Immunodetection of CP4 EPSPS Protein in Roundup Ready Flex Cotton MON 88913 Defatted, Raw Cottonseed Meal Following Heat Treatment

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Submitter: _____ Date: _____

Sponsor Representative: _____ Date: May 10, 2005

Study Director: _____ Date: May 10 2005

Quality Assurance Statement

Study Title: Immunodetection of CP4 EPSPS Protein in Roundup Ready Flex Cotton MON 88913 Defatted, Raw Cottonseed Meal Following Heat Treatment

Study Number: 04-01-45-09

Reviews conducted by the Quality Assurance Unit confirm that the final report accurately describes the methods and standard operating procedures followed and accurately reflects the raw data of the study.

Following is a list of reviews conducted by the Monsanto Regulatory Quality Assurance Unit on the study reported herein.

Dates of Inspection / Audit	Phase	Date Reported To:	
		Study Director	Management
01/07/2005	Heat Stability	01/10/2005	01/10/2005
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Joan M. Rejda-Heath
Quality Assurance Unit
Monsanto Regulatory, Monsanto Company

May 10, 2005
Date

Study Information

Study Number: 04-01-45-09

Title: Immunodetection of CP4 EPSPS Protein in Roundup Ready Flex Cotton MON 88913 Defatted, Raw Cottonseed Meal Following Heat Treatment

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Study Completion Date: May 10, 2005

Study Certification

The results reported in this Final Report accurately reflect the data generated under study number 04-01-45-09.

Approved By:

Andre Silvanovich, Ph.D.
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May 10, 2005
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Abbreviations[1] and Definitions

APS	Analytical protein standard
CFR	Code of Federal Regulations
COA	Certificate of Analysis
CP4 EPSPS	5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium tumefaciens* sp. strain CP4, encoded by the *cp4 epsps* gene
ECL	Enhanced chemiluminescence
E. coli	*Escherichia coli*
EPA	Environmental Protection Agency
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
LOD	Limit of detection
NFDM	Non-fat dry milk
SDS-PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
PBST	Phosphate buffered saline containing Tween-20
PCR	Polymerase chain reaction
ppm	Parts per million
PVDF	Polyvinylidene difluoride
SOP	Standard operating procedure
VOI	Verification of Identity

[1] Standard abbreviations, e.g. units of measure, concentration, mass, time, etc., are used without definition according to the format described in "Instructions to Authors" in The Journal of Biological Chemistry.

1.0 Summary

Monsanto has developed a second-generation cotton product, Roundup Ready® Flex cotton, MON 88913, which provides increased tolerance to glyphosate compared to the original Roundup Ready cotton product. MON 88913 was produced by the stable insertion of the *epsps* (5-enolpyruvylshikimate-3-phosphate synthase) coding sequence from *Agrobacterium sp.* strain CP4 into the genome of conventional cotton. The *cp4 epsps* encodes a glyphosate-tolerant EPSPS (CP4 EPSPS) that confers tolerance to glyphosate, the active ingredient in the Roundup® family of agricultural herbicides.

The purpose of this study was to assess the immunodetectability of the CP4 EPSPS protein in MON 88913 defatted, raw cottonseed meal after heat treatment. Following grinding and defatting, cottonseed meal from MON 88913 or control cotton, was heated in an autoclave to simulate the heating process used commercially to process cottonseed meal. Heated and unheated samples of defatted, raw cottonseed meal from the test and control substances were extracted with two buffers and the extracts were analyzed using the western blot method to detect the presence of the CP4 EPSPS protein. Image analyses of immunoreactive bands on blot films were used to provide a semiquantitative estimate of immunoreactive CP4 EPSPS protein levels in the heated and unheated extracts of cottonseed meal from MON 88913 and control cottonseed meal.

The results of this study demonstrate that the level of immunodetectable CP4 EPSPS protein present in extracts of the MON 88913 after heating was below the LOD, 2 ppm, or had decreased by greater than 98% from its original value, regardless of the buffer solution employed for extraction. These results clearly indicate that the heating of cottonseed meal, in a manner similar to the conditions employed for the commercial processing of cottonseed meal, results in the loss of immunodetectable CP4 EPSPS protein. This loss is likely due to protein degradation or aggregation into an insoluble complex as a result of heat treatment.

2.0 Introduction

Monsanto has developed a second-generation cotton product, Roundup Ready® Flex cotton, MON 88913, which provides increased tolerance to glyphosate compared to the original Roundup Ready cotton product. MON 88913 was produced by the stable insertion

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of the *epsps* (5-enolpyruvylshikimate-3-phosphate synthase) coding sequence from *Agrobacterium sp.* strain CP4 into the genome of conventional cotton. The *cp4 epsps* encodes a glyphosate-tolerant EPSPS (CP4 EPSPS) that confers tolerance to glyphosate, the active ingredient in the Roundup® family of agricultural herbicides.

Food crops that have been developed through agricultural biotechnology for commercial use are thoroughly assessed for their safety. A key issue in the safety assessment of such crops is the potential allergenicity of the proteins introduced into food crops. One aspect of this assessment includes evaluation of the heat stability of the target protein. Manufacturing process steps, such as heating, fermentation, or chemical treatment, may alter the allergenic potential of food by changing the physicochemical properties of proteins. For example, heating completely eliminates the allergenic potential of the patatin protein in potato (Koppelman et al., 2002) and chitinases present in fruits (Sanchez-Monge et al., 2000). Heating may also alter the digestibility of the proteins, thus, changing the potential allergenicity of food (Takagi et al., 2003). On the other hand, a number of food allergens, such as peanut allergen Ara h 1 (Koppelman et al., 1999), and the lipid-transfer proteins present in peach (Brenna et al., 2000) and maize (Pastorello et al., 2003), are extremely stable to heat treatment.

This study was performed to evaluate how heat treatment, which simulates the heating process used commercially to process cottonseed meal (Lee et al., 2001), affects the immunodetectability of the CP4 EPSPS protein present in MON 88913.

3.0 Purpose

The purpose of this study was to assess the immunodetectability of the CP4 EPSPS protein in MON 88913 defatted, raw cottonseed meal following heat treatment.

4.0 Materials

4.1 Test Substance

The test substance for this study was MON 88913. In this study, cottonseed containing CP4 EPSPS protein was evaluated. Cottonseed (LIMS 03CTSEO00086 produced from seed lot number GLP-0308-14234-S) was harvested from plants grown in the field under Production Plan 03-08-45-01. Records of test substance production were archived with the Production Plan 03-08-45-01. Cottonseed from the test substance were stored at room temperature prior to processing for the study.

4.2 Control Substance

The control substance was the negative segregant of MON 88913 cottonseed [MON 88913(-)] which does not contain the coding sequence for the CP4 EPSPS protein. Cottonseed (LIMS 03CTSEO00116 produced from seed lot number GLP-0308-14235-S) was harvested from plants grown in the field under Production Plan 03-08-45-01. Records of control substance production were archived with the Production Plan 03-08-45-01. Cottonseed from the control substance were stored at room temperature prior to processing for the study.

4.3 Reference Substance

The reference standard in this study was CP4 EPSPS protein, produced in and purified from *E. coli* (APS lot 20-100015). The reference standard was stored until use in a –80 °C freezer in a buffer solution [50mM Tris-Cl, pH 7.5, 50mM KCl, 2mM DTT, 25% (v/v) glycerol, 1mM benzamidine-HCl] at a total protein concentration of 3.8 mg/ml based on amino acid composition analysis.

Analytical reference standards (e.g. molecular weight markers for SDS-PAGE) used in this study are documented in the study data and are described in this final report.

4.4 Characterization of the Test, Control, and Reference Substances

Prior to study initiation, the identity of the test and control substances was confirmed by event specific polymerase chain reaction (PCR) analysis. Characterization documentation for the test substance is archived under lot GLP-0308-14234-S and for the control substance under lot GLP-0308-14235-S. Copies of the certificate of analysis (COA) for the test and control substance starting seed and verification of identity (VOI) for the harvested test and control seed are archived with this study. The reference standard was originally characterized under Characterization Plan 20-100015 using a battery of analyses. These analyses included protein concentration by amino acid analysis, molecular weight and purity estimation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), gel staining and densitometry, molecular weight estimation by MALDI analysis, identity determination by N-terminal sequence analysis, MALDI and immunoblot, and strength and identity determination by functional assay. A copy of the COA for the CP4 EPSPS protein is archived with this study. Copies of the information sheets for the analytical reference standards used are archived with this study.

5.0 Experimental Design

Raw cottonseed from the test and control substances was ground to a powder in liquid nitrogen and defatted by treatment with hexane. Subsamples of defatted, raw cottonseed meal from the test and control substances that had undergone heat treatment are referred to as "heated" samples. The remainder of the defatted, raw cottonseed meal from the test and control substances are referred to hereafter as an "unheated" sample. Extraction from heated and unheated samples was performed using phosphate buffered saline containing Tween 20 (PBST) or 2× Laemmli buffer (Laemmli, 1970). Extracts were subjected to electrophoresis on SDS-PAGE gels and analyzed by the western blot method for the presence of the CP4 EPSPS protein.

The western blot limit of detection (LOD) analysis for the CP4 EPSPS protein spiked into an extract prepared from unheated control substance was determined concurrently. The reference standard protein was run on each gel with extracts from test and control substances to positively identify the position of the CP4 EPSPS protein. Also, protein molecular weight standards were run on each gel to determine the approximate molecular weight of each immunoreactive band and assess protein transfer to the blot membrane. Signal intensity from the immunoreactive bands was determined for all samples, except for samples where signal was not observed. The heat treatment was considered to have an impact on the immunodetectability of the CP4 EPSPS protein if a greater than or equal to 20% difference in immunoreactivity was observed between heated and unheated samples.

6.0 Methods

The purpose of this study was to evaluate the effect of heat treatment on the immunologically detectable level of the CP4 EPSPS protein in defatted, raw cottonseed meal containing MON 88913 using the western blot method and image analysis of blot films. Defatted, raw cottonseed meal from either MON 88913, or control cotton, was heated in an autoclave for 20 min at 17 psi steam pressure followed by a 5 min drying to simulate heat processing used commercially to process cottonseed meal. Two separate buffers, PBST, representing a relatively mild aqueous extraction buffer with physiological ionic strength and pH, and 2× Laemmli buffer, that represents denaturing and reducing extraction conditions were used for sample extraction. Extracted proteins were separated by SDS-PAGE and analyzed for the presence of CP4 EPSPS protein by the western blot method using anti-CP4 EPSPS specific antibodies. The standard curves, representing different quantities of the CP4 EPSPS spiked into the PBST and 2× Laemmli buffer extracts of unheated control substance, were prepared and analyzed by

western blot method (see Sections 6.4 and 6.5). The amount of the CP4 EPSPS protein present in the PBST and 2× Laemmli buffer extracts of unheated and heated samples of test substance was estimated using these standard curves.

6.1 Grinding and Defatting of the Cottonseed

Approximately 5 g of the test substance, MON 88913, and the control were ground to a powder in the presence of liquid nitrogen. After grinding, the test and control substances were stored in a -80 °C freezer until defatting. The processed cottonseed meal from the test and control substances was transferred to separate flasks and defatted by three sequential extractions in hexane (pre-heated to approximately 50 °C) using periodic mixing. The hexane was removed by filtration after each extraction. Residual hexane in the grain powder was removed by evaporation at room temperature in a fume hood overnight. The defatted, raw cottonseed meal was then stored in a -80 °C freezer until use.

6.2 Heat Treatment of Cottonseed Meal

Samples (~ 2.0 g) of the defatted, raw cottonseed meal test and control substances were transferred to glass beakers and heated in an autoclave for 20 min at 17 psi steam pressure followed by 5 min drying time with a 150 °C temperature setting to release steam pressure. The aforementioned steam pressure yielded a heating temperature of ~123.1 °C based upon standard steam pressure tables. Following heat treatment, the test and control substances were stored in a -80 °C freezer until protein extraction.

6.3 Protein Extraction

Extraction was performed on the heated and unheated samples using two separate buffers: PBST [1 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4 and 0.05% (v/v) Tween 20] and 2× Laemmli buffer [composed of 50 µl β-mercaptoethanol + 950 µl of 62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 25% (v/v) glycerol, 0.01% (w/v) Bromophenol Blue per ml of buffer]. Heated and unheated samples of the test and control substances were extracted in PBST or 2× Laemmli buffer at 1:5 weight-to-volume (w/v) ratios and clarified by centrifugation. PBST extraction was performed for 60 min in a 4 °C refrigerator with gentle rocking on a Nutator. The 2× Laemmli sample buffer extraction was performed with gentle rocking on a Nutator for 10 min at room temperature. A 500 µl subsample of each clarified extract was then diluted by the addition of 500 µl PBST for the 2× Laemmli buffer extracts or 500 µl 2× Laemmli buffer for

the PBST extracts. The diluted extracts were heated for 5 min at ~100 °C and then stored in a -80 °C freezer.

The table below summarizes the coding system used to identify samples during the heating procedure, extraction, and subsequent western analysis.

Cottonseed Meal	Treatment	Extraction Buffer	Code
MON 88913(-) negative segregant control substance	Unheated	PBST	C-UH-P
		2× Laemmli buffer	C-UH-L
	Heated	PBST	C-H-P
		2× Laemmli buffer	C-H-L
MON 88913 test substance	Unheated	PBST	T-UH-P
		2× Laemmli buffer	T-UH-L
	Heated	PBST	T-H-P
		2× Laemmli buffer	T-H-L

6.4 Western Blot Analysis

Prior to western blot analysis, the test and control substance extracts were further diluted 20-fold (yielding a final dilution of 40-fold relative to the initial extract) with a solution composed of equal volumes of 2× Laemmli and PBST. Samples containing a CP4 EPSPS protein spike were diluted 20-fold as described above and then diluted an additional 1.2-fold by the addition of the CP4 EPSPS protein spike yielding a final dilution of 48-fold relative to the initial extract. Aliquots of the test and control samples, and control samples containing a CP4 EPSPS protein spike were separated by SDS-PAGE using pre-cast 12-well 4-20% polyacrylamide gradient Tris-glycine mini-gels and Tris-glycine SDS running buffer (Invitrogen, Carlsbad, CA and Bio-Rad, Hercules, CA, respectively). Electrophoresis was performed at 175 V for 60 minutes. Following electrophoresis, proteins were electrotransferred to a PVDF membrane (Invitrogen) for 60 min at a constant voltage of 25 V. Control extracts were analyzed on each gel to account for any nonspecific binding of the antibody. Pre-stained molecular weight markers (Precision Plus Dual Color, Bio-Rad) were used to verify electrotransfer of proteins to the membrane and estimate the molecular weight of immunoreactive bands. A lane containing CP4 EPSPS

protein standard spiked into a control extract background was included on each gel to positively identify the position of the CP4 EPSPS protein on the blots.

All membranes were blocked overnight at ~4 °C with 5% (w/v) non-fat dry milk (NFDM) in PBST. The membranes were incubated with a previously characterized goat anti–CP4 EPSPS serum (lot 6844572) at a dilution of 1:4000 in 1% (v/v) NFDM in PBST for 1 h. Incubation in the primary antibody and all subsequent steps were performed at room temperature. Excess serum was removed by three 10 min washes with PBST. The membrane was incubated with horseradish peroxidase (HRP)–conjugated anti-goat IgG (Sigma Chemical Co, St. Louis, MO) at a dilution of 1:10,000 in 1% (v/v) NFDM in PBST for 1 h and washed again with PBST (three 10 min washes). Immunoreactive bands on the membrane were visualized using the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia, Buckinghamshire, England) by exposing the membrane to Hyperfilm ECL film (Amersham Pharmacia) for 30 sec and 60 sec. Films were developed using a Konica SRX101A automated film processor (Tokyo, Japan).

To account for any matrix effect on immunodetectability of the CP4 EPSPS protein, the CP4 EPSPS LOD was determined by spiking 5 µl aliquots of various dilutions of the CP4 EPSPS protein reference standard into 25 µl aliquots of the unheated control extracts. The LOD was defined as the lowest detectable standard visible on the blots. Gels to determine the LOD were run concurrently with the gels for the test and control extracts and, after transfer to the PVDF membrane, subjected to the same western blot procedure as described above. The following amounts of CP4 EPSPS protein standard were used for the LOD western blot analysis: 10, 5, 2.5, 1, 0.5, 0.25, 0.1, and 0.05 ng.

6.5 Image Analysis of Immunoreactive Bands on Films

Image analysis of immunoreactive bands on the 60 sec exposure blot films was conducted using a Bio-Rad model GS-800 calibrated imaging densitometer (Hercules, CA) supplied with Quantity One software (version 4.4.0). The levels of signals for immunoreactive bands on the blot films were determined as band volumes (average band OD x band area in mm^2). Due to the close proximity of the bands to one another in adjacent lanes, the software was unable to recognize the bands as separate entities automatically; therefore, all bands were drawn manually. Consistent with the intent of the LOD blots, seven visible reference standard points (representing different amounts of CP4 EPSPS protein spiked into respective PBST or 2× Laemmli buffer extracts of the unheated control extract)

were plotted versus adjusted band volume data using the Quantity One software. Using a linear fit, coefficients of determination (R^2) of 0.991 and 0.980 were obtained for the PBST and 2× Laemmli buffer extracts, respectively. These values exceed the minimum acceptance criteria of $R^2 \geq 0.95$ established in SOP BR-ME-0932. Based on the acceptability of the standard curve, the volume quantity data for protein bands detected in the test substance samples were converted to nanograms (ng) of protein using the FORECAST worksheet function of Microsoft Excel software (SP3 Version 10.6730.6718). The quantity of detected protein for each sample was divided by the mg of cottonseed tissue to calculate the concentration of CP4 EPSPS protein in parts per million (ppm).

7.0 Data Rejected or Not Reported

The 30 sec exposures of blots containing the PBST and 2× Laemmli buffer extracts and the respective LOD blots yielded low CP4 EPSPS band intensity by visual examination. No further analysis was performed on these blots.

8.0 Control of Bias

Appropriate controls and standards were included with each analysis. The reference standard protein was spiked into the control substance to account for any possible influences of the matrix on the detection of the CP4 EPSPS protein. Electroblots of gels with standard protein samples for the determination of the LOD and with test and control extracts were run concurrently to minimize run-to-run variations.

9.0 Protocol Amendments

A typographical error was identified in the lot numbers for the cottonseed after the protocol was signed by the study director. The incorrect lot numbers were corrected by amendment. This amendment had no impact on the study outcome.

10.0 Results and Discussion

10.1 Analysis of the CP4 EPSPS Protein Levels in PBST Buffer Extracts of Heated and Unheated Cottonseed Meal

A single immunoreactive band on the western blot with an approximate molecular weight of 44 kDa was identified as the full-length CP4 EPSPS protein from PBST

buffered extracts (Fig. 1, lanes 2, 4 and 5). In addition, likely proteolytic breakdown products of CP4 EPSPS were observed as weaker intensity bands of 35-40 kDa (Fig. 1, lanes 4 and 5). Based on the standard curve, the amount of full length CP4 EPSPS protein in the unheated test substance extracts was estimated to be 5.20 ng and 8.50 ng in 5 and 10 µl of 40-fold diluted extract loads, respectively.

No CP4 EPSPS protein or the presumed CP4 EPSPS proteolytic fragments were detected in the extract of the heated test substance (Fig.1, lanes 7 and 8). The extracts of heated and unheated control samples were assessed concurrently with extracts of the test substance to confirm antibody specificity. As expected, CP4 EPSPS protein was not detected in the unheated or heated control substance extracts (Fig. 1, lanes 9 and 10).

A western blot was run concurrently to assess the LOD of the full length CP4 EPSPS protein spiked into the PBST buffer extract of unheated control substance. The LOD was defined as the lowest detectable standard visible on blots. The LOD was determined as described in Section 6.4 and was visually estimated to be 0.1 ng (Fig. 2 lane 9).

Calculations of the level of immunodetectable full length CP4 EPSPS protein in the test substance extracts in ppm (equivalent to ng protein per mg cottonseed meal or µg per g cottonseed meal) and the effect of heat treatment on the level of immunodetectable protein are summarized in Table 1. A total of 0.20 g of the cottonseed meal sample was extracted in a total volume of 1.0 ml PBST buffer, and then 500 µl of clarified extract was diluted by the addition of 500 µl 2× Laemmli buffer. This yielded a final concentration of approximately 0.1 g tissue per ml of extract or 0.1 mg tissue per µl of extract. Furthermore, prior to analysis, the extracts were diluted an additional 20-fold (yielding a final dilution of 40-fold relative to the initial clarified extract). Based on image analysis, 5.20 ng and 8.50 ng of the CP4 EPSPS protein was detected in 5 µl and 10 µl of 40-fold diluted extract, representing 0.025 mg and 0.050 mg of tissue, respectively. Therefore, the estimated protein concentration of the CP4 EPSPS protein was 208.0 ppm and 170.0 ppm calculated for 5 µl and 10 µl loads, respectively:

$$\frac{5.20\ ng\ CP4\ EPSPS\ protein}{0.025\ \ mg\ tissue} = 208.0\ \frac{ng}{mg}\ or\ 208.0\ ppm\ of\ CP4\ EPSPS\ protein$$

$$\frac{8.50\ ng\ CP4\ EPSPS\ protein}{0.050\ mg\ tissue} = 170.0\ \frac{ng}{mg}\ or\ 170.0\ ppm\ of\ CP4\ EPSPS\ protein$$

The average concentration of the CP4 EPSPS protein in the unheated test substance cottonseed meal was calculated as 189.0 ppm.

The LOD was estimated to be 0.1 ng of the CP4 EPSPS protein detected in diluted control substance extract representing 0.05 mg of tissue. Therefore, the LOD was calculated to be 2.0 ppm:

$$\frac{0.1\ ng\ CP4\ EPSPS\ protein}{0.050\ mg\ tissue} = 2.0\frac{ng}{mg}\ or\ 2.0\ ppm\ of\ CP4\ EPSPS\ protein$$

The LOD value represents 1.1% of the total protein loaded

$$\frac{2.0\ ppm}{189.0\ ppm} = 0.011\ or\ 1.1\%$$

Because CP4 EPSPS protein was not observed in the PBST extract of the heated test substance cottonseed meal, it can be inferred that as a result of heat treatment the level of immunodetectable CP4 EPSPS protein was below the LOD. Thus, immunodetectable protein had declined by greater than 98% $(100\% - 1.1\% = 98.9\%)$ from its original calculated level.

10.2 Analysis of the CP4 EPSPS Protein Levels in 2× Laemmli Buffer Extracts of Heated and Unheated Cottonseed Meal

Results obtained with 2× Laemmli buffer extract parallel the results obtained with PBST buffer extract. An immunoreactive band on the western blot, with an approximate molecular weight of 44 kDa was identified as the full-length CP4 EPSPS protein (Fig. 3, lanes 2, 4 and 5). In addition, likely proteolytic breakdown products of CP4 EPSPS were observed as weaker intensity bands of 35-40 kDa (Fig. 3, lanes 4 and 5). Based on the standard curve, the amount of full length CP4 EPSPS protein in the unheated test substance extracts was estimated to be 2.89 ng and 5.63 ng in 5 and 10 µl of 40-fold diluted extract loads, respectively.

No full length CP4 EPSPS protein or the presumed CP4 EPSPS proteolytic fragments were detected in the extract of heat-treated test substance (Fig. 3, lanes 7 and 8). The extracts of heated and unheated control samples were assessed concurrently with the extracts of heated and unheated test substance to confirm antibody specificity. As expected, CP4 EPSPS protein was not detected in unheated or heated control substance extracts (Fig. 3, lanes 9 and 10).

A western blot was run concurrently to assess the LOD of full length CP4 EPSPS protein spiked into the 2× Laemmli buffer extract of unheated control substance. The LOD was determined as described in Section 6.4 and was visually estimated to be 0.1 ng (Fig. 4, lane 9).

Calculations of the level of immunodetectable full length CP4 EPSPS protein in the test substance extracts (in ppm) and the effect of heat treatment on the level of immunodetectable protein were performed as described in Section 10.1 and are summarized in Table 1. A total of 0.20 g of the cottonseed meal sample was extracted in a total volume of 1.0 ml 2× Laemmli buffer, and then 500 µl of clarified extract was diluted by the addition of 500 µl PBST buffer. This yielded a final concentration of approximately 0.1 g tissue per ml of extract or 0.1 mg tissue per µl of extract. Furthermore, prior to analysis, the extracts were diluted an additional 20-fold (yielding a final dilution of 40-fold relative to the initial clarified extract). Based on image analysis, 2.89 ng and 5.63 ng of CP4 EPSPS protein was detected in 5 µl (equivalent to 0.025 mg of tissue) and 10 µl (equivalent to 0.050 mg of tissue) of extract. Therefore, the estimated protein concentration of the CP4 EPSPS protein in 2× Laemmli buffer extract was 115.6 ppm and 112.6 ppm in the 5 µl and 10 µl loads, respectively:

$$\frac{2.89\ ng\ CP4\ EPSPS\ protein}{0.025\ mg\ tissue} = 115.6\frac{ng}{mg}\ or\ 115.6\ ppm\ of\ CP4\ EPSPS\ protein,$$

$$\frac{5.63\ ng\ CP4\ EPSPS\ protein}{0.050\ mg\ tissue} = 112.6\frac{ng}{mg}\ or\ 112.6\ ppm\ of\ CP4\ EPSPS\ protein$$

The average concentration of the CP4 EPSPS protein in the unheated test substance cottonseed meal was calculated as 114.1 ppm.

The LOD was estimated to be 0.1 ng of the CP4 EPSPS protein detected in diluted control substance extract representing 0.05 mg of tissue. Therefore, the LOD was calculated to be 2.0 ppm:

$$\frac{0.1\ ng\ CP4\ EPSPS\ protein}{0.05\ mg\ tissue} = 2.0\ \frac{ng}{mg}\ or\ 2.0\ ppm\ of\ CP4\ EPSPS\ protein$$

The calculated LOD value represents 1.8% of the average total protein loaded:

$$\frac{2.0\ ppm}{114.1\ ppm} = 0.018\ or\ 1.8\%$$

Because the CP4 EPSPS protein was not observed in the 2× Laemmli buffer extract of the heated test substance cottonseed meal, it can be inferred that as a result of heat treatment the level of immunodetectable CP4 EPSPS protein was below LOD. Thus, immunodetectable protein had declined by greater than 98% $(100\% - 1.8\% = 98.2\%)$ from its original calculated level.

11.0 Conclusions

The results of this study demonstrate that the level of immunodetectable CP4 EPSPS protein present in extracts of MON 88913 after heating was below the LOD, or had decreased by greater than 98% of its original value, regardless of the buffer solution employed for extraction. These results clearly indicate that the heating of defatted, raw cottonseed meal in an autoclave to simulate heat processing used commercially to process cottonseed meal results in the loss of immunodetectable CP4 EPSPS protein likely due to protein degradation or aggregation into an insoluble complex as a result of heat treatment.

12.0 References

Brenna, O., Pompei, C., Ortolani, C., Pravettoni, V., Farioli, L., and Pastorello, E.A. (2000). Technological process to decrease the allergenicity of peach juice and nectar. *J. Agric. Food Chem.* **48**: 493-497.

Koppelman, S. J., Bruijnzeel-Koomen, C. A. F. M., Hessing, M., and de Jonghm, H. H. J. (1999). Heat-induced conformational changes of *Ara h 1*, a major peanut allergen, do not affect its allergenic properties. *J. Biol. Chem.* **274:** 4770-4777.

Koppelman, S. J., van Kpningsveld, G. A., Knulst, A. C., Gruppen, H., Pigmans, I. G. A. J., and de Jonghm, H. H. J. (2002). Effect of heat-induced aggregation on the IgE binding of patatin (Sol t 1) is dominated by other potato proteins. *J. Agric. Food Chem.* **50**: 1562-1568.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227**: 680-685.

Lee, T. C., Lee, J. L. and Astwood, J. D. (2001). Effect of Heat Treatment on the Immunodetection of Cry2Ab2, GUS, Cry1Ac and NPTII Proteins produced in Cotton Event 15985. Monsanto Technical Report, St. Louis, MSL-16810.

Pastorello, E. A., Pompei, C., Pravettoni, V., Fariolo, L., Calamari, A. M., Scibilia, J., Robino, A. M., Conti, A., Iametti, S., Fortunato, D., Bonomi, S., and Ortolani, C. (2003). Lipid-transfer protein is the major maize allergen maintaining IgE-binding activity after cooking at 100 °C, as demonstrated in anaphylactic patients and patients with positive double-blind, placebo-controlled food challenge results. *J. Allergy Clin. Immunol.* **112**: 775-783.

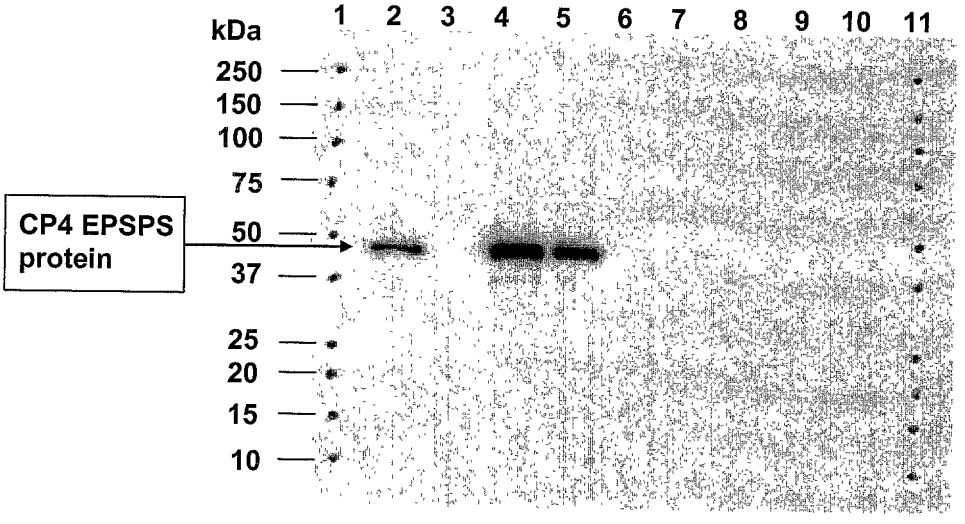
Sanchez-Monge, R., Blanco, C., Perales, A. D., Collada, C., Carrillo, T., Aragoncillo, C., and Salcedo, G. (2000). Class I chitinases, the panallergens responsible for the latex-fruit syndrome, are induced by ethylene treatment and inactivated by heating. *J. Allergy Clin. Immunol.* **106**: 190-195.

Takagi, K., Teshima, R., Okunuki, H., and Sawada, J. (2003). Comparative study of in vitro digestability of food proteins and effect of preheating on the digestion. *Biol. Pharm. Bull.* **26**: 969-973.

Table 1. Summary of the CP4 EPSPS Protein Concentrations in PBST and 2x Laemmli Buffer Extracts of Heated and Unheated MON 88913

Extraction Buffer	LOD		Heat Treatment	Test substance sample quantitation				
	ng	ppm		Volume loaded (µl)	CP4 EPSPS detected (ng)	Amount of tissue loaded (mg)	CP4 EPSPS concentration (ppm)	Average CP4 EPSPS concentration (ppm)
PBST	0.1	2.0	Unheated	5	5.20	0.025	208.0	189.0
				10	8.50	0.050	170.0	
			Heated	5	not detected[1]	0.025	<LOD	<LOD
				10	not detected[1]	0.050	<LOD	
2x Laemmli Buffer	0.1	2.0	Unheated	5	2.89	0.025	115.6	114.1
				10	5.63	0.050	112.6	
			Heated	5	not detected[1]	0.025	<LOD	<LOD
				10	not detected[1]	0.050	<LOD	

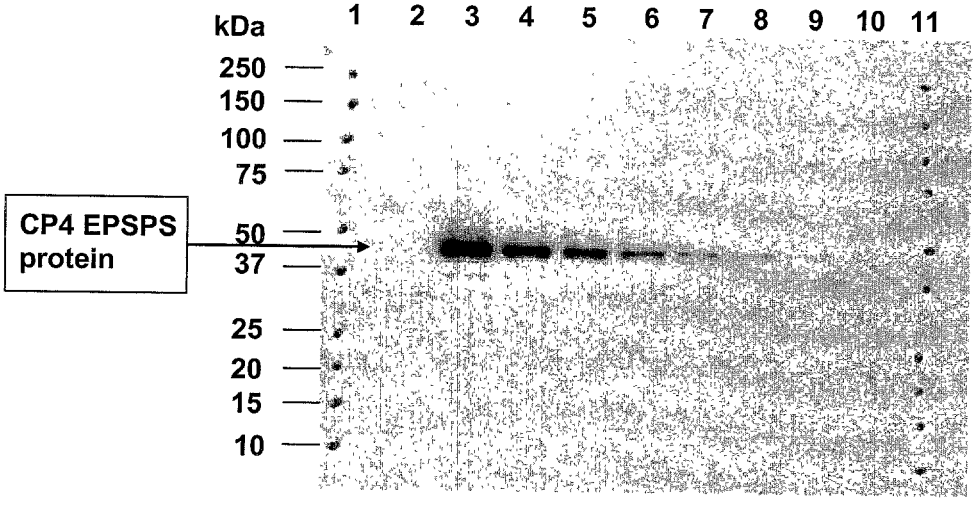
[1] Signal from CP4 EPSPS protein was not observed on the western blot films.



Lane	Sample	Volume Loaded (µl)
1	Molecular weight markers	10
2	1 ng CP4 EPSPS protein reference standard in unheated control extract	12
3	Empty	—
4	Unheated test substance PBST extract	10
5	Unheated test substance PBST extract	5
6	Empty	—
7	Heated test substance PBST extract	10
8	Heated test substance PBST extract	5
9	Unheated control substance PBST extract	10
10	Heated control substance PBST extract	10
11	Molecular weight markers	10

Figure 1. Western Blot Demonstrating the Effect of Heat Treatment on the Immunodetectability of the CP4 EPSPS Protein in PBST Extracts of Heated and Unheated MON 88913

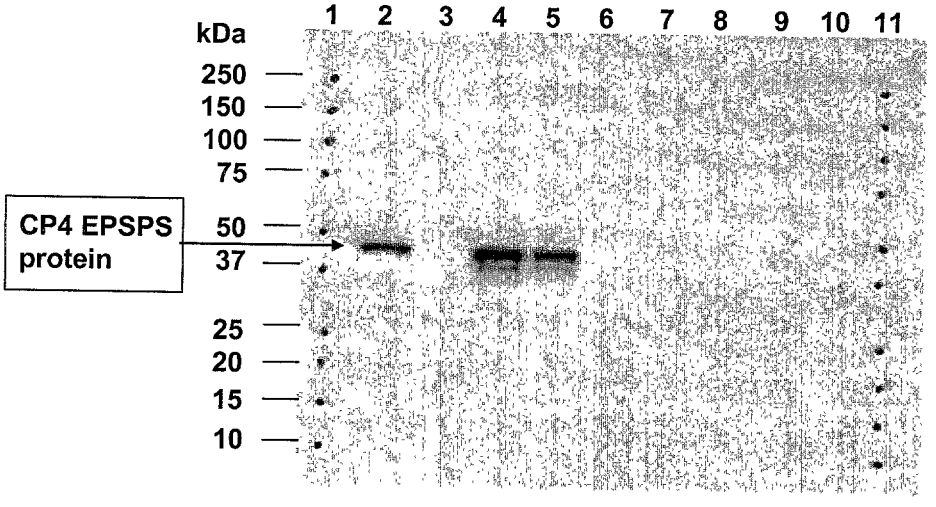
PBST extracts of the test and control substance samples were separated by a 4-20% polyacrylamide gradient Tris-glycine gel under reducing and denaturing conditions and transferred to a PVDF membrane by electroblot. Western blot analysis was conducted as described in Section 6.4. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in lanes 1 and 11. Lane 12 which is empty has been cropped in this figure.



Lane	Sample	Volume Loaded (µl)	Amount Loaded (ng)
1	Molecular weight markers	10	—
2	Unheated control substance extract	10	—
3	CP4 EPSPS spiked into unheated control extract	12	10
4	CP4 EPSPS spiked into unheated control extract	12	5
5	CP4 EPSPS spiked into unheated control extract	12	2.5
6	CP4 EPSPS spiked into unheated control extract	12	1
7	CP4 EPSPS spiked into unheated control extract	12	0.5
8	CP4 EPSPS spiked into unheated control extract	12	0.25
9	CP4 EPSPS spiked into unheated control extract	12	0.1
10	CP4 EPSPS spiked into unheated control extract	12	0.05
11	Molecular weight markers	10	—

Figure 2. Western Blot Demonstrating Limit of Detection of the CP4 EPSPS Protein Reference Standard Spiked into the PBST Extract of Unheated Control Substance MON 88913 (-)

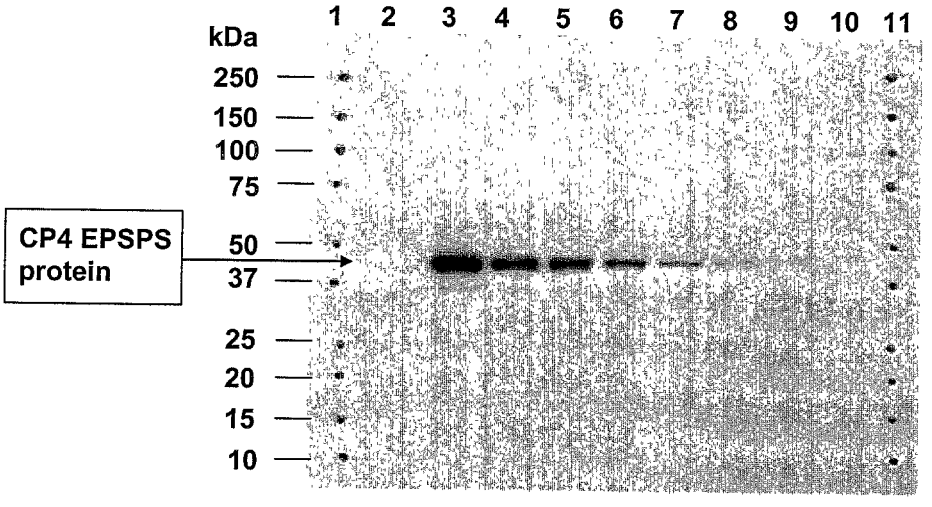
Samples were separated by a 4-20% polyacrylamide gradient Tris-glycine gel under reducing and denaturing conditions and transferred to a PVDF membrane by electroblot. Western blot analysis was conducted as described in Section 6.4. Amount Loaded (ng) refers to the quantity of the CP4 EPSPS protein reference standard spike. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in lanes 1 and 11. Lane 12 which is empty has been cropped in this figure.



Lane	Sample	Volume Loaded (µl)
1	Molecular weight markers	10
2	1 ng CP4 EPSPS protein reference standard in unheated control extract	12
3	Empty	—
4	Unheated test substance 2× Laemmli buffer extract	10
5	Unheated test substance2× Laemmli buffer extract	5
6	Empty	—
7	Heated test substance 2× Laemmli buffer extract	10
8	Heated test substance 2× Laemmli buffer extract	5
9	Unheated control substance 2× Laemmli buffer extract	10
10	Heated control substance 2× Laemmli buffer extract	10
11	Molecular weight markers	10

Figure 3. Western Blot Demonstrating the Effect of Heat Treatment on the Immunodetection of the CP4 EPSPS Protein in 2× Laemmli Buffer Extracts of Heated and Unheated MON 88913

2×Laemmli buffer extracts of the unheated and heated test and control substance samples were separated by a 4-20% polyacrylamide gradient Tris-glycine gel under reducing and denaturing conditions and transferred to a PVDF membrane by electroblot. Western blot analysis was conducted as described in Section 6.4. Amount loaded (ng) refers to the quantity of CP4 EPSPS protein reference standard. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in lanes 1 and 11. Lane 12 which is empty has been cropped in this figure.



Lane	Sample	Volume Loaded (µl)	Amount Loaded (ng)
1	Molecular weight markers	10	—
2	Unheated control substance extract	10	—
3	CP4 EPSPS spiked into unheated control extract	12	10
4	CP4 EPSPS spiked into unheated control extract	12	5
5	CP4 EPSPS spiked into unheated control extract	12	2.5
6	CP4 EPSPS spiked into unheated control extract	12	1
7	CP4 EPSPS spiked into unheated control extract	12	0.5
8	CP4 EPSPS spiked into unheated control extract	12	0.25
9	CP4 EPSPS spiked into unheated control extract	12	0.1
10	CP4 EPSPS spiked into unheated control extract	12	0.05
11	Molecular weight markers	10	—

Figure 4. Western Blot Demonstrating Limit of Detection of the CP4 EPSPS Protein Reference Standard Spiked into 2× Laemmli Buffer Extract of Unheated Control Substance MON 88913(-)

Samples were separated by a 4-20% polyacrylamide gradient Tris-glycine gel under reducing and denaturing conditions and transferred to a PVDF membrane by electroblot. Western blot analysis was conducted as described in Section 6.4. Amount Loaded (ng) refers to the quantity of the CP4 EPSPS protein reference standard spike. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in lanes 1 and 11. Lane 12 which is empty has been cropped in this figure.

Appendix

List of Applicable SOPs

BR-ME-0388-02	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
BR-ME-0392-01	Western Blot Analysis (Immunoblotting)
BR-ME-0924-01	Electrotransfer of Proteins to Membranes
BR-ME-0932-01	Assessment of Immunoreactive Bands from Western Blots Exposed to X-Ray Films Using Bio-Rad GS-710 and GS-800 Densitometers