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Validation of gel-free, label-free quantitative proteomics approaches: Applications for seed allergen profiling

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ARTICLE DATA

Keywords:

Peanut

Allergen

Spectral counting

Label-free proteomics

Quantitative proteomics

Seed biology

ABSTRACT

Plant seeds provide a significant portion of the protein present in the human diet, but are also the major contributors of allergenic proteins that cause a majority of the reported cases of food-induced anaphylaxis. New varieties of grains and nuts as well as other seeds could be screened for allergen content before they are introduced as cultivars for food production using mass spectrometry-based quantitation approaches. Here, we present a practical comparison of gel-free and label-free methods, peak integration and spectral counting, using a linear trap mass spectrometer. The results show that both methods are linear and reproducible with protein standards from 5–200 ng, however, bioinformatic analysis for spectral counting is much simpler and therefore more amenable to high-throughput sample processing. We therefore applied spectral counting towards the analysis of transgenic peanut lines targeting the reduction of a prominent allergen. Spectral count analysis of an Ara h 2 (conglutinin-7) RNA-silenced line confirmed reduction of this allergen as well as Ara h 6 (conglutinin), which was further confirmed by quantitative immunoblotting. Other collateral changes include an increase in Ara h 10 (oleosin 1) in one of the three lines, a decrease in conarachin as well as increased 13-lipoxygenase and Ahy-3 (arachin) in two of three lines.

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1. Introduction

Proteins are responsible for many allergic reactions suffered by people including the most threatening one, anaphylaxis, of which 80% are caused by peanuts or tree nuts in the U.S. [1]. It has been reported that as much as 6–8% of children 4 years old or younger are allergic to certain foods [2]. In peanut, Ara h 1–3 and 6 are considered the major allergens because they elicit immune responses in over 50% of peanut-allergic people [3,4]. Because seeds (nuts and legumes) are the major provider of oils and bulk protein for the world, and new varieties are continually being created or discovered, a fast and accurate

method for measuring seed allergen content before they enter the market is paramount.

Quantitation of peptides by mass spectrometry includes chemical labeling and label-free approaches with some variations on these two themes [5]. Quantitation by chemical labeling has been achieved by feeding stable isotopes of amino acids to cells in culture (SILAC) [6], or by covalently binding isotopically-coded affinity tags to cysteines of denatured proteins or peptides (ICAT) [6–8]. Peptide labeling has also been demonstrated by linking isobaric tags to primary amines (iTRAQ), or by ¹⁸O-labeling during proteolytic digestion [5,9,10]. These isotopic labeling methods benefit the

Abbreviations: LT-MS, linear trap-mass spectrometry; MM, molecular mass; RNAi, RNA interference; SILAC, stable isotope labeling with amino acids in cell culture.

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doi:10.1016/j.jprot.2008.11.005

researcher by allowing sample pooling thereby minimizing sample-to-sample variation and decreasing mass spectrometry use time [11]. However, chemical labeling is not 100% efficient, and depending on the method, does not evenly represent all proteins or peptides [12,13]. Chemical labeling is also relatively expensive and offers a limited dynamic range of quantitation [8]. Label-free methods overcome some of these limitations and are therefore gaining acceptance as an alternative for quantitative proteomics. Label-free technology allows the researcher to quantitate peptides in a relative or absolute manner. Relative quantitation methods quantify peptides using spectral characteristics such as peak area (peak integration) or frequency of peptide fragment spectra during LC-MS/MS analysis (spectral counting). Absolute quantitation requires sample spiking with synthetic, stable-isotopic peptide standards for comparing ion current peak areas [9,14]. Label-free methods are aided by recent advances in mass spectrometry instrumentation. Spectral counting benefits from faster scan rates, higher sensitivity and faster MS to MS/MS conversions, while peak integration benefits from stable and precise LC systems and high accuracy mass analyzers [5,11,15].

In the current study, we compared two label-free methods (peak integration and spectral counting) using a 40-fold dilution series of a protein standard. The methods were compared for response linearity, reproducibility (coefficient of variance) and ease of use. Spectral counting was comparable to peak integration for the first two criteria, however, in our hands post-analysis time for spectral counting was much lower than peak integration. Spectral counting was therefore employed to analyze transgenic peanut lines reduced in peanut allergens Ara h 2 and 6 by RNAi in order to test the effectiveness of spectral counting with a complex biological sample. Along with corroborating the knockdown of Ara h 2 and 6, spectral counting was able to provide a nearly complete view of the relative quantities of other major peanut allergens (Ara h 1, 2, 3/4, 6, 7, 8 and 10 found in allergen.org), showing drastic increases in Ara h 10 (oleosin 1) as well as collateral decreases in a conarachin (gi 52001225). However, most of these collateral changes were not observed in all three lines suggesting variation among transgenic events that will ultimately require confirmation from multiple seed from different generations. This proof-of-principle study demonstrates that gel-free, label-free proteomics could be a useful, high-throughput approach to profile seed allergens, enabling the researcher to perform rapid screening of numerous transgenic or breeding lines at minimal cost.

2. Materials and methods

2.1. Preparation of protein standard for mass spectrometry

For protein standard curve serial dilutions a protein standard (PeppermintStick™, Molecular Probes, Eugene, OR) containing stoichiometrically equal amounts of pure proteins was diluted from stock (0.5 µg/µL) to 0.2 µg/µL with de-ionized 18 MΩ water, three separate times (biological replicates). Each standard was made to 8 M urea by adding urea powder. Disulfide bonds were

reduced with 10 mM DTT (100 mM stock in 100 mM Tris-HCl pH 8.0), at 25 °C for 1 h. Reduced cysteines were alkylated with 40 mM iodoacetamide (200 mM stock in 100 mM Tris-HCl pH 8.0), at 25 °C in the dark for 1 h. Iodoacetamide was quenched by adding additional DTT to 30 mM (100 mM stock in 100 mM

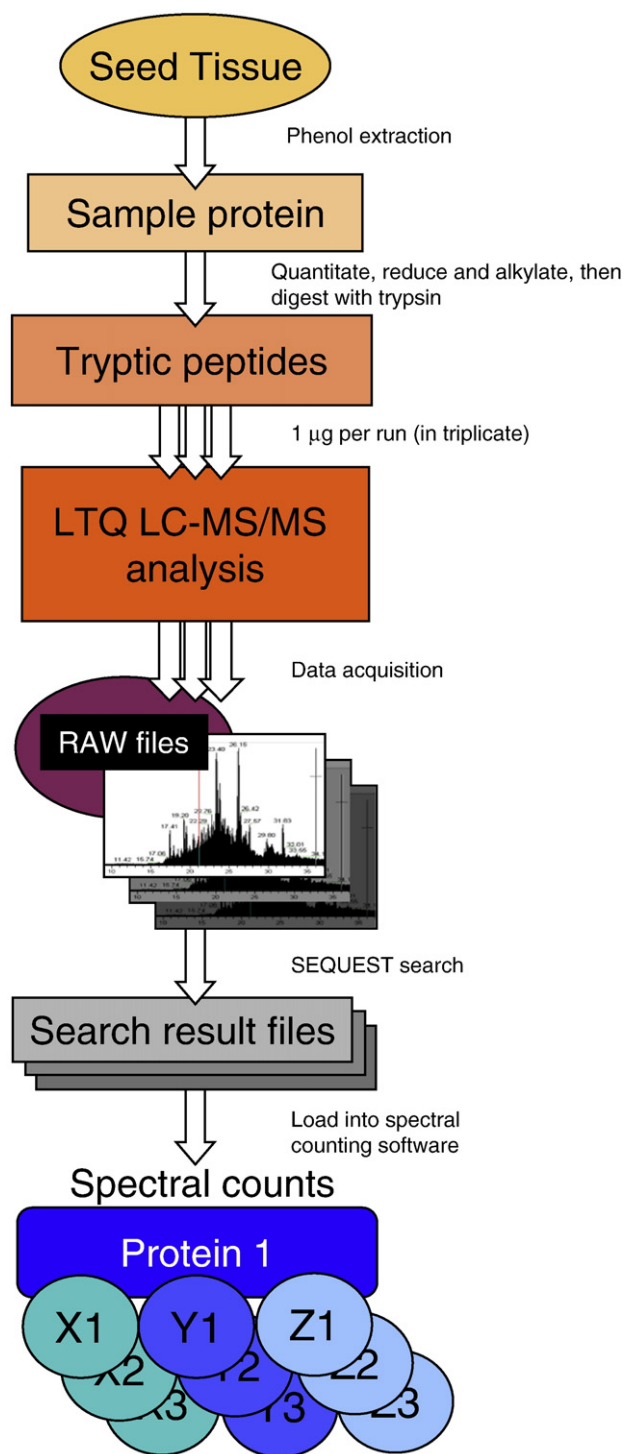


Fig. 1 – Workflow for biological sample preparation and LC-MS/MS analysis of total protein using in-solution digestion and spectral counting.

Tris-HCl pH 8.0) and incubating at 25 °C for 30 min. Urea was brought to 1 M by dilution with 100 mM Tris-HCl pH 8.0. Trypsin (Sequencing Grade Modified-Promega, Madison, WI) was reconstituted to 0.02 µg/µL and activated in the provided re-suspension buffer per manufacturer's instructions, then added to samples at a ratio of 1:50 (w/w) (trypsin:sample). Samples were incubated at 37 °C for 16 h. Digests were lyophilized to dryness. Dried peptides were reconstituted in 0.1% (v/v) formic acid to a concentration of 20 ng/µL. Serial dilutions were performed in a polypropylene 96 v-well plate with 0.1% (v/v) formic acid to attain 10 ng/µL, 5 ng/µL and 0.5 ng/µL concentrations.

2.2. Plant materials and protein extraction

Ara h 2 and 6 were simultaneously silenced in peanut by an RNAi construct containing a 222 bp inverted repeat with 81% nucleotide sequence identity between the two genes [16]. Since transgenic lines were expected to segregate at the T1 generation, embryos of four seeds from each of the three transgenic lines were used for protein extraction [17] and separation of 10 µg of protein on 15% polyacrylamide gels. After staining with Coomassie blue, the presence or absence of Ara h 2 double bands was used to differentiate silenced from non-silenced seeds. A single cotyledon from one Ara h 2 silenced and one Ara h 2 non-silenced segregant seeds from each of the three lines were used for subsequent analyses. Each cotyledon was ground by mortar and pestle in liquid nitrogen, then further ground with 15 mL of a 1 to 1 mixture of extraction buffer (0.9 M sucrose, 0.1 M Tris-HCl pH 8.8, 10 mM EDTA and 0.4% (v/v) 2-mercaptoethanol) and phenol (pH 8.0). The frozen powder was transferred to a 15 mL conical tube and allowed to thaw at 25 °C. The samples were then incubated at 4 °C with gentle agitation for 1 h. Phases were separated by centrifugation at 5000 g and 4 °C for 20 min. Phenol was removed and dissolved protein was precipitated at –20 °C overnight with 5 volumes of 100% methanol containing 0.1 M ammonium acetate. Protein precipitate was collected by centrifugation at 4000 g and 4 °C for 20 min. Protein pellets were washed 3 times by re-suspending in the above methanol ammonium acetate solution using brief sonication and vortexing. Protein pellets were then washed with chilled 80% (v/v) acetone (twice), 70% (v/v) ethanol (once) and then stored in 80% (v/v) acetone at –20 °C. Fig. 1 shows a workflow for the entire MS-based quantitative method beginning with protein isolation.

2.3. Protein preparation for mass spectrometry

Precipitated protein in 80% (v/v) acetone was collected by centrifugation at 4000 g and 4 °C. Pellet was dissolved in reconstitution buffer (30 mM Tris-HCl, 7 M urea, 2 M thiourea, 2% (w/v) CHAPS at pH 8.8) by gentle vortexing. Samples were quantified using the Bradford method using ovalbumin as standard [18] and are the average of five replicate readings. The average standard deviation across all samples was less than 3.8%. A 20 µg portion of protein from each sample was removed and diluted to 1 µg/µL with reconstitution buffer (see above). As an internal standard, 200 ng of BSA was added to give a protein ratio of 1% (w/w) BSA protein. Processing was performed as stated above for

the internal standard, but here urea was diluted to 1 M by addition of 50 mM ammonium bicarbonate. Trypsin was added to samples at a ratio of 1:25 (trypsin:sample), and allowed to proceed at 37 °C for 16 h. Digests were lyophilized to dryness. Dried peptides were reconstituted in 0.1% formic acid (v/v) by vortexing, and each brought to 0.1 µg/µL. Before adding to a polypropylene 96 v-well plate, samples were centrifuged at 13 k rpm at 4 °C for 20 min and the top 95% removed to a new tube.

2.4. Mass spectrometry analysis

Protein samples were centrifuged at 13 k rpm at 4 °C for 10 min to remove insoluble debris. Fifteen µL portions from each dilution were aliquoted into a polypropylene 96 v-well plate, covered with adhesive film, briefly centrifuged to collect samples at bottom of well, and placed in the cooled tray of the LC auto-sampler. For analysis, 10 µL injections were run on an LTQ ProteomeX linear ion trap LC-MS/MS instrument (Thermo Fisher, San Jose, CA). Peptides were concentrated and desalted on in-line C₈ Captraps (Michrom Bioresources, Auburn, CA) prior to final separation by C₁₈ column using an acetonitrile gradient 0–90% solvent B (100% acetonitrile with 0.1% (v/v) formic acid), in solvent A (deionized 18 MΩ water with 0.1% (v/v) formic acid) for a duration of 80 min. The peptide trap and C₁₈ column were then re-equilibrated for 5 min with 100% solvent A before applying the next sample.

LC separation was performed using fused silica nanospray needles, 10 cm length, (360 µm outer diameter, 150 µm inner diameter; Polymicro Technologies, Phoenix, AZ), that were packed with “Magic C18” (100 Å, 5 µm particles; Michrom Bioresources) in 100% methanol. Before sample acquisition, columns were equilibrated for 3–4 h at 200 nL/min (at the column tip) with a 60:40 mix of solvent B to solvent A. Samples were analyzed in the data-dependent positive acquisition mode on the LC-MS/MS instrument, using normal scan rate (enhanced for standard dilution series experiment) for precursor ion analysis, and dynamic exclusion enabled (1 repeat count, 30 s repeat duration, list size of 50, and 30 s exclusion). Following each full scan (400–2000 *m/z*), a data-dependent triggered MS/MS scan for the three most intense parent ions were acquired. The nanospray column was held at ion sprays of 3.1 kV and a flow rate of 100 µL/min, with 200 nL/min at the column tip at 80% solvent B.

2.5. Database searching and spectra analysis

RAW files were searched using Bioworks “SEQUEST batch-search” against a FASTA database containing only the proteins present in the protein standard; gi|13397826 (avidin), gi|47825389 (lysozyme), gi|114939 (beta-galactosidase), gi|1351907 (serum albumin precursor), gi|45384056 (ovalbumin), gi|162805 (beta-casein) or “Plant Protein database” created using the National Center for Biotechnology Information's non-redundant protein database filtered via Bioworks using keywords: *Brassicaceae*, *Arachis hypogaea*, peanut, soy, arabidopsis. After excluding unwanted entries the database ended with a mostly plant specific database containing 61,458 entries.

2.6. Peak integration quantitation by DeCyder MS

The search results files (.dta and .out files) were imported into DeCyder MS (GE Healthcare, Piscataway, NJ) using the “Import” module. Peptides were detected using charge state assignments from 1–4, mass resolution of 0.45, LC peak shape tolerance of 20.0, m/z shift tolerance of 0.5 u, m/z shape tolerance of 10% and using a s/n threshold of 3.0 (other settings were left as default). The base ion current chromatograms (MS1) were cropped to include only peaks from 10–50 min, and were retention-time aligned. For peptide matching, retention time and m/z tolerances of 1.0 min and 0.5 Da, respectively, were used (other settings were default). All peptide intensity data was exported to Excel for graphing and analysis.

2.7. Spectral counting quantitation by Scaffold

The search results files (.srf) were imported into Scaffold™ (Proteome Software, Portland, OR- release 1.0 or 2.0) and analyzed as described below and illustrated in Fig. 1.

2.7.1. Database searching

Tandem mass spectra were extracted by BioWorks version 3.0. All MS/MS samples were analyzed using SEQUEST (ThermoFinnigan, San Jose, CA; version 2.7). SEQUEST was set up to search the protein standard database indexed for trypsin digestion. SEQUEST was searched with a fragment ion mass tolerance of 1.0 Da and a parent ion tolerance of 2.0 Da. Iodoacetamide derivative of cysteine (+57) was specified in SEQUEST as a fixed modification. Oxidation of methionine (+16) was specified in SEQUEST as a variable modification.

Scaffold 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 PeptideProphet™ algorithm. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 1 identified peptide. Protein probabilities were assigned by the PeptideProphet™ algorithm. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped.

Counted spectra are provided by Scaffold in five different forms (four for release 1.0, and one for release 2.0); 1) unique peptides, 2) unique spectra, 3) number of identified spectra and 4) percent of total spectra, along with unweighted spectral counts for version 2.0. Each of these methods was used to analyze the standard dilution series by plotting their respective data sets in Excel. Normalized, un-weighted spectral counts were normalized to the sum of all spectra from the sample with the highest number of spectra.

2.8. PAGE, western blotting and quantitative analysis

Aliquots of acetone-powdered protein samples were dissolved in 1X sample buffer (SB, 0.06 M Tris-HCl (pH 6.8), 0.06 M sodium dodecyl-sulfate, 10 mM DTT, 5% glycerol (v/v) and 0.0012 mM bromophenol blue) and quantified using a BCA kit (Pierce, Rockford, IL) per manufacturer's instructions for 96-

well assays. Standard curves were made using ovalbumin dilutions of 0.5, 1.0, 2.0, 4.0, and 6.0 mg/mL dissolved in 0.2X SB. Absorbance was determined using a Multiskan MCC plate reader (Thermo Electron corp., Waltham, MA) at 560 nm. For electrophoresis, protein (20 μ g) in 1X sample buffer was brought to 30 mM DTT, and heated to 95 °C for 5 min before loading. Protein was separated using a 12% polyacrylamide gel at 30 mA until the dye-front reached the bottom of the gel.

For blotting, gels were rinsed with water for 10 min, then put into transfer buffer (25 mM Tris, 192 mM glycine, and 10% (v/v) methanol), 10 min before setting up transfer.

Blotting was performed at 200 mA for 6 h, to Hybond-LFP PVDF (GE Healthcare Ltd, UK). Blots were rinsed in PBS-T (10 mM NaH_2PO_4 , 150 mM NaCl, 0.3% (w/v) Tween 20) adjusted to pH 7.2) for 10 min, blocked for 1 h in PBS-TM (PBS-T with 3% (w/v) non-fat dried milk), probed with primary at 1:8000 (Ara h 1, 2, 3 and 6 from chicken generously provided by Dr. Sohelia Maleki, USDA-ARS-SSRC, New Orleans) in PBS-TM for 16 h at 4 °C. Blots were rinsed in PBS-TM twice for 5 min, rinsed in PBS-T twice for 5 min, blocked in PBS-TM for 1 h, and then incubated with secondary antibody (anti-chicken IGG-HRP conjugate) at 1:20,000 for 2 h in PBS-TM. Blots were washed three times in PBS-TM, 5 min each, and once in PBS-T before visualizing. Probes were visualized using ECL reagents from Pierce (Rockford, IL) and autoradiography. Developed film was scanned (16 bit, 300 dpi) and densitometry analysis performed using ImageQuant TL (GE Healthcare). For each probe, all bands were measured separately, and their volumes summed. For the RNAi lines, the areas next to the bands from the wild type samples were measured.

3. Results

To be considered quantitative, spectral counting and peak integration must provide a linear signal across a serial dilution series, and the results must be reproducible. To test the efficacy of each approach we compared a 40-fold dilution series (5–200 ng) of a commercial protein standard containing six proteins of varying molecular masses (116.25–14.4 kDa) at equimolar concentrations. The dilutions were treated, digested and analyzed by LC-MS/MS. Spectral data were then analyzed with different label-free quantitation programs, one for each label-free method, DeCyder MS (peak integration) and Scaffold (spectral counting).

3.1. Peak integration using DeCyder MS

Protein quantitation by peak integration, as performed by DeCyder MS, requires several steps performed by separate program modules. First, the RAW files produced from the LTQ mass spectrometer are converted into a “.dcms” format by the Import module. During this process retention times and mass/charge scales can be “cropped” and resampling can be adjusted. This process creates a new version of the RAW file which is then opened by the PepDetect module. The PepDetect module displays the LC and m/z data in a pseudo-2D gel format, showing mass to charge ratio on the y-axis and retention time on the x-axis. Settings for resolution and charge state identification as well as signal to noise ratios

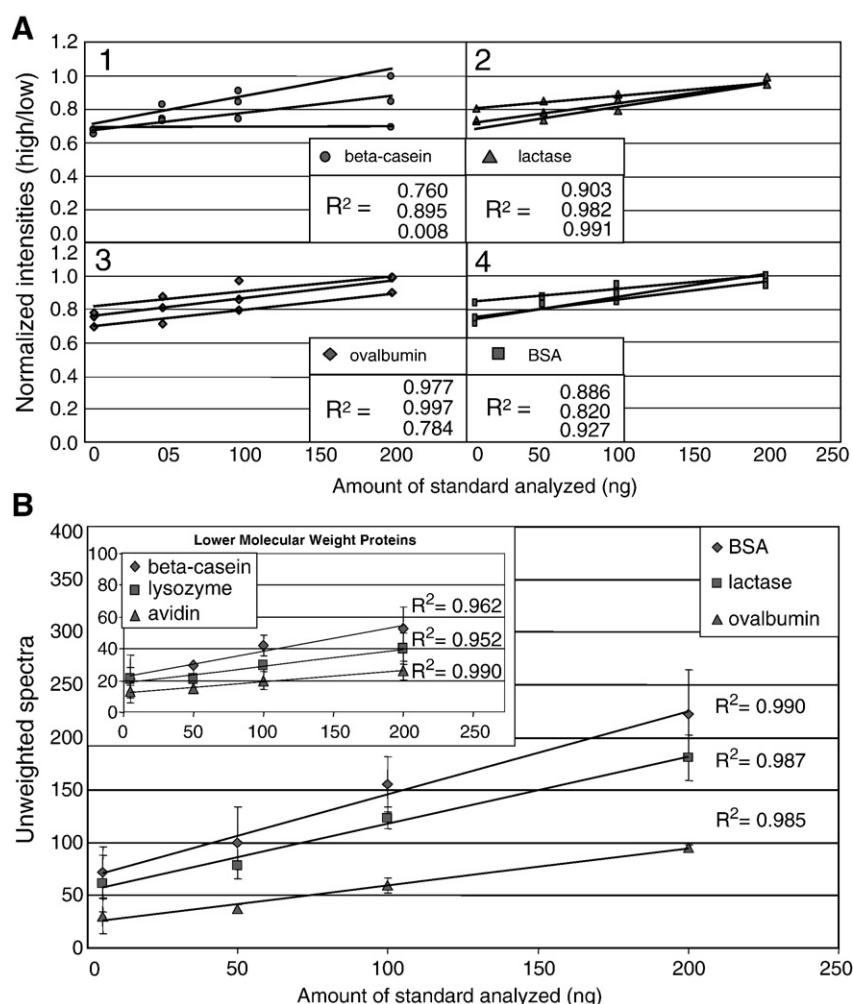


Fig. 2—Comparison of spectral counting and peak integration using a protein standard dilution series. A, Results of peak integration method used to quantify a protein standard dilution series. In this plot each protein is represented by three different peptides each with a set of four values (depicted with the same symbol) for each concentration in the dilution series. Data are the average of three biological replicates; with three technical replicates each (nine total replications). Values were normalized to the highest intensity for each peptide. Average errors associated with this analysis are shown in Supplemental Table 1. Trend lines represent the changing intensity for peptides observed across the dilution series, R^2 values are provided. B, Results of spectral counting method to quantify a protein standard dilution series. Unweighted spectral counts were plotted for each protein at each dilution in the series. R^2 values are provided. Other representations of spectral counts are shown in Supplemental Fig. 1. The inset shows the low MM proteins in the protein standard using a different y-axis scale. Error bars represent standard deviation between three biological replicates, and are shown in Supplemental Table 1.

can be adjusted to optimize detection of masses by this module. These PepDetect files can be batch processed and are later matched across datasets by peptide retention time characteristics in the PepMatch module. Product ion masses are then searched by SEQUEST for protein identification. Quantitation is achieved by comparing the areas of the precursor ion peaks measured during LC elution. Peptides that elute at similar times and produce the same product ions during MS/MS analyses can be compared. A quantitative relationship can only be made between peaks that correspond to the same peptides in different samples, i.e. peptide A can only be compared to peptide A from another sample. Also, intensities of different peptides belonging to the same protein cannot be averaged within a biological sample due to differences in ionization properties.

Peak integration analysis of the protein standard dilution series is shown in Fig. 2A. The smallest proteins in the standard mix, lysozyme and avidin, did not provide many high resolution spectra and were removed from this analysis. The dilution series was prepared three separate times to simulate biological replicates. Each of the biological replicates was analyzed three times to make three technical replicates for a total of nine replicates per dilution. Three ions (peptides) were chosen to represent each protein in the standard, and intensity values for each ion at each dilution were plotted. Data plotted are average biological, normalized to the highest intensity value for each protein. Plots show a positive slope from the highest to the lowest dilutions for four of the six proteins in the cocktail as would be expected if the peak areas correlate with peptide abundance. R^2 values range

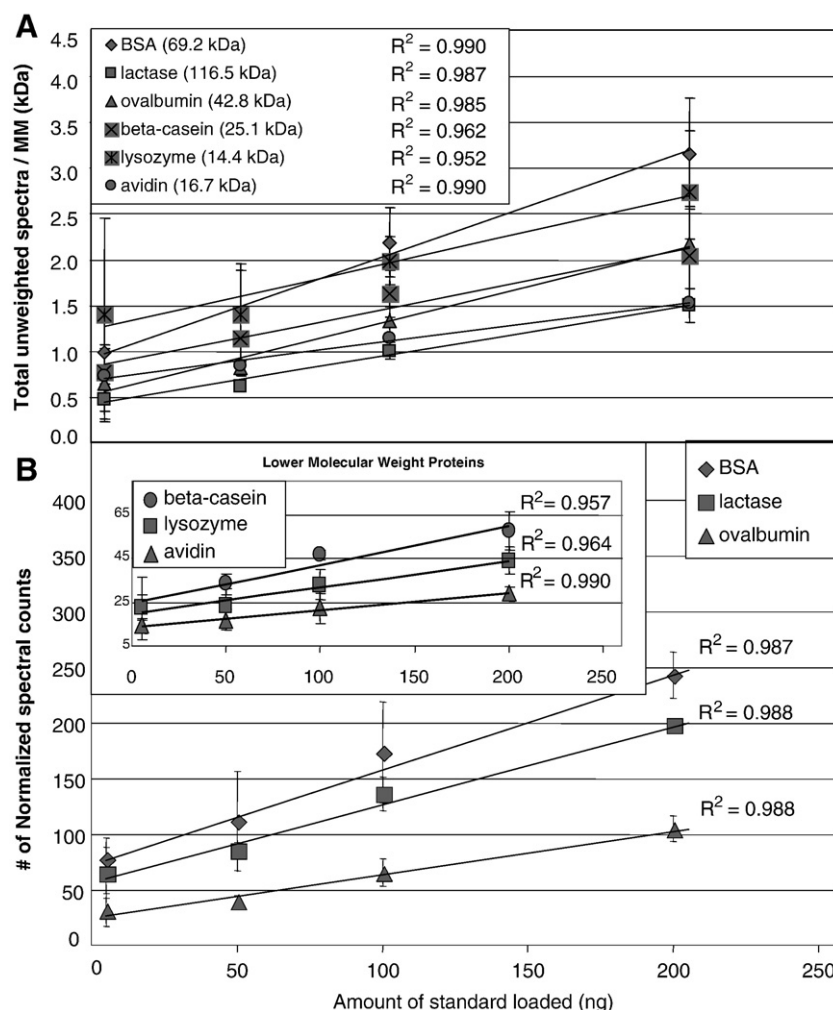


Fig. 3 – Normalization methods for spectral counting. A, Unweighted spectral counts for each protein divided by their respective molecular masses (kDa). This technique helps to normalize between proteins with differing molecular masses, which consequently have different amounts of possible spectra. B, Unweighted spectral counts for each protein which were normalized by multiplying the counts for each protein in each biological replicate by a normalization factor. The normalization factor is the ratio of counts in a biological replicate (sum of all counts for all proteins in the replicate that has the highest) to that for the other biological replicates. Error bars are standard deviation between biological replicates. R^2 values are provided.

from 0.008 to 0.997 with an overall average of 0.828, excluding the lowest value. The protein with the lowest R^2 values, beta-casein, averaged 0.554 and is the smallest protein of those analyzed. DeCyder MS calculated intensity values ranged from a little over 22 for the most concentrated down to 14 for the most dilute standard. This produced shallow slopes for most lines, uncharacteristic of a 40-fold dilution series.

3.2. Spectral counting using Scaffold

Protein quantitation by spectral counting involves searching a collection of MS/MS spectra (for example dta files contained in a Thermo RAW file) against a database using a peptide matching algorithm like SEQUEST. The combined “dta” and subsequent “out” files are imported into Scaffold as search result files or “sdfs”, and the number of dta/out spectra that correspond to a given peptide are culled into “counts” associated to a specific protein. Quantitation by spectral counting operates with the

assumption that if a protein is twice as abundant in one sample as it is in another, then this will lead to twice the number of spectra during analysis. This is feasible if the populations are equally represented (loading amounts are identical) and that sampling is unbiased. Unlike peak integration, spectral counting with low-abundance proteins (peptides) will produce very few spectra leading to small numbers that are unreliable. For example, if we find 1 spectra for protein B in a sample, and 3 spectra for protein B in another sample, we cannot immediately assume that the protein is 3-fold higher in the second sample.

Results from spectral count analysis of the protein standard dilution series are shown in Fig. 2B. Values plotted are the average counts for peptides found in three technical and three biological replicates. As with peak integration, this method showed a linear relationship for all proteins across the serial dilution series. R^2 values for spectral count data were over 0.9 in nearly all cases indicating the high degree of

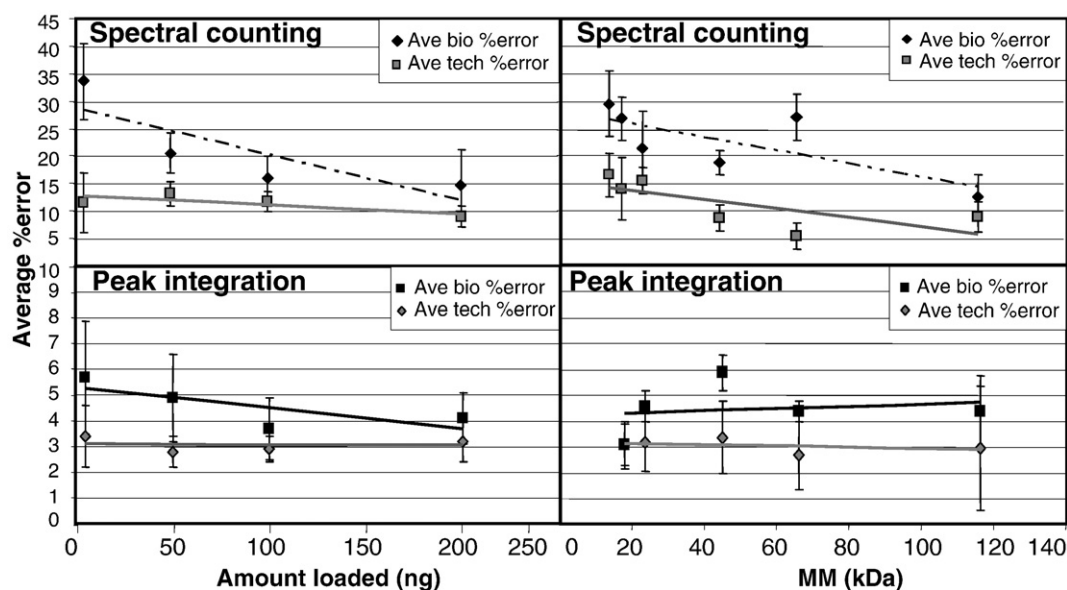


Fig. 4—Peak integration and spectral counting show greater error associated with pipetting than MS instrumentation. Graphs are plots of % error values obtained during the analysis. Biological and technical errors are defined here as the percent error between independently prepared samples and between multiple injections of the same sample, respectively. Error bars represent standard deviations between replicates. Error is plotted both as a function of protein loading amount and protein molecular mass (MM).

linearity for points plotted in our dilution series regardless of intact protein mass (Fig. 2B). Counts for proteins ranged from around 200 for the most concentrated dilution, down to around 50 for the most dilute. Similar to peak integration this produced a relatively shallow slope, not reflective of a 40-fold dilution series. It was also observed that high molecular mass (MM) proteins produced more spectra than low MM proteins, as previously noted [19–21]; this is because lower MM proteins produce fewer tryptic peptides. This disparity between peptide abundances leads to more shallow line slopes for low mass proteins (inset Fig. 2B).

To correct for mass bias, spectral counts can be normalized to the MM of the protein (Fig. 3A); by doing so, the slopes for each protein are more congruent. A potential issue with spectral counting is run-to-run sampling differences, which can be problematic if nanospray is unstable. Run-to-run sampling differences can be accounted for by multiplying counts for each protein by the ratio of total counts of the sample with the highest counts to that of the sample with the lowest counts as described by Carvalho et al. [22]. Standard curves utilizing this normalization method are shown in Fig. 3B. Comparison with Fig. 2B which shows unweighted spectral counts reveals no difference in R^2 values, suggesting run-to-run variation for this specific LC-MS/MS configuration is minimal.

Besides the non-normalized method of counting spectra (unweighted spectra comparisons), spectral abundances can be compared in at least four other ways. Total spectra can be divided into “unique spectra”, “unique peptides”, “% of total spectra” and “number of identified spectra”. The same data for the protein standards was plotted using each of these methods (Supplemental Fig. 1). Plotting “% of total spectra” produced the lowest R^2 values, while the other three approaches yielded similar values as “unweighted spectra.”

3.3. Variation versus protein loading and molecular mass for peak integration and spectral counting

Variation or error for technical and biological replicates of the protein standard dilution series is presented as coefficient of variance (Supplemental Table 1). Technical replicate is defined as multiple injections of the same sample, while biological replicate is defined as an independently prepared sample. The average technical and biological variation for all six proteins at each dilution was 3.1% and 4.5%, respectively, for peak integration and 9.1% and 24% (unweighted spectra), respectively, for spectral counting.

Variation was also calculated as a function of sample loading and MM. Technical variation measures the reproducibility of the auto-sampler, LC system and mass spectrometer. For spectral counting and peak integration average technical CV values did not vary with the dilution series, and was consistently 11% and 3%, respectively (Fig. 4). In contrast, biological variation for both methods was higher (33.6 and 5.7%) at the most dilute protein concentrations, indicating manual pipetting is a greater source of error than automated LC-MS/MS analyses. When technical and biological variation was plotted versus MM it was again apparent that biological variation was 75–120% higher than technical variation (i.e. LC-MS/MS instrumentation; Fig. 4). Therefore, methodological workflows that minimize manual sample handling and the number of steps prior to LC-MS/MS analysis will produce lowest variation. Interestingly, both technical and biological variations were higher for low MM proteins versus high MM proteins for spectral counting but not for peak integration (Fig. 4). This is likely a reflection of the reduced number of tryptic peptides for low MM proteins, ultimately producing lower overall spectral counts.

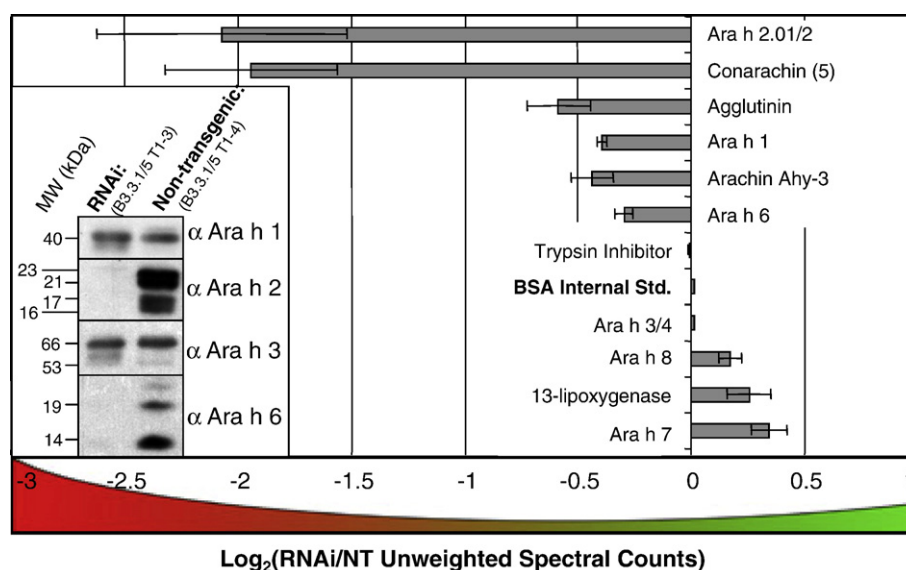


Fig. 5 – Proteome screening of peanut line B3.3.1/5 T1-3 (RNAi) and 4 (non-transgenic control, NT). Bars show the $\log_2$ ratios (RNAi/NT) of spectra found for each protein. Protein names are adjacent to each bar. Error bars are average standard deviations for technical replicates (three replicates for NT, four for RNAi). Standard deviation for $\log_2$ data was calculated by finding the average coefficient of variance (Stdev/Ave) for non-transgenic and RNAi values, then multiplying that by the $\log_2$ value. All values were normalized to an internal BSA standard (added whole before processing) by multiplying the raw data by a normalization factor. Normalization factors are the ratio of BSA counts in NT to that in RNAi. Inset panel shows quantitative western blot data obtained for Ara h 1, 2, 3, and 6. The headings Ara h 3/4 and Ara h 1, for the purposes of comparing spectral counts to the western blot data, represent all the hits found during the analysis that were >70% identical to gi21314465 (Ara h 3/4) or gi1168391 (Ara h 1) as they would likely not be distinguished by western blotting. Genbank accession numbers and experimental data are provided in Supplemental Tables 2–4.

3.4. Spectral counting analysis of Ara h 2/6 in RNA-silenced peanut lines and comparison with non-transgenic controls

Since spectral counting and peak integration yielded mostly comparable results with a dilution series of standards, but spectral counting was easier and more intuitive to perform we chose this approach to perform comparative analysis of

transgenic peanut Ara h 2/6 RNAi lines versus non-transgenic controls. As an alternative to spectral count normalization we chose to include BSA as an internal standard to control for run-to-run variation. BSA was chosen as an internal standard because it has an average MM (66 kDa), is inexpensive, and typically provides greater than 40% sequence coverage. In two out of three sets of analyses the

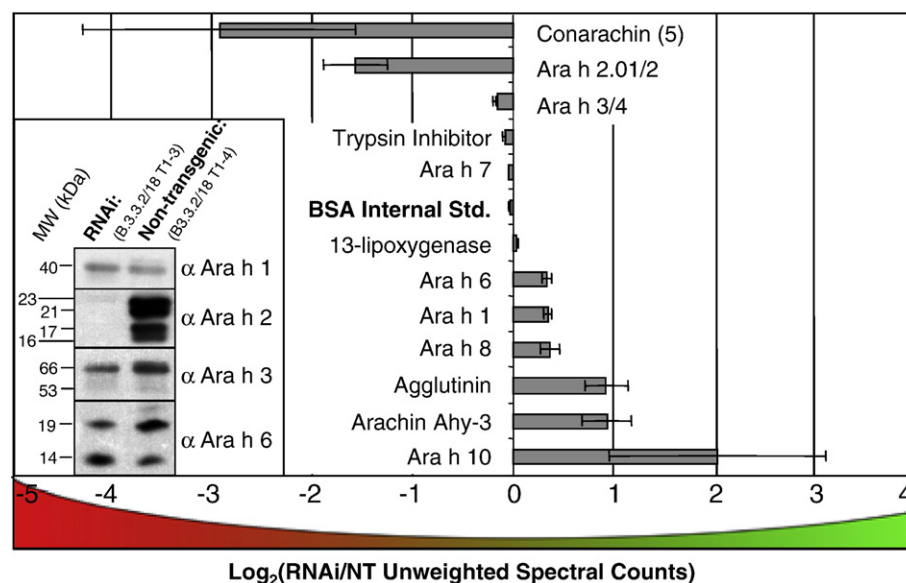


Fig. 6 – Proteome screening of peanut line B3.3.2/18T1-3 (RNAi) and 4 (non-transgenic control, NT). See Fig. 5 legend for further information.

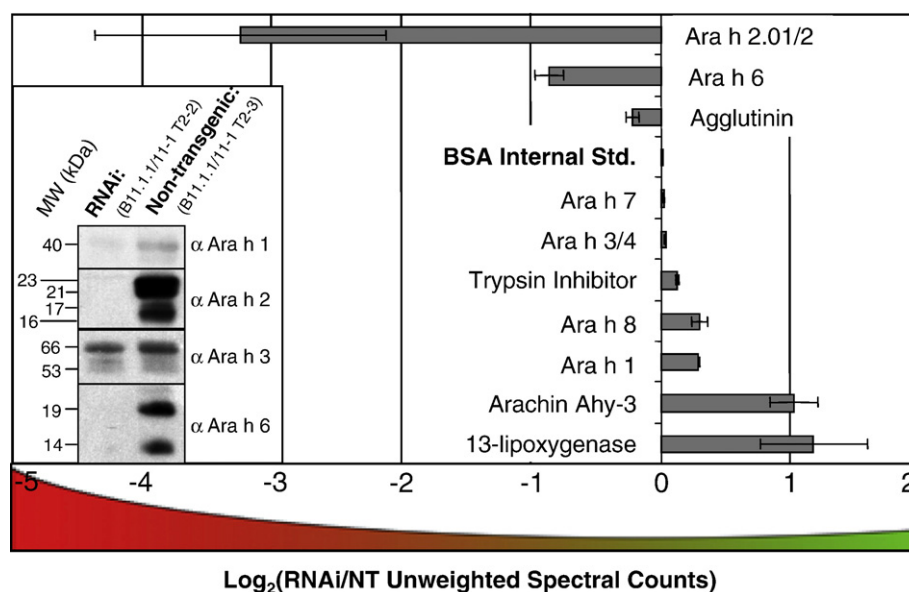


Fig. 7 – Proteome screening of peanut line B11.1.1/11-1T2-2 (RNAi) and 3 (non-transgenic control, NT). See Fig. 5 legend for further information.

log₂ BSA ratio was near zero, suggesting nanospray and LC were both stable with this system.

Gel-free, LC-MS/MS analysis of 1 µg of protein yielded over 100 protein identifications, however, a majority of these were single peptide assignments or multiple peptides with low spectral counts, neither of which produce high-confidence quantitative results. Based upon technical %error values of ≤60%, and a minimum total peptide count value of 20, quantitative information was obtained for 13 proteins in RNAi line 3.3.1/5, 14 proteins for line B3.3.2/18, and 12 proteins for line B11.1.1/11-1 (Figs. 5–7). Figs. 5–7 show the log₂ ratios of proteins identified in both RNAi and non-transgenic (NT) samples that were collected during the analyses, with error bars denoting standard deviation.

For the purpose of comparing spectral counting data to western blot data, proteins that were greater than 70% identical to Ara h 3/4 (gi21314465) were grouped together. Conarachin (gi46560478, hereafter referred to as conarachin 4) was grouped under Ara h 1 because their sequences are 99% identical as shown by BLAST analysis (data not shown). Supplemental Fig. 2 provides an alignment of the proteins grouped under the Ara h 3/4 heading, and highlights the peptides that were counted for each. Supplemental Fig. 3 lists these peptides and indicates which peptides were used to quantitate Ara h 3/4 paralogs along with redundant peptides. Redundant peptides could theoretically have come from any of the Ara h 3/4-like proteins or paralogs, necessitating their grouping under one heading. Other proteins present in the graphs did not display the same redundancy and were therefore not merged. However, nomenclature was adjusted to account for allergen annotation, but the original protein name is provided in Supplemental tables. The quantitative western blot results (densitometry scanning) agreed with the spectral counting data in the trends though not in their magnitudes. For Ara h

1, quantitative immunoblotting showed log₂ fold changes of 0.12, 0.28 and –1.6 for peanut lines 3.3.1, 3.3.2, and 11.1.1 respectively. Spectral counting Ara h 1 log₂ ratios were similar for the first two lines with –0.16, 0.55, but quite different for line 11.1.1 with a value of 0.29. For Ara h 2, densitometry showed log₂ fold changes of –9.0, –5.1 and –7.0 for peanut lines 3.3.1, 3.3.2, and 11.1.1 respectively, while spectral counting provided –1.7, –1.4 and –3.2. For Ara h 3/4 densitometry provided log₂ fold changes, of 0.14, –0.81 and –0.38, while spectral counting gave log₂ fold changes of 0.012, –0.16 and –0.035. Ara h 6 had the most variable results. Densitometry for Ara h 6 gave log₂ fold changes of –7.4, –0.43 and –14 for peanut lines 3.3.1, 3.3.2, and 11.1.1 respectively. Spectral counting gave log₂ fold changes of –0.30, 0.33, and –0.86. Accession numbers for all proteins in Figs. 5–7 as well as the total counts and technical standard deviations and CVs are present in Supplemental Tables 2, 3 and 4. P-values calculated by Student's t-test are also presented to qualify the log₂(RNAi/NT) ratio results.

Along with the quantitation of Ara h 1, 2, 3/4 and 6 allergens, spectral counting provided quantitative ratios for other proteins that we were unable to confirm by western blotting, but displayed differential expression compared to non-transgenic. Conarachin (gi52001225, referred to as conarachin 5) showed large negative log₂ fold changes in lines 3.3.1 and 3.3.2 (Supplemental Tables 2, 3) suggesting concomitant down-regulation with Ara h 2. Conarachin 5 does not share any significant homology with Ara h 2 or 6, which suggests its down-regulation isn't due to direct co-silencing. Also, in line 3.3.2, Ara h 10 (oleosin 1), agglutinin (galactose-binding lectin) and Ahy-3 (an 11S seed storage protein) are induced. Peanut line 11.1.1 showed collateral changes in the proteome including significant increases in 13-lipoxygenase and Ahy-3 proteins.

4. Discussion

4.1. Error and R^2 values for serial dilution analysis by spectral counting and peak integration

Our results for the serial dilution of a protein standard show that spectral counting and peak integration are both reproducible methods for relative quantitation using linear trap-mass spectrometry (LT-MS). The largest differences were seen in the % error. At best, spectral counting gave technical CV values of 9% considering unweighted spectral counts, while peak integration provided 3% (Supplemental Table 1). This might be because the same data set was used for both techniques with the scan rate settings catering to peak integration. Because peak integration (using DeCyder MS) requires isotopic resolution in the MS scan, it was necessary to set the scan rate to the slower “enhanced” acquisition mode. This means that the number of MS scans acquired per second is lower and may potentially compromise the accuracy of spectral counting particularly for low abundance proteins.

The response linearity for each method based on the data from the protein standard dilution series was compared by the R^2 values generated by fitting data to a line. On average, peak integration produced an R^2 value of 0.902 (with the outlier in Fig. 2A-1 removed), while spectral counting gave 0.980. Both methods produced linear responses, though spectral counting was slightly better and provided data for all proteins in the standard protein set. The biggest problem for peak integration was the lack of a complete data set for all proteins in the mixture. In some instances for dilute samples, the MS scan failed to provide quantifiable peaks for low MM proteins. This made the data set incomplete, suggesting that peak integration may have its own limitations particularly for low MM proteins. Because the scan rate had already been set to the best mode for peak integration, this inability to provide quantitative MS data for a simple protein mixture shows a potential limitation for LT-MS. However, more sensitive acquisition modes are available to analyze target ions including selected ion monitoring.

4.2. Data throughput and applications

We chose spectral counting as our preferred quantitative method for several reasons. Firstly, peak integration by DeCyder MS proved to be a much more arduous method for quantitation as the processing time was considerably longer than that for spectral counting. Peak integration processes such as chromatogram alignment and peptide matching between datasets took many hours even for a simple protein mixture. After database searching, time spent on spectral counting workflows is minimal. Also, our objective was to create a quick and simple screening method that could identify differences in seed proteomes. LTQ instruments are well suited for spectral counting because of their fast scan rates, and we demonstrate here that spectral counting is comparable to peak integration for reproducibility and response linearity. Secondly, dynamic range was also limited in the peak integration approach. For a 40-fold change in our dilution series, we were only able to detect a 1.5 fold difference

in intensity. Spectral counting was also unable to provide accurate fold changes, but did better than peak integration (with LT-MS), producing up to 4-fold differences. Thirdly, peak integration does not allow data from different peptides mapping to the same protein to be readily merged. Peptide ionization is unique, and therefore signals are only comparable to each other. For spectral counting, all peptides matching to a protein can be used to quantitate that protein. Also, spectral counting is able to provide quantitative data between different proteins if their counts are normalized to their MM or their number of potential tryptic peptides (Fig. 3).

4.3. Differentially expressed peanut proteins

Screening of the peanut proteome by reverse-phase LC-MS/MS using spectral counting provided results that indicate the capabilities and potential weaknesses of the method for relative quantitation of seed proteins. Down regulation of both Ara h 2 and 6 agreed with quantitative western blots, and accurately portrayed the levels of those allergens in the peanut RNAi mutant when compared to non-transgenic lines. Along with these results, other proteins showed differential expression as a potential consequence of expression of the introduced RNAi transgene.

In peanut line 3.3.2/18, oleosin was higher in transgenic versus non-transgenic control, suggesting collateral changes associated with the down-regulation of Ara h 2 and 6 by RNAi (Fig. 6). Oleosins are the major protein constituent of oil bodies during seed development [25–27]. Their role during oil accumulation is to regulate the size of the oil body in order to maintain surface to volume ratios that presumably facilitate access by lipases during germination [25,28,29]. Along with the targeted decrease in Ara h 2 and 6, a marked decrease in conarachin 5 was also noted. BLAST analysis of conarachin 5 shows that it contains conserved barrel domains of the cupin family and is not homologous to other glycinins or vicilins like conarachin 4 (Ara h 1 [30]). Its sequence was deduced by Yan et al. while analyzing ESTs from peanut cotyledon [31].

Increasing protein along with lipids in plant seed has been difficult because these storage reserves are inversely proportional [32–34], i.e. when plants are bred for one trait the other recedes. As an example, Siloto et al. showed that knocking down oleosin expression in *Arabidopsis thaliana* caused a decrease in lipid accumulation with a concomitant increase in total protein [23]. If oleosin is higher in line 3.3.2/18, this may be a sign that oil has increased. This potential increase in oil may have resulted from the drastic decrease in two storage proteins brought about by RNAi. If this is the case, monitoring oleosin and storage protein expression (using high-throughput proteomics) could be a rapid “protein phenotyping” strategy to monitor oil and protein content within seed.

Another protein that was up-regulated as a consequence of Ara h 2/6 RNAi transgene expression was a lipoxygenase. The most drastic increase in 13-lipoxygenase (13-LOX) showed a $\log_2$ (RNAi/NT) change of +1.2 (Fig. 7). Lipoxygenases play roles in plant-microbe interactions, and have been shown to be induced by biotic and abiotic stimuli [35,36] including colonization by *Aspergillus*. Lipoxygenases produce oxylipins

through oxygenation of polyunsaturated fatty acids (specifically linolenic (18:3) for 13-LOX) [25] which can go on to produce jasmonic acid [36]. Jasmonates are signaling molecules involved in regulation of defense-related gene expression [37]. The increases observed in line 11.1.1/11-1 suggest that this variety may have increased amounts of 13-lipoxygenase activity assuming the seeds weren't under attack during development. Alternatively, higher 13-LOX could facilitate increased mobilization of stored TAGs during germination [38], an attractive possibility given the increase in oleosin. However, during seed development, increased amounts of hydroperoxylinoleic acids could lead to an altered susceptibility to *Aspergillus* infection as they have been shown to affect sporogenesis by Calvo et al. [39] but with differential effects depending on concentration and the position of the hydroperoxide on linoleic acid. Where an increase in sporulation occurs, a concomitant increase in aflatoxin production may not always follow because methyl jasmonate (produced from 13S-hydroperoxides) is an inhibitor of aflatoxin biosynthesis [35].

4.4. Conclusions and considerations

Validation studies for label-free proteomic approaches, peak integration and spectral counting, suggest these techniques hold promise for high-throughput protein quantitation. However, neither method produced consistent results for low mass proteins presumably due to the paucity of proteotypic peptides for quantitation. Lack of diagnostic peptides, particularly for low mass proteins, was also problematic for resolving paralogous proteins. Ara h 2 and 6 are relatively small proteins (172 and 144 aa respectively), and as conglutins in the prolamin family [40] share homology with each other and other conglutins which can lead to ambiguous assignments. To avoid this, manual confirmation of peptide assignments and redundancy should be made to ensure accurate assignment or at least minimize ambiguity. It is also recommended that initial validation of approach and biological system involve confirmation of differential proteins with alternative proteomics approaches such as quantitative western blotting, as demonstrated here.

Although western blotting is an extremely valuable technique it has recently been described as semi-quantitative at best [41]. Even if care is taken to quantitate within the linear range of development, other concerns exist, such as antibody cross-reactivity. This is particularly problematic for polyploid crop plants harboring multiple paralogous genes that are frequently greater than 90% identical at the amino acid level. For gene families, antibodies are rarely capable of distinguishing between the multiple, highly similar protein forms. In contrast, mass spectrometry is theoretically capable of discriminating multiple genes and gene products (that exhibit at least one non-isobaric amino acid substitution within a proteotypic peptide) if peptide quantitation data are carefully scrutinized at the post-analysis phase, as shown for the multiple forms of Ara h 3 and 4.

The spectral counting, label-free method used in this study is an example of a quick and effective method for detecting large and subtle differences in protein expression from a biological sample. The higher overall level of biological

variation observed with the peanut samples versus a defined protein standard suggests that other factors such as seed maturation, environmental conditions, or sample size must be considered (and controlled) to ascertain the source of this "extra" biological variation. Although a fast and reasonably accurate profiling approach, the results of spectral counting should be confirmed by more focused and accurate techniques such as absolute quantitation (AQUA) technology which uses synthetic, stable isotopic peptides as internal standards for signal quantitation [10]. Whereas spectral counting is a global, anonymous profiling strategy, AQUA quantitation is best applied to a small subset of proteins due to cost and time considerations.

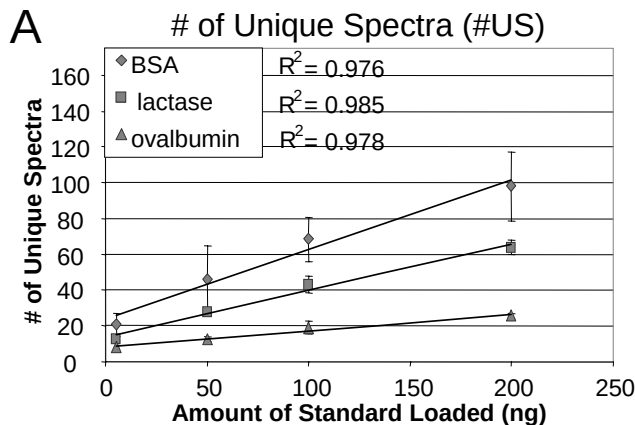
Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.jprot.2008.11.005](https://doi.org/10.1016/j.jprot.2008.11.005).

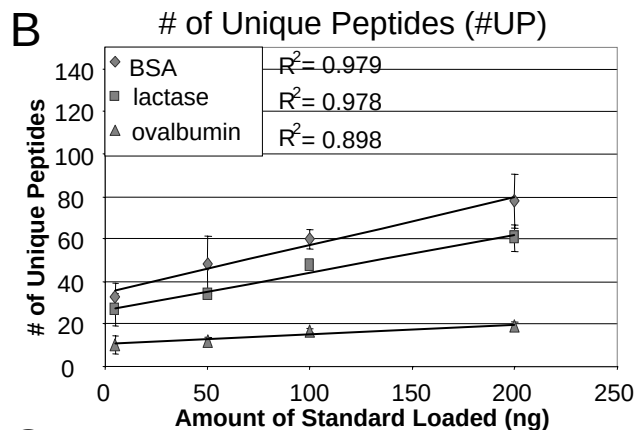
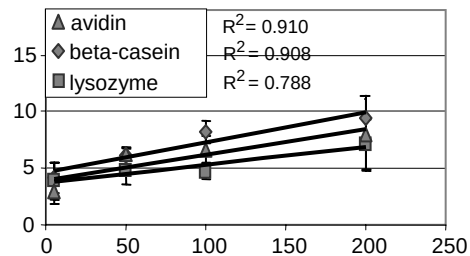
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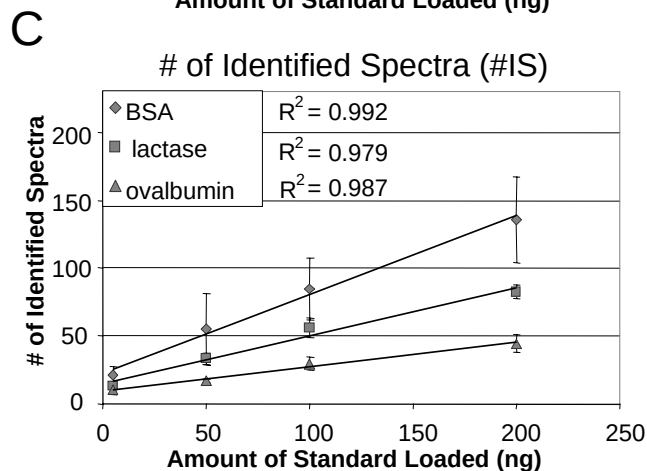
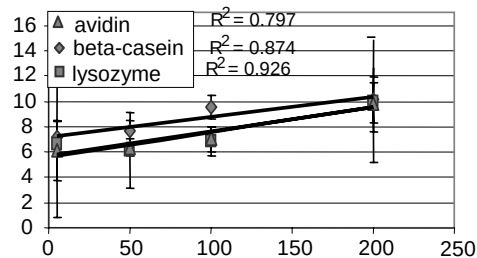
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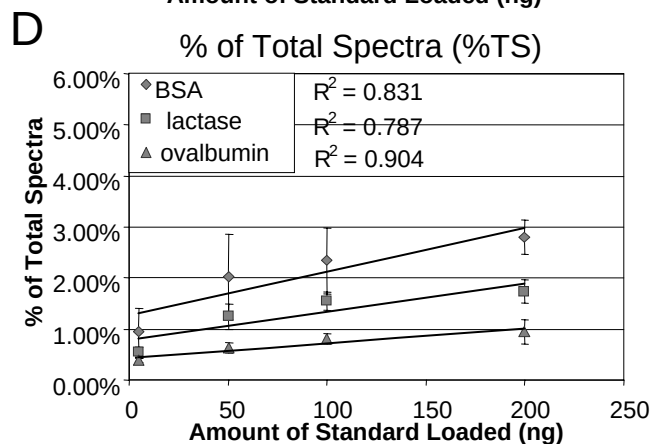
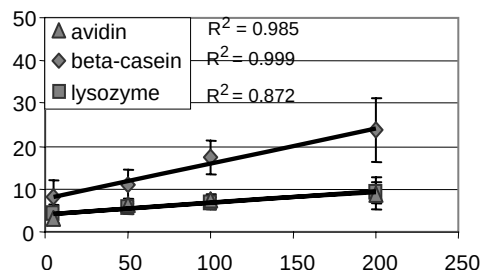
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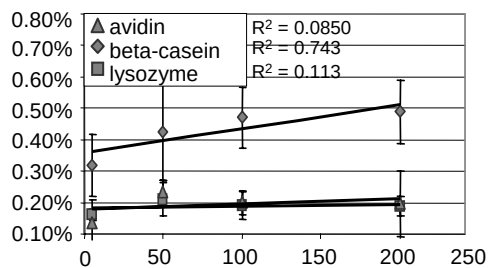
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Lower Molecular Mass Proteins (#IS)



Lower Molecular Mass Proteins (%TS)



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gi|37789212|gb|AAR02860.1| AIVTVRGGLRILSPDRKRQQYERPDEEEYDEDEYDEEERQQDRRRG 343
gi|3703107|gb|AAC63045.1| AIVTVRGGLRILSPDRKR-----GADEEEYDEDEYDE-----EDRRRG 314
gi|9864777|gb|AAG01363.1| AIVTVRGGLRILSPDRKR-----GADEEEYDEDEYDE-----EDRRRG 336
gi|5712199|gb|AAD47382.1| AIVTVRGGLRILSPDGR-----GADEEEYDEDQYEH-----QDGRRG 337
gi|118776570|gb|ABL14270.1| AIVTVRGGLRILSPDRK-----SPDEEEYDEDEYAEER-----QDWRRG 336
gi|47933675|gb|AAT39430.1| AIVTVRGGLRILSPDEDESSRSPSRREEFDEDRSRPQ-----RGKYD 316

gi|21314465|gb|AAM46958.1|AF51 RGSRGSGNGIETICTASFKKNIGNRSPDIYNPQAGSLKTANELQLNLL 393
gi|37789212|gb|AAR02860.1| RGSRGSGNGIETICTASFKKNIGNRSPDIYNPQAGSLKTANEL--NLL 391
gi|3703107|gb|AAC63045.1| RGSRGSGNGIETICTASAKKNIGNRSPDIYNPQAGSLKTANDL--NLL 362
gi|9864777|gb|AAG01363.1| RGSRGSGNGIETICTASVKKKNIGNRSPDIYNPQAGSLKTANDL--NLL 384
gi|5712199|gb|AAD47382.1| RGSRGSGNGIETICTACVKKKNIGNRSPHIYDPQRWFTQNCNDL--NLL 385
gi|118776570|gb|ABL14270.1| RGSRGSGNGIETICTATVKKKNIGNRSPDIYNPQAGSLKTANEL--NLL 384
gi|47933675|gb|AAT39430.1| ENRRGYKNGIETICSASVKKNLGRSSNPDIYNPQAGSLRSVNEL--DLP 364

ILRWLGLSAEYGNLYRNALFVPHYNTNAHSIIYALGRAHVQVVDNSNGDR 443
ILRWLGLSAEYGNLYRNALFVPHYNTNAHSIIYALGRAHVQVVDNSNGRI 441
ILRWLGPSAEYGNLYRNALFVAHYNTNAHSIIYALGRAHVQVVDNSNGNR 412
ILRWLGLSAEYGNLYRNALFVPHYNTNAHSIIYALGRAHVQVVDNSNGNR 434
ILRWLGLSAEYGNLYRNALFVPHYNTNAHSIIYALGRAHVQVVDNSNGNR 435
ILRWLGLSAEYGNLYRNALFVPHYNTNAHSIIYALGRAHVQVVDNSNGNR 434
ILGWLGLSAQHGTIYRNAMFVPHYTLNAHTITVVALNGRAHVQVVDNSNGNR 414

VFDEELQEGHVLVVPQNF~~AVAGK~~SQSENFEYVAFKTD~~SRPS~~IANLAGENS 493
 VFDEELQEGHVLVVPQNF~~AVAGK~~SQSENFEYVAFKTD~~SRPS~~IANLAGENS 491
 VFDEELQEGHVLVVPQNF~~AVAGK~~SQSENFEYVAFKTD~~SRPS~~IANLAGENS 462
 VFDEELQEGHVLVVPQNF~~AVAGK~~SQSDNFEYVAFKTD~~SRPN~~IANFAGENS 484
 VFDEELQEGHVLVVPQNF~~AVAGK~~SQSENFEYVAFKTD~~SRPS~~IANFAGENS 485
 VFDEELQEGHVLVVPQNF~~AVAGK~~SQSENFEYVAFKTD~~SRPS~~IANLAGENS 484
 VFDEELQEGHVLVVPQNF~~AVAAKAQ~~SENFEYLAFTKTD~~SRPS~~IANLAGENS 464

FIDNLP~~EEVVANSYGLPREQARQLKNNNPFKFFVPPS-EQSLRAVA~~ 538
FIDNLP~~EEVVANSYGLPREQARQLKNNNPFKFFVPPS-EQSLRAVA~~ 536
VIDNLP~~EEVVANSYGLPREQARQLKNNNPFKFFVPPS-QQSPRAVA~~ 507
IDNLP~~EEVVANSYGLPREQARQLKNNNPFKFFVPPS-QQSLRAVA~~ 529
FIDNLP~~EEVVANSYGLPREQARQLKNNNPFKFFVPPF-QQSPRAVA~~ 530
FIDNLP~~EEVVANSYGLPREQARQLKNNNPFKFFVPPF-QQSPRAVA~~ 529
IDNLP~~EEVVANSYGLPREQARQLKNNNPFKFFVPPFDHDSMREVA~~ 510

gij21324465	gij118776570	gij3703107	gij37789212	gij47933675	gij5712199	gij9864777
AGQEQENEGGNIFSGFTPEFLAQAFQVDDR	AGQEQENEGGNIFSGFTSEFLAQAFQVDDR	ADEEEYDEDEYEDDEDR	AYFGLFLGCPSTYEEPAQQGR	AQSENIEYLAFK	FFVPPFQQSPR	AGQEQENEGGNIFSGFTPEFLAQAFQVDDR
AHVQVDSNGDR	GENESEEQGAIVTK	FFVPPSQSPR	SPDIYNPQAGSLKTANELNLILR	FFVPPFDHQSMT	QIVQNLWGENESEEQGAIVTVR	AHVQVDSNGNR
FFVPPSEQSLR	KSPDEEEYDEDEYAEER	GETESEEQGAIVTVR	TANELNLILR	GENEREQGAIVTVK	SQSENFEYVAFK	FFVPPSQSLR
GENESDEQGAIVTVR	KSPDEEEYDEDEYAEERQQR	RADEEEYDEDEYEDDEDR		IESEGGIETWNPNNQEFQCAGVALSR		FNLAGNHEQFLR
QILQNL	QIVQNLGENESEEQGAIVTVK	RADEEEYDEDEYEDDEDR		NAMFVPHYTLNAHTIVVALNGR		GADEEEYDEDEYEDDEDR
QILQNLGENESDEQGAIVTVR	QIVQNLGENESEEQGAIVTVKGGLR	RQPEENACQFQR		QGGEENECQFQR		GADEEEYDEDEYEDDEDR
QQYERPDDEEEYDEDEYDEER	SPDEEEYDEDEYAEERQQR			RFYLAGNHEQFLR		GADEEEYDEDEYEDDEDR
RQYERPDDEEEYDEDEYDEER	SPDEEEYDEDEYAEERQQR			SSNPDIYNPQAGSLR		GENESEEQGAIVTK
VFDEELQEGHVLVVPQNFVAGK				SVNELDLPILGWLGLSAQHGTIYR		GYFGLIFPGCPSTYEEPAQQGR
				TDSRPSIANLAGENSIIDNLPPEEVANSYR		GYFGLIFPGCPSTYEEPAQQGR
				VYDEELQEGHVLVVPQNFVAAK		KRGADDEEEYDEDEYEDDEDR
						LESEGGIETWNPNNQEFECAGVALSR
						NALFVPHYNTNAHSIYALR
						NALRRPFYSNAPQEIFIQGR
						NRSPDIYNPQAGSLK
						QIVQNL
						QIVQNLGENESEEQGAIVTVK
						QIVQNLGENESEEQGAIVTVKGGLR
						QLKNNNPFKFFVPPSQSLR
						QPEENACQFQR
						RFEGEDSQQQQDSHQK
						RFNLAGNHEQFLR
						RGADDEEEYDEDEYEDDEDR
						RGADDEEEYDEDEYEDDEDR
						RPFYSNAPQEIFIQGR
						SPDIYNPQAGSLK
						SQSDNFEYVAFK
						TANDNLILR
						VYDEELQEGHVLVVPQNFVAGK
						WLGLSAEYGNLYR

	Spectral Counting												Peak Integration		
	# Unique Spectra		% Total Spectra	# Unique Peptides	# Identified Spectra		Unweighted Spectra (normalized)		Unweighted Spectra (un-normalized)						
MM (kDa)	Coefficient of Variance by Molecular Weight														
	Biological	Technical	Biological	Technical	Biological	Technical	Biological	Technical	Biological	Technical	Biological	Technical	Biological	Technical	
	116.3-lactase	8.3±2.0	10.7±6.6	16.2±4.2	9.4±6.9	6.9±1.7	9.9±4.7	12.3±4.3	10.2±2.0	14.0±14.2	NA	17.2±18.3	4.1±1.1	4.4±2.4	3.0±1.4
	66.2-BSA	27.1±10.7	3.0±1.3	32.5±16.3	3.7±4.2	21.2±9.6	3.9±1.5	31.5±10.8	8.1±13.5	24.5±12.7	NA	26.0±9.4	7.7±2.1	4.4±1.3	2.7±0.40
	45.0-ovalbumin	16.5±11.4	9.0±2.7	15.9±6.7	7.7±5.4	21.0±14.5	10.0±4.2	18.2±10.3	11.5±16.9	21.3±16.3	NA	20.0±23.7	5.2±2.6	5.9±1.4	3.4±0.70
	23.6-casein	19.1±9.5	15.6±4.9	26.9±7.7	12.1±6.8	15.7±10.4	18.8±8.3	32.2±9.6	15.0±11.3	15.0±8.4	NA	20.0±13.8	16.0±6.8	4.6±1.1	3.2±0.60
	18.0-avidin	26.3±16.1	12.9±9.8	34.6±20.6	17.8±7.1	24.6±15.2	12.6±7.0	25.9±14.6	20.7±17.4	23.6±8.5	NA	26.0±4.3	5.8±2.6	3.1±0.90	3.1±0.80
	14.4-lysozyme	28.6±11.9	13.5±1.3	19.2±6.4	17.1±12.9	28.6±11.9	13.5±1.3	30.4±12.1	23.1±16.3	33.8±22.1	NA	36.7±25.7	15.6±11.1	ND	ND
Standard (ng)	Coefficient of Variance by Amount of Standard Loaded														
	Biological	Technical	Biological	Technical	Biological	Technical	Biological	Technical	Biological	Technical	Biological	Technical	Biological	Technical	
	200.0	20.4±13.4	8.1±3.5	21.3±16.2	10.0±8.0	19.5±12.4	7.9±2.9	23.4±13.9	12.1±7.7	10.6±4.6	NA	17.4±8.7	7.5±5.0	4.1±1.0	3.2±0.80
	100.0	15.2±3.2	13.3±5.8	19.6±6.3	12.1±14.4	12.3±4.0	12.0±6.0	20.0±5.3	13.2±12.9	18.7±9.5	NA	15.5±6.7	8.7±5.6	3.7±1.2	2.9±0.50
	50.0	19.0±13.4	14.5±6.8	25.5±12.4	12.8±6.8	16.0±10.8	15.9±7.4	21.6±16.8	12.9±5.5	22.8±13.8	NA	18.9±15.1	10.2±7.1	4.9±1.7	2.8±0.60
	5.0	30.8±12.4	7.2±6.5	27.5±18.5	10.3±3.2	30.8±13.5	10.0±7.1	31.2±10.5	21.0±6.7	36.1±16.0	NA	45.3±7.0	10.0±10.8	5.7±2.2	3.4±1.2
Overall Average CV	21.1±7.0	10.8±3.9	23.9±7.0	11.3±4.1	19.7±7.3	11.4±4.2	24.9±6.8	14.8±5.1	21.0±13.8	NA	24.3±9.7	9.1±4.1	4.5±0.90	3.1±0.30	

**Screening of Peanut Lines:
B3.3.1/5 T1-3 (RNAi) and B3.3.1/5
T1-4 (WT)**

Protein name	Accession #	RNAi (4 Technical Replicates)			WT (3 Technical Replicates)			Students T- test (p-value)	Sum of Counts (Used in Analysis)
		Average Tech Counts	Technical SD	Technical CV	Average Tech Counts	Technical SD	Technical CV		
13-lipoxygenase	gi 68161358	6	1.73	0.3149	5	2.08	0.5677	0.611	33
Agglutinin	gi 1708792	5	1.26	0.2649	7	1.53	0.2696	0.101	36
Arachin Ahy-3 precursor	gi 75253181	7	1.29	0.1986	9	2.00	0.2857	0.168	47
Ara h 1	gi 1168391	100	6.40	0.0750	131	6.35	0.0664	0.001	713
	gi 46560478	15	1.50	0.1017	11	3.79	0.4368	0.181	85
Ara h 2.01/2 (conglutin-7)	gi 147639154	4	1.15	0.2887	17	4.04	0.3031	0.004	56
Ara h 3/Ara h 4 (glycinin)	gi 21314465	285	24.76	0.0869	282	15.82	0.0705	0.903	1812
	gi 37789212	3	0.50	0.1818	5	0.58	0.1575	0.010	22
	gi 3703107	5	1.41	0.2828	3	2.00	1.0000	0.154	26
	gi 5712199	12	2.06	0.1755	8	3.00	0.5000	0.114	65
	gi 118776570	15	1.29	0.0890	10	3.51	0.4581	0.086	81
	gi 47933675	62	14.84	0.2414	63	7.51	0.1491	0.857	397
	gi 9864777	178	16.06	0.0902	185	2.08	0.0142	0.516	1152
Ara h 6 (Conglutin)	gi 17225991	18	1.71	0.0962	22	4.04	0.2332	0.187	123
Ara h 7 (2S protein 2)	gi 52001231	4	0.50	0.1176	3	1.15	0.4330	0.295	25
Ara h 8 (PR-10 protein)	gi 52547774	4	1.26	0.2961	4	1.00	0.3333	0.644	26
BSA Internal Std	gi 1351907	34	2.22	0.0647	34	5.20	0.1925	0.945	218
Conarachin (5)	gi 52001225	4	0.58	0.1650	13	3.06	0.2864	0.003	46
Trypsin Inhibitor	gi 2914482	5	1.15	0.2309	5	1.73	0.4330	0.978	32

Screening of Peanut Lines: B3.3.2/18 T1-3 (RNAi) and B3.3.2/18 T1-4 (WT)		RNAi (4 Technical Replicates)			WT (3 Technical Replicates)			Students T- test (p-value)	Sum of Counts (Used in Analysis)
Protein name	Accession #	Average Tech Counts	Technical SD	Technical CV	Average Tech Counts	Technical SD	Technical CV		
13-lipoxygenase	gi 68161358	4	0.96	0.255	4	1.53	0.458	0.928	25
Agglutinin	gi 1708792	6	0.96	0.153	3	1.00	0.333	0.012	34
Arachin Ahy-3 precursor	gi 75253181	10	2.22	0.227	5	1.53	0.327	0.030	53
Ara h 1	gi 1168391	115	15.26	0.133	90	7.02	0.085	0.056	705
	gi 46560478	13	0.96	0.075	14	3.06	0.241	0.535	89
Ara h 2.01/2 (Conglutin-7)	gi 147639154	7	0.50	0.074	20	6.51	0.355	0.012	82
Ara h 3/Ara h 4	gi 21314465	243	22.43	0.092	271	14.19	0.057	0.126	1715
	gi 37789212	3	0.50	0.182	4	1.73	0.433	0.150	23
	gi 3703107	4	1.26	0.296	6	0.58	0.102	0.059	34
	gi 5712199	6	2.38	0.433	8	1.53	0.199	0.133	45
	gi 118776570	13	1.50	0.118	11	1.00	0.100	0.145	81
	gi 47933675	62	8.41	0.136	65	12.42	0.209	0.725	426
	gi 9864777	146	10.80	0.074	167	6.08	0.040	0.034	1040
Ara h 6 (Conglutin)	gi 17225991	26	3.50	0.133	21	2.65	0.139	0.083	162
Ara h 7 (2S protein 2)	gi 52001231	4	0.50	0.118	4	0.00	0.000	0.663	29
Ara h 8 (PR-10 protein)	gi 52547774	4	0.96	0.225	3	1.00	0.333	0.271	26
Ara h 10 (Oleosin)	gi 113200509	3	0.82	0.272	1	0.58	0.866	0.011	14
BSA Internal Std.	gi 1351907	34	6.24	0.186	34	6.66	0.213	0.872	228
Conarachin (5)	gi 52001225	1	0.96	0.766	10	1.53	0.176	4.0E ⁻⁴	31
Trypsin Inhibitor	gi 2914482	5	0.58	0.128	5	0.58	0.133	0.605	31

Screening of Peanut Lines: B11.1.1/11-1 T2-2 (RNAi) and B11.1.1/11-1 T2-3 (WT)		RNAi (4 Technical Replicates)			WT (3 Technical Replicates)			Students T- test (p-value)	Sum of Counts (Used in Analysis)
Protein name	Accession #	Average Tech Counts	Technical SD	Technical CV	Average Tech Counts	Technical SD	Technical CV		
13-lipoxygenase	gi 68161358	3	0.82	0.272	1	0.58	0.433	0.031	16
Agglutinin	gi 1708792	4	0.82	0.204	5	1.15	0.247	0.408	30
Arachin Ahy-3 precursor	gi 75253181	7	1.26	0.186	3	0.58	0.173	0.008	37
Ara h 1	gi 1168391	118	4.03	0.034	96	4.16	0.043	0.001	760
	gi 46560478	12	1.91	0.167	11	1.15	0.108	0.539	78
Ara h 2.01/2 (Conglutin)	gi 147639154	2	0.96	0.426	21	5.57	0.265	0.001	72
Ara h 3/Ara h 4	gi 21314465	255	35.87	0.141	248	13.05	0.053	0.792	1763
	gi 37789212	4	1.71	0.455	4	1.53	0.353	0.661	28
	gi 3703107	4	0.96	0.225	5	0.58	0.124	0.539	31
	gi 5712199	7	2.22	0.329	4	1.73	0.433	0.137	39
	gi 118776570	13	1.73	0.139	13	1.53	0.121	0.900	88
	gi 47933675	63	12.31	0.196	57	4.73	0.083	0.462	421
	gi 9864777	154	23.33	0.151	156	7.77	0.050	0.890	1086
Ara h 6	gi 17225991	14	1.83	0.130	25	3.06	0.121	0.002	132
Ara h 7 (2S protein 2)	gi 52001231	5	1.26	0.265	5	1.15	0.247	0.932	33
Ara h 8 (PR10 protein)	gi 52547774	5	1.29	0.287	4	0.58	0.158	0.352	29
BSA Internal Std.	gi 1351907	29	3.56	0.123	29	2.52	0.088	0.896	202
Trypsin Inhibitor	gi 2914482	4	0.00	0.000	4	0.58	0.158	0.286	27

