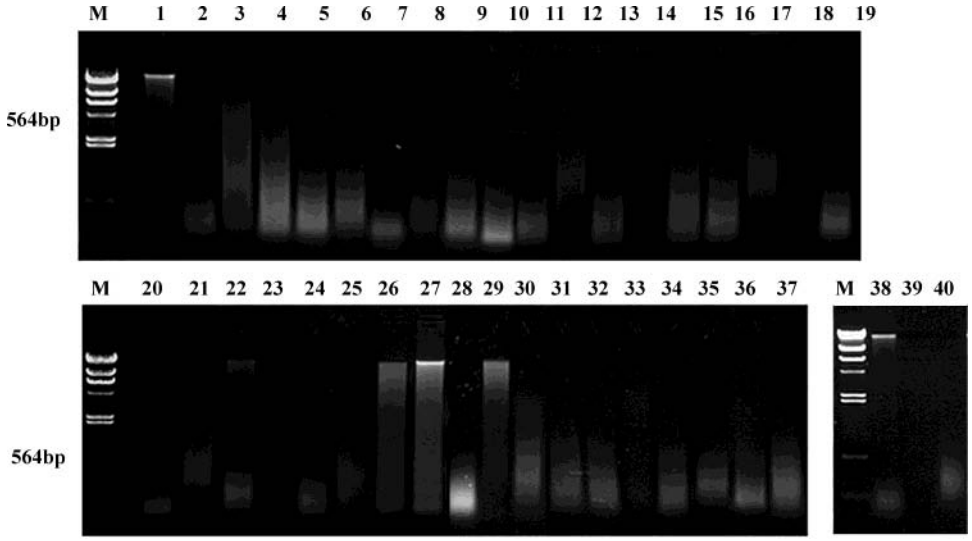


Figure 1. Agarose gel electrophoresis of nucleic acids extracted from the soy foods using the modified Wizard method unless otherwise stated: (lane 1) dried (organic) soybean; (lane 2) soy nut spread; (lane 3) tofu; (lane 4) bread 2; (lane 5) bread 1; (lane 6) meat alternative 1; (lane 7) soybean pâté; (lane 8) cracker 2; (lane 9) meat alternative 2; (lane 10) soup; (lane 11) meat alternative 3; (lane 12) yogurt; (lane 13) infant formula 1; (lane 14) meal replacement beverage; (lane 15) protein bar 2; (lane 16) protein bar 1; (lane 17) coffee whitener; (lane 18) soy beverage; (lane 19) infant formula 2; (lane 20) miso (Qiagen Stool Kit); (lane 21) TVP; (lane 22) gravy mix 2; (lane 23) frozen dessert; (lane 24) soy nut snack; (lane 25) nutritional supplement bar; (lane 26) soup mix; (lane 27) gravy mix 1; (lane 28) boiled soybean; (lane 29) simulated bacon bits; (lane 30) crispbread; (lane 31) cracker 1 (Qiagen Stool Kit); (lane 32) cheese; (lane 33) cheesies (Qiagen Stool Kit); (lane 34) roasted soy nuts; (lane 35) natto; (lane 36) biscuit; (lane 37) flour; (lane 38) S14M7 soybean; (lane 39) soy sauce (Qiagen Stool Kit); (lane 40) chocolate pudding (Qiagen Stool Kit); (lane M) molecular weight marker II (Roche).

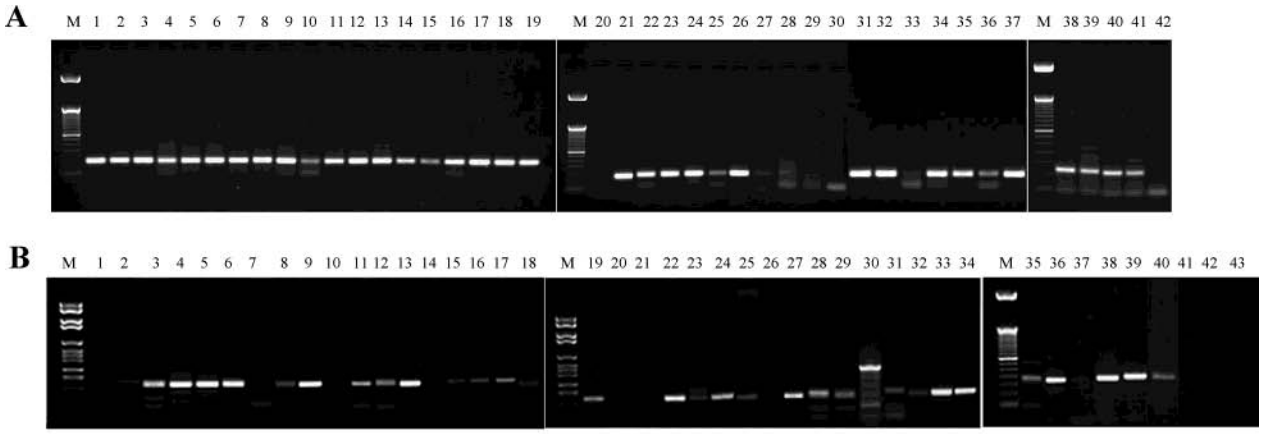


Figure 2. (**A**) Qualitative PCR analysis of lectin and Roundup Ready DNA in food samples. The 118 bp PCR product represents the amplified region of the lectin gene using primers GMO3 and GMO4: (lane 1) dried (organic) soybean; (lane 2) soy nut spread; (lane 3) tofu; (lane 4) bread 2; (lane 5) bread 1; (lane 6) meat alternative 1; (lane 7) soybean pâté; (lane 8) cracker 2; (lane 9) meat alternative 2; (lane 10) soup; (lane 11) meat alternative 3; (lane 12) yogurt; (lane 13) infant formula 1; (lane 14) meal replacement beverage; (lane 15) protein bar 2; (lane 16) protein bar 1; (lane 17) coffee whitener; (lane 18) soy beverage; (lane 19) infant formula 2; (lane 20) miso; (lane 21) TVP; (lane 22) gravy mix 2; (lane 23) frozen dessert; (lane 24) soy nut snack; (lane 25) nutritional supplement bar; (lane 26) soup mix; (lane 27) gravy mix 1; (lane 28) cracker 1; (lane 29) boiled soybean; (lane 30) simulated bacon bits; (lane 31) natto; (lane 32) biscuit; (lane 33) chocolate pudding; (lane 34) soy sauce; (lane 35) positive control (S14M7); (lane 36) flour; (lane 37) cheesies; (lane 38) cheese; (lane 39) crispbread; (lane 40) roasted soy nuts; (lane 41) positive control (S14M7); (lane 42) water control (no DNA); (lane M) 50 bp DNA ladder (Invitrogen). (**B**) The 180 bp PCR product represents the CTP4–CP4EPSPS junction of the Roundup Ready transgene using primers RR04 and RR05: (lane 1) dried (organic) soybean; (lane 2) soy nut spread; (lane 3) tofu; (lane 4) bread 2; (lane 5) bread 1; (lane 6) meat alternative 1; (lane 7) soybean pâté; (lane 8) cracker 2; (lane 9) positive control (S14M7); (lane 10) soup; (lane 11) meat alternative 3; (lane 12) yogurt; (lane 13) infant formula 1; (lane 14) meal replacement beverage; (lane 15) protein bar 2; (lane 16) protein bar 1; (lane 17) infant formula 2; (lane 18) cheesies; (lane 19) gravy mix 2; (lane 20) soy nut snack; (lane 21) nutritional supplement bar; (lane 22) soup mix; (lane 23) miso; (lane 24) cracker 1; (lane 25) TVP; (lane 26) meat alternative 2; (lane 27) water (no DNA); (lane 28) coffee whitener; (lane 29) soy beverage; (lane 30) cheese; (lane 31) crispbread; (lane 32) roasted soy nuts; (lane 33) gravy mix 1; (lane 34) frozen dessert; (lane M) molecular weight marker VI (Roche); (lane 35) boiled soybeans; (lane 36) simulated bacon bits; (lane 37) natto; (lane 38) biscuits; (lane 39) positive control (S14M7); (lane 40) flour; (lane 41) chocolate pudding; (lane 42) soy sauce; (lane 43) water (no DNA); (lane M) 50 bp ladder (Invitrogen).

not shown). **Figure 2A** shows the amplified lectin band (118 bp) for each of the soy foods. Lectin PCR products from miso, gravy mix 1, cheesies, and natto were weak, and there were no clear amplification products of the expected size from the

cracker 1 sample. These foods were re-extracted using the Qiagen Stool Kit, which improved amplification from cheesies, miso, and cracker 1 but not from natto or gravy mix 1 (**Figure 2A**).

The 39 food samples were subsequently analyzed using primers RR04 and RRO5 (15) specific for the CTP4−CP4EPSPS junction of the RR transgene. Of these 39 samples, 28 tested positive for RR (**Figure 2B**). The LOD for this assay was 10 pg (eight genome copies) of RR soy DNA (data not shown). Three samples, gravy mix 1, miso, and cheesies, which amplified poorly with the lectin-specific primers, weakly amplified with the RR specific primers. RR could not be detected in natto and cracker 1, which were also only weakly amplified with the lectin primers. The dried (organic) soybean, soybean pâté, soup, meal replacement beverage, soy nut snack, nutritional supplement bar, crispbread, chocolate pudding, and soy sauce, which strongly amplified with the lectin primers, also tested negative in the RR PCR. Bands with a higher molecular weight than expected were observed in miso, cracker 1, coffee whitener, and crispbread. In previous experiments, we found that amplification from rye and wheat using the RR-specific primers gave amplified products of sizes similar to those in the crispbread and cracker 1, respectively, suggesting nonspecific amplification from rye or wheat in these samples (unpublished results).

Quantitative PCR. The 28 food samples that tested positive for the RR trait were further analyzed by quantitative real-time PCR. The standard curves for both the lectin and the RR assays were made from serially diluted S14M7 DNA (see Material and Methods). Dilutions in the range of 200−0.06 ng were used to generate the standard curves. Nine interassays for lectin and RR were performed, and the mean efficiencies (*E*) were calculated to be 2.11 and 2.07, respectively (**Table 3**). Data were linear through the range of dilutions; therefore, 0.06 ng (53 genome copies) was defined as the limit of quantification (LOQ). This limit is well above the experimentally determined LOQ of 30 copies reported by Berdal and Holst-Jensen (*4*, *15*). For a pure (100%) soy product, the LOQ expressed as %RR/soy can be defined as the lowest quantifiable amount of RR soy DNA (0.06 ng) divided by the highest quantifiable amount of soy DNA (200 ng) multiplied by 100 (e.g., 0.06 ng/200 ng $\times$ 100 = 0.03%). The LODs for the lectin and RR quantitative assays were not determined. The total soy DNA in the foods was determined using primers LecLC-F and LecLC-R and probes LecLCFITC-1 and LecLCR705-1, and the total RR DNA in the food products was determined using primers RRLC-F20 and RRLC-R15 and probes RRSoyLCFITC-1 and RRSoyLC640-1 (**Table 2**).

Initially, three serial dilutions of the DNA food extracts (25−200 ng) were analyzed to determine *E* for both the lectin and RR assays. A subsequent assay in duplicate for each food extract was used to calculate the %RR DNA relative to total soy DNA. **Table 3** summarizes the results for the foods that were above the LOQ for the RR quantitative PCR assay. The nanograms of target DNA was determined by comparing the mean crossing point of each sample to the standard curve. Cheesies, miso, and gravy mix 1 had a weak lectin amplicon in the qualitative PCR (**Figure 2A**) and were below the limit of quantification for the lectin in the real-time PCR (data not shown). For this reason, the amount of RR soy in these samples was not quantified. Fourteen of the 36 food samples contained quantifiable levels of RR soy that ranged from 0.03 to 87%. Foods that contained levels of RR soy >5% included TVP, frozen dessert, infant formula 1, simulated bacon bits, bread 1, bread 2, biscuit, and gravy mix 2. Foods that contained <5% RR soy included meat alternative 1, meat alternative 3, tofu, protein bar 1, protein bar 2, and soup mix. In order for the quantitative results for these foods to fall within the RR standard curve, 4−20 times more DNA had to be added to the RR reactions compared to the lectin reactions. Eleven samples contained only trace amounts of amplifiable RR-specific DNA, which could not be quantified in the real-time PCR assays. These included yogurt, boiled soybean, flour, cheese, infant formula 2, soy beverage, roasted soy nuts, cracker 2, coffee whitener, meat alternative 2, and soy nut spread.

Table 4. Lectin and RR PCR Efficiencies for the Soy Foods

food	lectin E[a]	ΔE[b]	RR E[c]	ΔE[d]	$\Delta\Delta\lvert E\rvert$[e]
TVP	1.95	0.16	1.85	0.22	0.06
meat alternative 1	2.11	0.00	2.31	−0.24	0.24
soup mix	1.93	0.18	1.81	0.26	0.08
meat alternative 3	2.05	0.06	1.88	0.19	0.13
protein bar 1	1.95	0.16	2.18	−0.11	0.27
protein bar 2	1.90	0.21	2.13	−0.06	0.27
tofu	1.98	0.13	1.83	0.24	0.11
simulated bacon bits	2.08	0.03	1.94	0.13	0.10
frozen dessert	1.81	0.30	1.77	0.30	0.00
infant formula 1	2.01	0.10	1.96	0.11	0.01
bread 1	2.01	0.10	2.05	0.02	0.08
bread 2	1.98	0.13	1.74	0.33	0.20
biscuit	1.97	0.14	1.97	0.10	0.04
gravy mix 2	1.95	0.16	1.86	0.21	0.05
5% Fluka	1.97	0.14	1.95	0.12	0.02

[a] *E*, efficiency of the lectin PCR calculated from the slope of logarithm of the ng of template versus Cp for three dilutions of the sample ($E = 10^{(-1/\text{slope})}$). [b] ΔE, difference in efficiency between the food sample and the lectin standard curve (2.11, calculated from the slope in **Table 3**). [c] *E*, effciency of the RR PCR for three dilutions of the sample. [d] ΔE, difference in efficiency between the food sample and the RR standard curve (2.07, calculated from the slope in **Table 3**). [e] $\Delta\Delta E$, difference in efficiency of the food samples between the lectin and RR PCR.

A positive control containing 5% RR soy (Fluka) was analyzed together with the samples and calculated to contain 4.6% RR/total soy DNA. GeMMA sample GeM18, meat pâté, was used as a processed food control. GEM18 was given an assigned value of 8.5% RR soy by GeMMA and was calculated in this study to contain 6.7% RR soy.

Calculations of %RR soy in the food samples were based on the assumption that the real-time PCR amplification *E* values of the standards and samples were the same. To determine the validity of this assumption, *E* values of the food samples were calculated and compared to the standards. Whereas *E* values for the standards are based on a standard curve consisting of six dilutions spanning a little more than 3 orders of magnitude (200−0.06ng) repeated nine times for a total of 54 data points, each food sample consisted of only three dilutions spanning <1 order of magnitude repeated three times for a total of nine data points. The amplification efficiencies of the food samples ranged from $1.81 \leq E \leq 2.11$ for lectin and from $1.74 \leq E \leq 2.31$ for RR (**Table 4**). For the lectin reaction, all samples had *E* values equal to or less than the standard. For the RR assay, meat alternative 1, protein bar 1, and protein bar 2 had *E* values greater than the standard ($E = 2.31$, 2.18, and 2.13) with the other food samples having *E* values equal to or less than the standard (**Table 4**). The difference in *E* between food sample and standard for each of the lectin and RR assays is expressed as ΔE. For most of the foods, ΔE values for the RR assay deviated from the standard to a greater degree than the lectin assay. The difference in ΔE values between the lectin and RR assays for each food sample is expressed as $\Delta\Delta E$. A low value of $\Delta\Delta E$ indicates that the efficiencies for the lectin and RR

assays for a sample deviated from the standard to the same degree. Eight food samples and the 5% Fluka standard had $\Delta\Delta E$ values of ≤ 0.1. The remaining six foods had $\Delta\Delta E$ values > 0.1 (**Table 4**). For food samples with $\Delta\Delta E$ values > 0.1, a correction factor (*29*)

$$Cp_{corr} = Cp_{meas} \times \frac{\log (E_f)}{\log (E_s)} \quad (1)$$

where E_f is the efficiency measured for the food sample and E_s is the efficiency for the corresponding standard, was used to calculate corrected percent RR values (**Table 3**). When this correction factor was applied, meat alternative 1 and protein bars 1 and 2 were no longer within the LOQ, for bread 2 the %RR values rose from 76 to >2400%, and for one sample (meat alternative 3) the %RR soy changed significantly (from 0.39 to 5.0%) which could alter the classification of this food with respect to a proposed Canadian labeling threshold of 5%.

DISCUSSION

In anticipation of food labeling for genetically modified products in Canada, a pilot project was undertaken to detect and quantify RR soy in a variety of soy-containing foods available in the Canadian marketplace. RR soy was chosen because the transgenes for this event are well characterized, a number of published tests for the detection and quantification are available, and it is widely grown and used in both Canada and the United States and was therefore expected to be detectable in a wide range of food products. From the survey, soy could be detected in 100% of the 39 food samples tested, and RR soy was detected in 28 (72%) of these foods. Of the foods testing positive, 11 foods (28%) contained quantifiable levels of RR soy, 8 of which (21%) contained levels of RR soy >5% and would be candidates for labeling.

Assay Controls. To test the accuracy of quantitative tests, two known controls were analyzed. The 5% RR soybean powder (Fluka) IRMM certified reference standard was calculated to contain 4.6% RR soy, an error of only 8%. The second control, GeM18 meat pâté sample, is more representative of the processed foods analyzed in this study. To summarize, the composition and preparation of this sample was as follows. A 6−7% RR soy (w/w) of total soy flour mix was added to other pâté ingredients (pig liver, pork belly, water, skimmed milk powder, salt, sorbic acid, sodium polyphosphate, pepper, onion powder, and monosodium glutamate) at a level of 5%, mixed, and baked at 130 °C for 1 h and then at 150 °C until a core temperature of 80 °C was obtained. Homogeneity testing of the pâté samples by GeMMA (10 subsamples, 2 replicates of each) gave a mean RR soy/total soy value of 5.5% with a range from 3.8 to 8.9%. The assigned %RR value for the pâté, based on the consensus mean of 81 laboratories participating in the GeM18 proficiency panel, was 8.5%, with acceptable individual results (based on *z* scores) ranging from 3.4 to 21.4%. In this study, the meat pâté sample was calculated to contain 6.7% RR soy/total soy, which is close to both the mean value determined by homogeneity testing and the assigned value. Close agreement of the experimentally determined and accepted values for both the RR flour and processed food controls gives confidence that the values obtained for the unknown processed food samples are accurate.

Food-Processing Effects. The food samples tested contained soy as an ingredient ranging from low (whole bean or flour) to highly processed (fermented bean). In general, it can be assumed that increasing levels of processing result in a decrease in the amount and quality of soy DNA that can be extracted due to degradation of the DNA caused by the different processing methods. This can have a significant effect on the ability to detect/quantify the amount of soy in these foods by PCR. Agarose gel electrophoresis of the DNA extracted from the food samples showed that many contained highly degraded DNA with average fragment sizes of <564 base pairs (bp). The four separate PCR assays used in this study amplified fragments from 114 to 180 bp in length, which are small enough to amplify from DNA that has been significantly degraded. For two of the most highly processed food products tested, miso and cheesies, both lectin and RR could be detected but not quantified. This is due to the very low yield of DNA obtained, which could not be quantified spectrophotometrically. To obtain sufficient DNA for quantitative analysis, larger amounts of food sample would have to be used in the DNA extraction procedure. For both miso and cheesies this would likely have resulted in relatively high levels of RR because both could be detected in the small amount of DNA extracted. However, it should be noted that the LOD of the RR PCR is lower than the lectin PCR (8 copies versus 44 copies).

Extraction Efficiencies and DNA Purification. Previous studies have reported difficulty extracting amplifiable DNA from soy sauce (*6*, *15*, *21*); however, using 1 g of dried soy sauce and the Qiagen Stool Kit, enough DNA could be obtained for spectrophotometric quantification and subsequent amplification. DNA from food products containing chocolate have been shown to require additional purification steps to remove the cocoa plant secondary metabolites thought to inhibit PCR (*1*, *15*, *20*−*22*). Although the lectin-specific PCR did not produce a product from the Wizard extracted chocolate pudding, DNA obtained from chocolate pudding using the Qiagen Stool Kit could be amplified. The Qiagen Stool Kit was designed to effectively remove known inhibitors of PCR and has been demonstrated by Tengel et al. (*22*) to be effective in removing PCR-inhibitory compounds in cocoa products. However, for protein bars 1 and 2, which list cocoa as an ingredient, DNA extracted using the modified Wizard method could be amplified in the PCR assays. The proportion of cocoa in the protein bars may be low enough that the PCR-inhibitory compounds are not enough to significantly affect the PCR. The Qiagen Stool Kit also improved the quantitative PCR from cheesies, miso, and cracker, although the amount of DNA obtained was too low to be accurately measured spectrophotometrically for cheesies and miso. Lectin could be weakly detected in gravy mix 1, natto, and cracker 1, and RR could be detected in only gravy mix 1. Soy in the form of oil/hsp in gravy mix 1 likely contains only low amounts of degraded soy DNA, which does not correspond to the high molecular weight DNA extracted from gravy mix 1 (**Figure 1**). The majority of DNA from these samples probably originates from ingredients other than soy. Alternatively, these extracts may contain PCR inhibitors not removed using the Qiagen Stool Kit. Infant formulas 1 and 2 contained the same type of soy ingredients, soy milk, isp, and oil, and both showed comparable DNA fragment sizes on the agarose gel (**Figure 1**). Infant formula 1 contained 63% RR soy, whereas infant formula 2, which contained about half as much total soy as infant formula 1, was below the LOQ for the RR assay. It would therefore appear that very little of the soy in infant formula 2 was RR soy, whereas in infant formula 1 a high percentage of the soy was derived from RR soy. There is a large difference in the quality of DNA in the extracts among the foods that were shown to contain higher levels of RR soy. For example, the DNA extracted from the frozen dessert is so degraded that it is not

visible on the agarose gel, whereas breads 1 and 2 have a greater abundance of higher molecular weight DNA fragments (**Figure 1**).

These results highlight problems of accurately measuring the amount of soy that is highly processed and/or present in a complex food product. In the first situation, highly processed soy products require a very large sample in order to obtain sufficient DNA for analysis. In the second situation, where soy is an ingredient of a complex food product, the total amount of DNA extracted may not be limiting, but the quality and quantity of the DNA to be amplified are limiting. If the food contains soy as a highly processed ingredient together with other food ingredients, these two factors are compounded. This has significant implications for compliance labeling purposes. It has been suggested that certain processed food ingredients that contain little or no amplifiable DNA be exempt from testing/labeling (*30*). However, this raises additional questions on how products to be exempt are to be defined. An alternative solution would be to test for genetically modified soy before processing or before it is added as a food ingredient.

Amplification Efficiencies. For precise quantification by real-time PCR, amplification efficiencies of the standards and sample must be the same (*4*, *15*). For many quantitative PCR experiments this assumption is made but not tested. When the source material for the standards and the samples are of the same matrix (e.g., both source and sample DNA are extracted from whole soybean), this assumption is probably close enough to be valid. Calculations show that differences in PCR efficiencies of only 5% can result in a 2-fold difference in measurement after 26 cycles (*31*). In this study, the standards consisted of high molecular weight DNA extracted from unprocessed soybean. The only current certified reference standards available on the market for quantitative PCR analysis of RR soy are ground flour mixtures produced by the Joint Research Centre, Institute for Reference Materials and Measurement. In contrast, many of the food samples analyzed in this study contained degraded soy DNA as a result of processing. In addition, soy was often only one of several food ingredients in a complex food sample. Essentially, each food represents a different matrix, often very different from the unprocessed soybean standard, used in this study. Each food potentially contains compounds that are not removed during the DNA extraction procedure, which could affect the efficiency of the PCR despite the use of DNA extraction procedures that can remove known PCR inhibitors. Therefore, for the quantitative PCR analysis of foods for genetically modified ingredients, the assumption cannot be made that efficiencies of standards and samples are the same.

Several methods have been reported to compare differences in PCR efficiencies (E) between standard and sample. A series of multiplex reactions can be used to calculate E (*32*); E values can be determined on the basis of the kinetics of the individual sample reactions (*33*) or calculated on the basis of a series of dilutions (*29*). In this study, E values were based on a series of dilutions. The mean calculated values for the standards were $E = 2.11$ (lectin) and $E = 2.07$ (RR). Each value is based on a total of 54 data points spanning a dilution range of 3×10^3, which is statistically twice the number required for an accurate determination of E over this range. E values for the food samples, however, are based on only nine data points for over a dilution range of <1 order of magnitude. As the dilution range decreases, the number of data points that are required for an accurate determination of E increases. Whereas a dilution range of 10^3 requires only 27 data points, a dilution range of 10^1 would require 240 data points, which is clearly not practical. It is also not possible to significantly expand the dilution range by further diluting the sample due to the limited amount of soy DNA extracted from the foods or by increasing the total amount of DNA used in the assay due to inhibitory effects. As a result, calculated E values for the foods are an approximation only and most likely overestimate the difference in E values between the standards and foods. E values for the foods differed from the standards (ΔE) by as much as 0.30 (lectin) and 0.33 (RR). In most cases, E values for the food samples were less than that of the standard. This suggests that inhibitors were present in the food DNA extractions. If the reduction in E of the sample was due to the presence of inhibitors, it might be expected that both the lectin and RR assays would be equally affected. This can be determined from the difference in the ΔE values of the lectin and RR assays ($\Delta\Delta E$). A small $\Delta\Delta E$ for a food sample indicates that the E values for both the lectin and RR assays differed from the E values of the standard by the same amount. Eight foods and the 5% Fluka standard had $\Delta\Delta E$ values of ≤ 0.1. For example, in the frozen desert sample, Cp values for lectin and RR are 26.44 and 28.73 and ΔE values were 0.30 and 0.30, respectively. For both reactions this would result in a calculated underestimation of the amount of lectin and RR by $\sim 80\times$. However, the $\Delta\Delta E$ value is 0.00. Therefore, because the efficiencies of the lectin and RR assays deviate from the mean value of the standards by the same degree, the ratio of the measured amounts of each (percent RR) is still valid, even if the absolute values are incorrect. Some of the foods had relatively high $\Delta\Delta E$ values (>0.10) which could indicate that the %RR determined is incorrect. High $\Delta\Delta E$ could be due to a number of factors, for example, differences in the robustness of the lectin and RR assays. A greater variation in the RR assay for the standards and the food samples was observed compared to the lectin assay, which could indicate that the RR assay is less robust. However, greater variations in the food quantitative RR assay may also be because the amount of RR relative to the total amount of soy can be much lower. Meat alternative 1, protein bar 1, and protein bar 2 contained low measured amounts of RR (0.04−0.39%) and had the largest $\Delta\Delta E$ values (0.24−0.27). To compensate for foods with $\Delta\Delta E$ values of >0.10, a correction factor was applied (eq 1). When this correction factor was applied to meat alternative 1 and protein bars 1 and 2, the amount of RR present was calculated to be below the LOQ. It has been demonstrated that low concentrations of target can lead to a calculation artifact with the LightCycler (*34*). The curve of Cp versus log of concentration, at low target concentrations, is no longer linear but parabolic, and calculations of E within this range would give artificially high values if a correction factor was not used. This appears to be the case for meat alternative 1 and protein bars 1 and 2.

Applying the correction factor to bread 2 ($\Delta\Delta E = 0.20$) resulted in an increase in %RR from 76 to 2477%, which clearly is not possible. This result is probably due to an error in the calculation of E for bread 2 due to an insufficient number of data points. Bread 2 contained the least amount of soy of all foods quantified (<1% of the total DNA extracted could be attributed to soy); slight errors in measurement at these concentrations would have a large effect on calculated E values. The correction factor was also applied to meat alternative 3 ($\Delta\Delta E = 0.13$) and tofu ($\Delta\Delta E = 0.11$), resulting in a measured increase in %RR of $\sim 10\times$ from 0.39 to 5.0% and from 0.03 to 0.43%, respectively. For all corrected samples, the change in calculated %RR would not have affected the classification and subsequent labeling under the newly proposed Canadian voluntary labeling guide for genetically modified products. The

usefulness of the correction factor is directly related to the accuracy with which *E* for the foods can be determined. Unfortunately, there is not a fully satisfactory and practical method for determining *E* for the foods. The method used here gives only an approximation that probably overestimates the change in *E* relative to the standards. As such it presently could be useful in determining the amount of error in the final percent values until more accurate methods of determining *E* for the foods have been developed.

Sampling Issues. The food samples tested were individual items that represent a random sampling. The results obtained apply only to those items, and no statement about any particular product can be inferred with respect to the presence or amount of RR soy. For labeling purposes, it would be necessary to test a population of samples from any one batch of products in order to make a meaningful statistical statement concerning the level of RR soy present. What has been demonstrated is a practical method for the detection and quantification of RR soy in a wide range of real world food products that have undergone various degrees of processing. Both qualitative and quantitative PCR methods are strongly influenced by the amount, quality, and presence of inhibitors in the DNA extractions from these foods. In some cases, the amount/quality of DNA extracted was too low for quantification either because the soy was highly processed and the DNA degraded or because the amount of soy present in the food was too low. Factors that alter the efficiencies of the quantitative PCRs unequally can dramatically affect the end results; however, it is possible, in theory, to compensate using a correction factor. Further work needs to be done to test whether the use of a correction factor is valid when applied to food samples.

Summary. For the majority of foods tested, unequivocal results could be obtained for the presence/absence of soy and RR soy. In the quantitative assays, two main groups were observed: foods with trace amounts of RR soy, $\leq 0.4\%$, suggesting adventitious contamination of soy with RR soy, and foods with relatively high levels of RR soy, $\geq 37\%$, suggesting RR soy was an intended ingredient. Of the 39 foods tested, only 2 samples were close to the proposed 5% level for labeling (soup mix and meat alternative 3). Many questions remain to be answered before reliable routine testing of foods can take place. For example, what is the accuracy and how much error is in the calculated %RR when testing foods. Although most of the foods tested fell into two broad groups that contained either trace amounts of RR soy or relatively high amounts of RR soy and could be easily separated as either above or below 5%, categorizing foods that test close to this level is more problematic until there is a better understanding of the amount of error present in the calculation. In particular, methods need to be developed for a more accurate determination of *E* for the foods being tested so that the correction factor can be more confidently applied. Failing that, an alternative would be to develop reference standards that are more representative of the foods being tested. These questions are difficult to answer without knowing the true %RR in the food samples, and studies using defined nontransgenic/transgenic flour mixtures are not truly representative of real-world food samples. Therefore, further real-time quantitative PCR experiments using defined flour mixtures processed into foods will be necessary.

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