Variability of Individual Allergenic Protein Levels in Crops

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Outline

- Why the concern with endogenous allergen levels? Is it warranted?
- Natural variability- genotype versus environment
- What do we know for specific allergenic proteins in specific crops? (maize, soybean, apple, peach, strawberry)
- Conclusions

Endogenous Allergens- Why?

- For GM soybean crops, a robust safety assessment is performed according to regulatory recommendations (Codex, 2009; EFSA, 2011). This evaluation addresses the possibility that the genetic modification may modulate the level of endogenous allergens and thus increase the allergenic potential of the GM crop compared to its non-GM counterpart.

- Individuals with food allergies may minimize their symptoms by selecting varieties with low allergen content.

Is the Evaluation of Endogenous Allergens Warranted?

- Allergic individuals attempt to avoid offending foods completely (GM or non-GM)
    - How important is a change in the level of allergen(s) in a GM food if the conventional food is already known to be allergenic and is regulated as such (e.g., soy).

- Comparisons of food allergen concentrations across different cultivars of a crop have rarely been attempted because protein threshold for eliciting an allergic reaction have typically not been determined and vary by individual.

- Natural variability in allergen levels in non-GM already in food supply.

- INTERPRETATION of any differences in terms of allergenicity (does change in protein levels = change in allergenicity?)

Is the Evaluation of Endogenous Allergens Warranted?

Consequences of higher allergen exposure

- How much of an increase in allergen concentrations within a cultivar is a safety concern? There is a lack of identification of safe consumption levels.
  - The quantity of food consumed is not subject to regulation – further sources of exposure variability

- What is the hypothesis you are testing? Higher exposure to a protein in individuals results in a higher frequency of sensitization within a population? Not so straightforward!

  - Greater exposure at a young age to allergens can have the opposite result-
    - Countries where infant-appropriate peanut snacks are available, peanut allergy is rare (Burks et al., 2008; Du Toit et al., 2008)
    - Similar relationships observed for certain milk, egg, and fish allergens where early exposure reduces sensitization rates (Katz et al., 2010; Koplin et al., 2010).

Altered allergenicity of whole GM food

GM soybean scenario

- Soy is recognised as an allergenic food (mandatory declaration applies) regardless of whether GM or not, and regardless of levels of endogenous allergenic proteins
- New non-GM soy varieties can be/ are introduced into the food supply with no pre-market safety assessment required
- No information on whether or not allergen levels are altered
- This is because, from a regulatory perspective, soy is considered to be a safe food for the general population
- The risk to allergic consumers is acknowledged, but it is addressed through mandatory declaration requirements (apply equally to GM and non-GM soy)

Slide courtesy of Lynda Graf Senior Scientist Food Standards Australia New Zealand

Measurement of endogenous allergens in food crops: What do we already know?

- Scientific literature (*soy, apple, peach*)
- ILSI-HESI Protein Allergenicity Technical Committee: international workshops, symposia with allergy experts, publications.
- Research efforts: recent advances in the quantitation of **soybean** allergens

  - large natural variation in allergen composition of non-GM soybean varieties as a function of germplasm and environmental factors (agricultural conditions, geographical location, etc)

  - possible with new techniques to significantly improve the limit of detection (LOD) of specific proteins

Houston et al. (2011) Quantitation of Soybean Allergens Using Tandem Mass Spectrometry. *J. Proteome Res.* 10(2), 763-773.

Slide courtesy of Lynda Graf Senior Scientist Food Standards Australia New Zealand

Where allergen concentrations have been characterized across crop cultivars, they have been found to vary considerably.

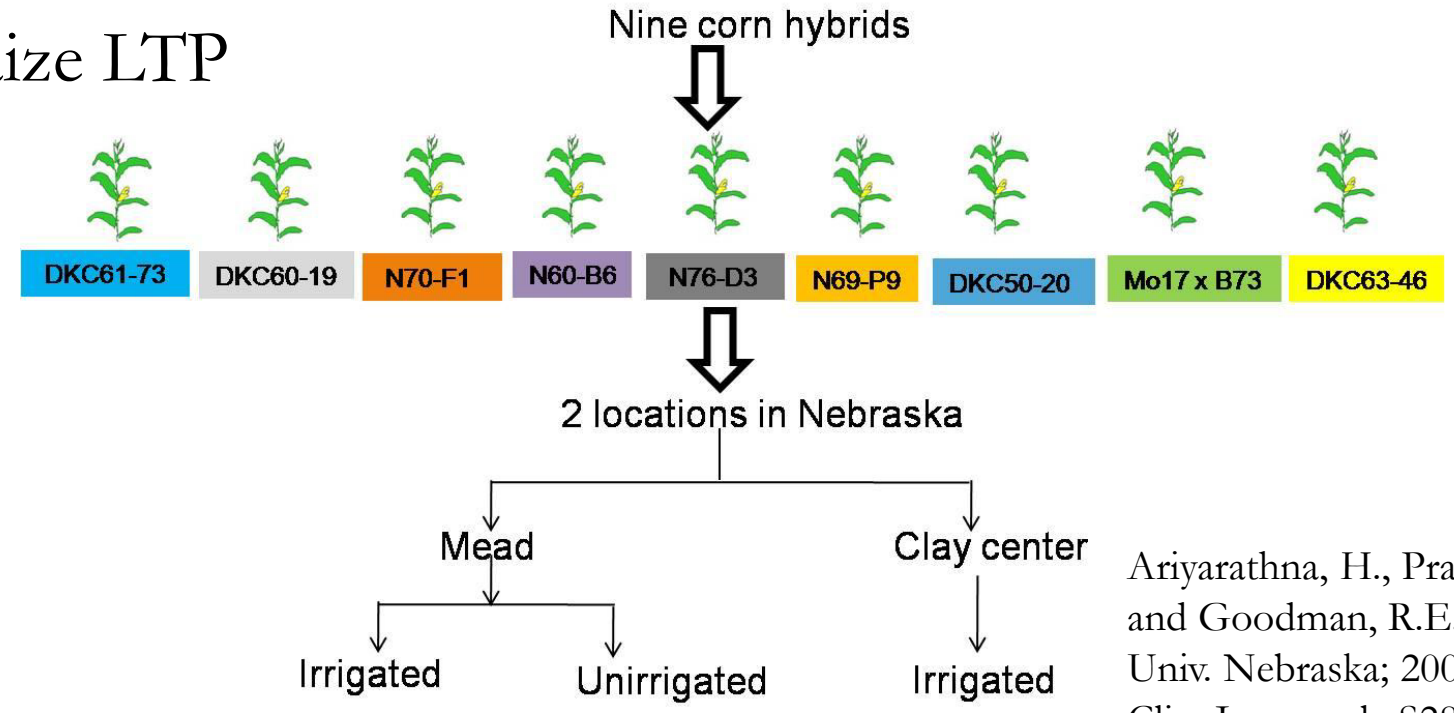
1. Differences among cultivars (genotype)

2. Natural variability in allergen concentrations can occur in response to differing environmental conditions (e.g., temperature, moisture, nutrients, plant pathogens, insect loads), harvest timing, or storage conditions.

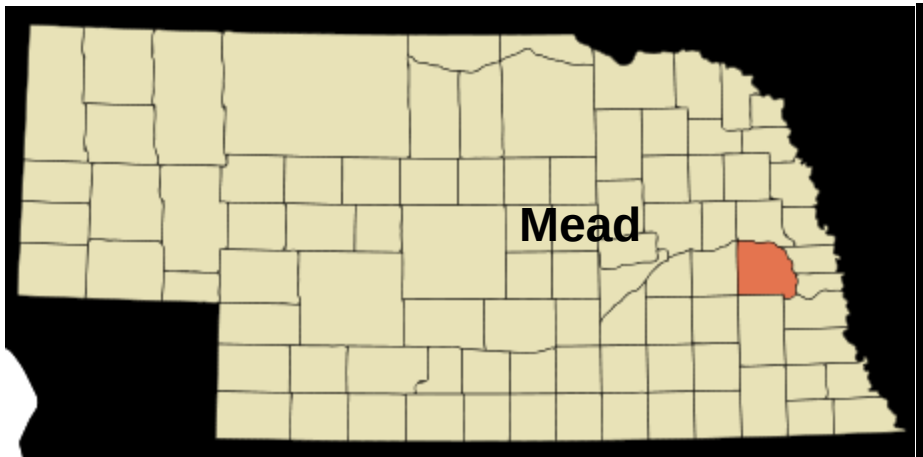
Some specific examples….

- Maize (LTP)
- Soybean allergenic proteins
- Apple (Mal d 1, Mal d 3)
- Peach

Maize LTP

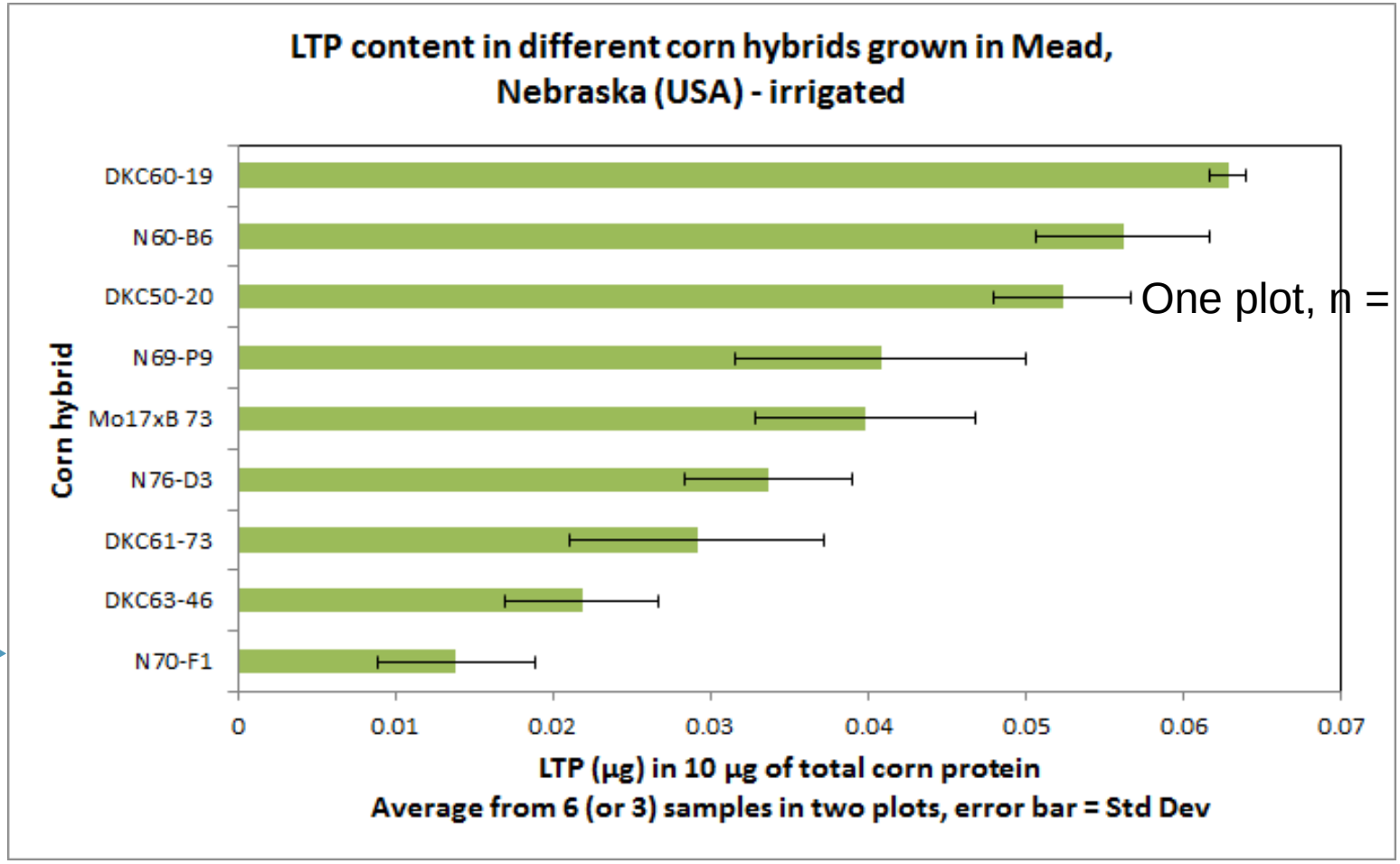


Ariyarathna, H., Pramod, S.N., and Goodman, R.E., FARRP, Univ. Nebraska; 2009, J. Allergy Clin. Immunol., S28, abstract 93



Mead irrigated: LTP determined by dot blotting with anti-maize LTP peptide specific Ab, using a standard dilution curve of purified maize LTP to estimate concentrations

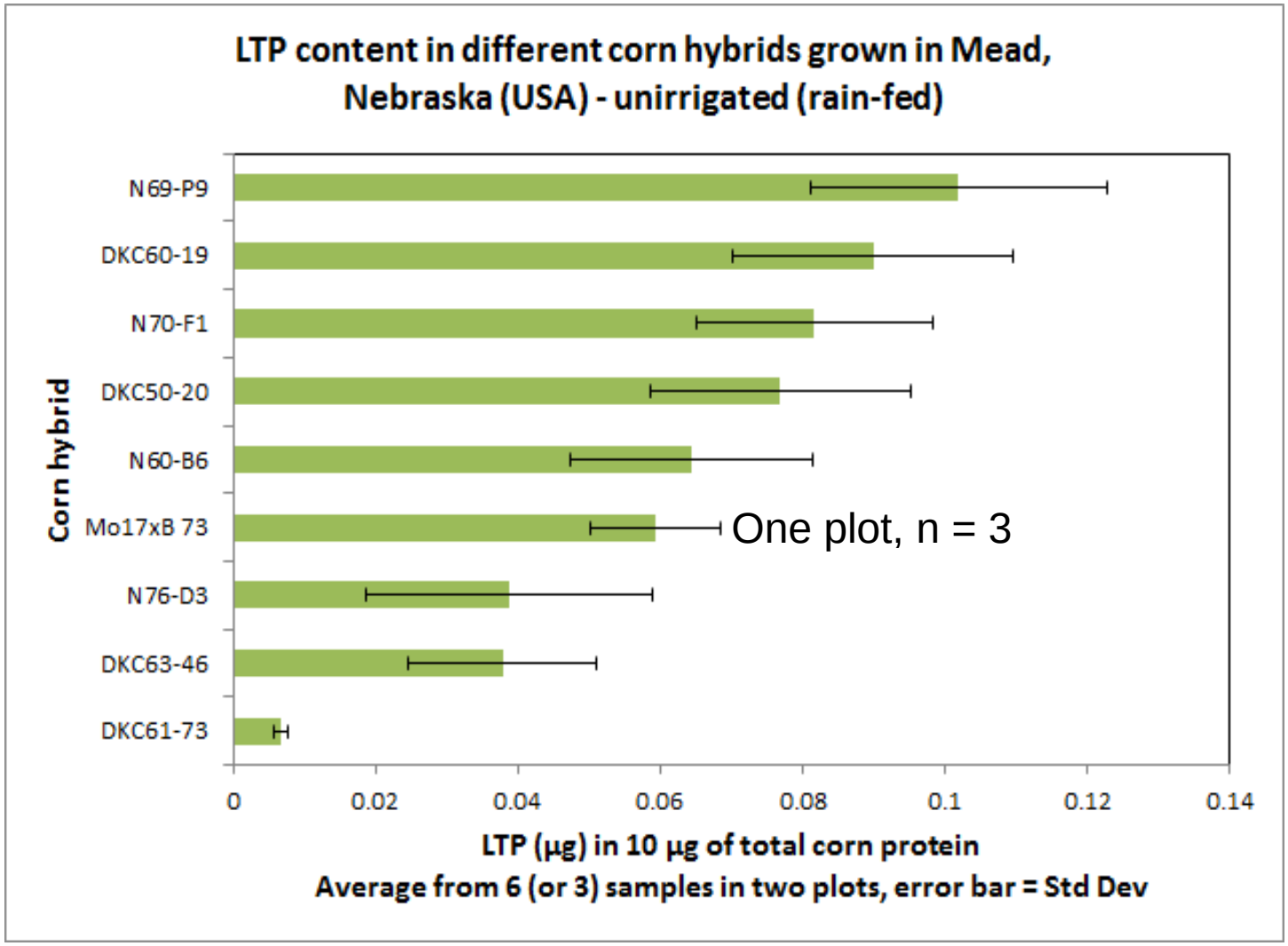
5-fold



Ariyarathna, H., et al., 2009, J. Allergy Clin. Immunol., S28, abstract 93

Mead unirrigated:

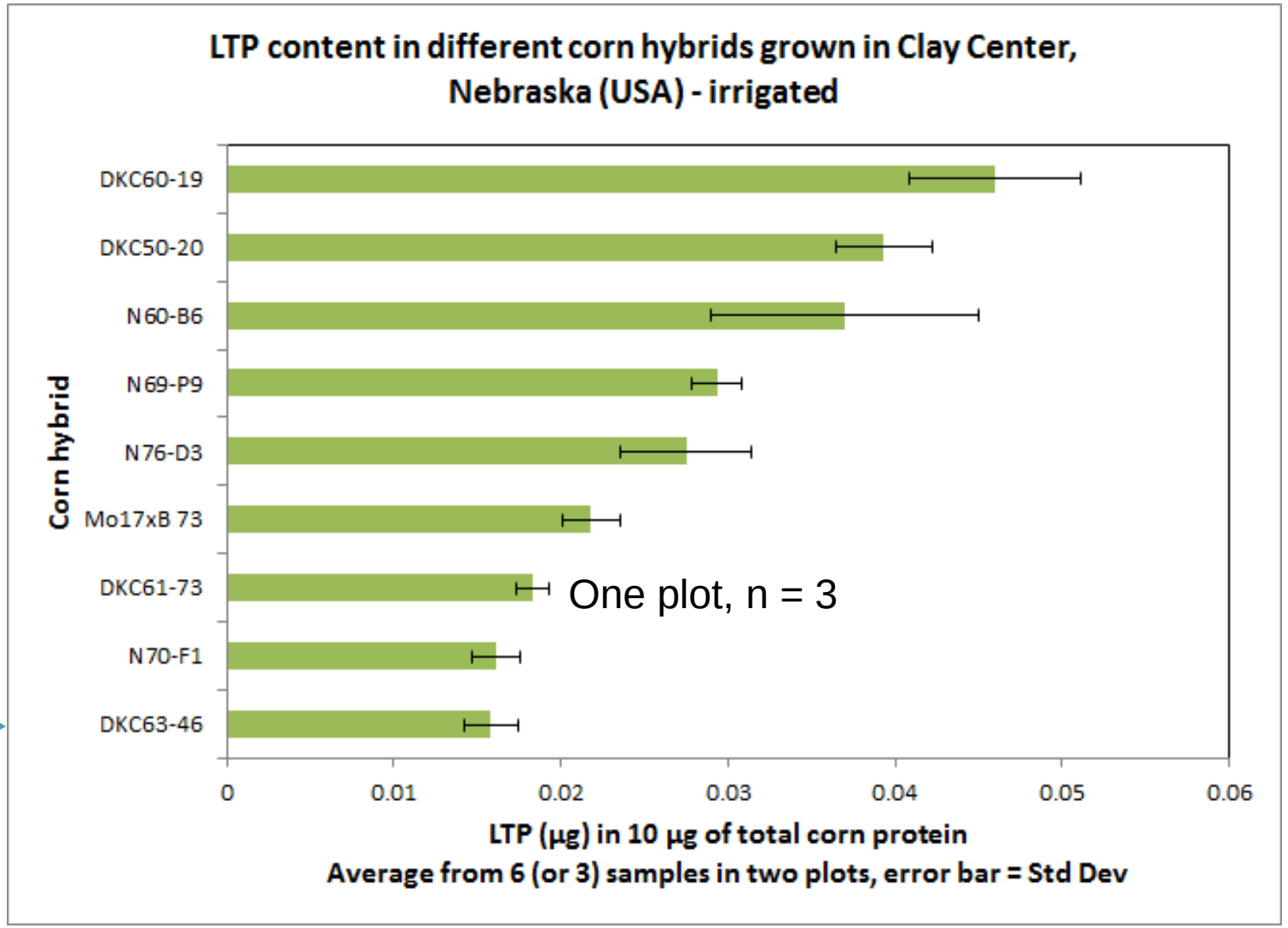
15-fold



Ariyarathna, H., et al., 2009, J. Allergy Clin. Immunol., S28, abstract 93

Clay Center irrigated:

4-fold



Ariyarathna, H., et al., 2009, J. Allergy Clin. Immunol., S28, abstract 93

Study Conclusions

Marked differences (up to 15-fold) were found in LTP abundance from corn grain samples, which suggests significant differences in allergenic activity of food products might be expected from similar products.

Except for one hybrid all the other hybrids showed increased amount of LTP in un-irrigated location compared to two irrigated locations.

Statistically significant differences were found between LTP content in nine non-transgenic corn hybrids grown in two locations with/without irrigation (p value <0.0001).

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Quantification of Maize Lipid Transfer Protein

- LTP levels quantitatively evaluated in 14 different commercially available non-GM maize lines

- Liquid chromatography-ultraviolet/mass spectrometry method

- The level of LTP varied between 58 and 678 µg/g (approx. 12-fold)

Kuppannan et al. (2011) Anal. Chem., 83:516-524.

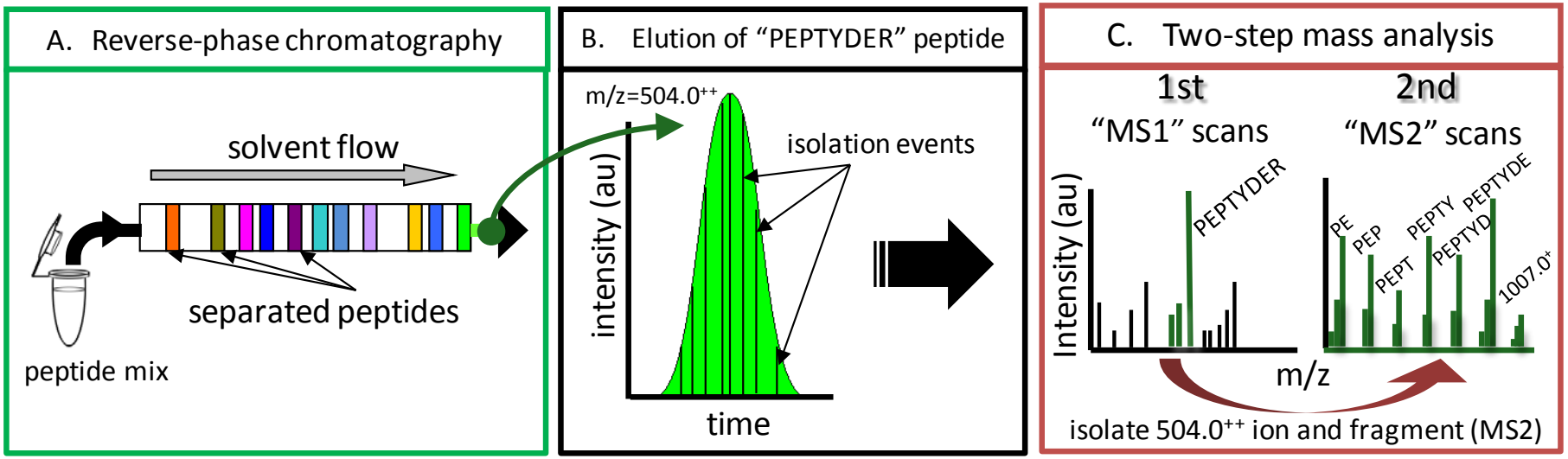
Variation of LTP content in cultivars or hybrids of allergenic fruits

- Apple LTP(Mal d 3)
  - Highest difference between 2 cultivars 100 fold
  - Sancho et al., 2008
- Peach LTP(Pru p 3)
  - Highest difference between 2 cultivars 39 fold
  - Pastorello et al., 2002

Absolute Quantitation of Soybean Allergenic Protein Levels using LC and Tandem Mass Spectrometry

HESI PATC
DuPont Pioneer

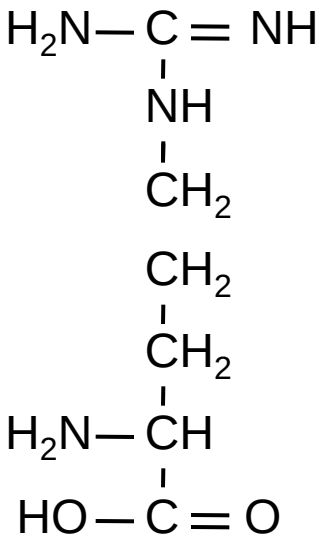
LC-MS/MS for protein identification



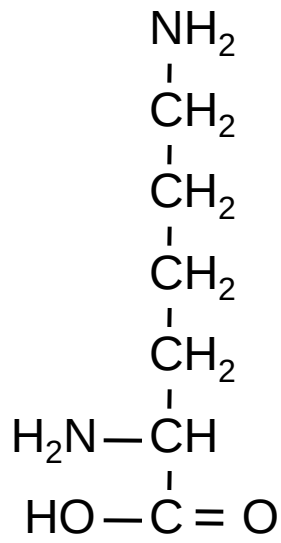
1. Proteins are digested into peptides
2. Peptides are separated to reduce complexity
3. Peptides are "weighed" and sequenced by mass spectrometry
4. Peptide sequence provides protein identification through bioinformatics

Severin E. Stevenson, Norma L. Houston, and Jay J. Thelen, 2010. *Regul. Toxicol. Pharmacol. 58, 3, S36-41.*

AQUA peptide analysis uses synthetic peptides that contain isotope-labeled amino acids



Arginine (R) – 174.2



Lysine (K) – 146.19

Isotope labeled	$^{13}C_6H_{14}{}^{15}N_4O_2$ – 184.2	$^{13}C_6H_{14}{}^{15}N_2O_2$ – 154.19
Native peptide	LSAEFGSLR – 978.51	VSDDEFNNYK – 1229.52
AQUA peptide	LSAEFGSLR – 988.51	VSDDEFNNYK – 1237.52

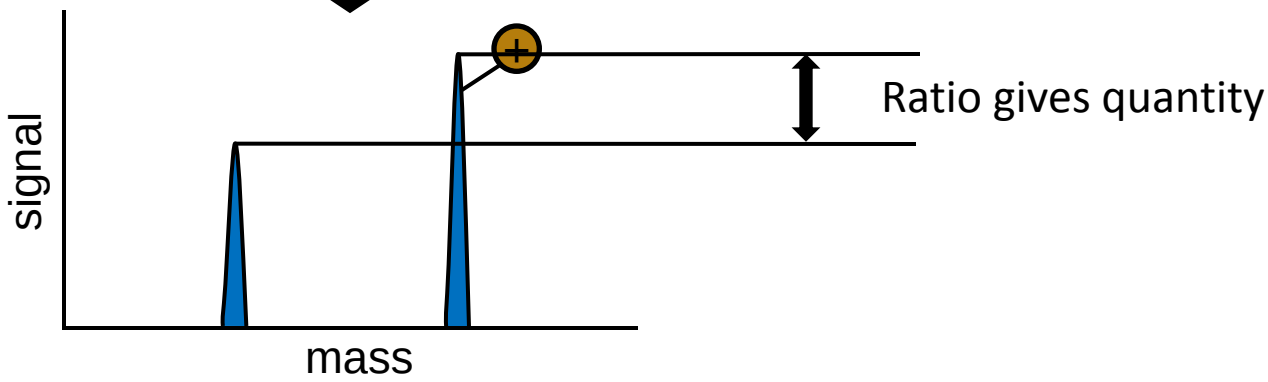
How does it work?

Seed allergen (peptides)

Labeled internal standard

Mix

mass spectrometry can distinguish them



Primary inventor: Jay J. Thelen, PhD
thelenj@missouri.edu

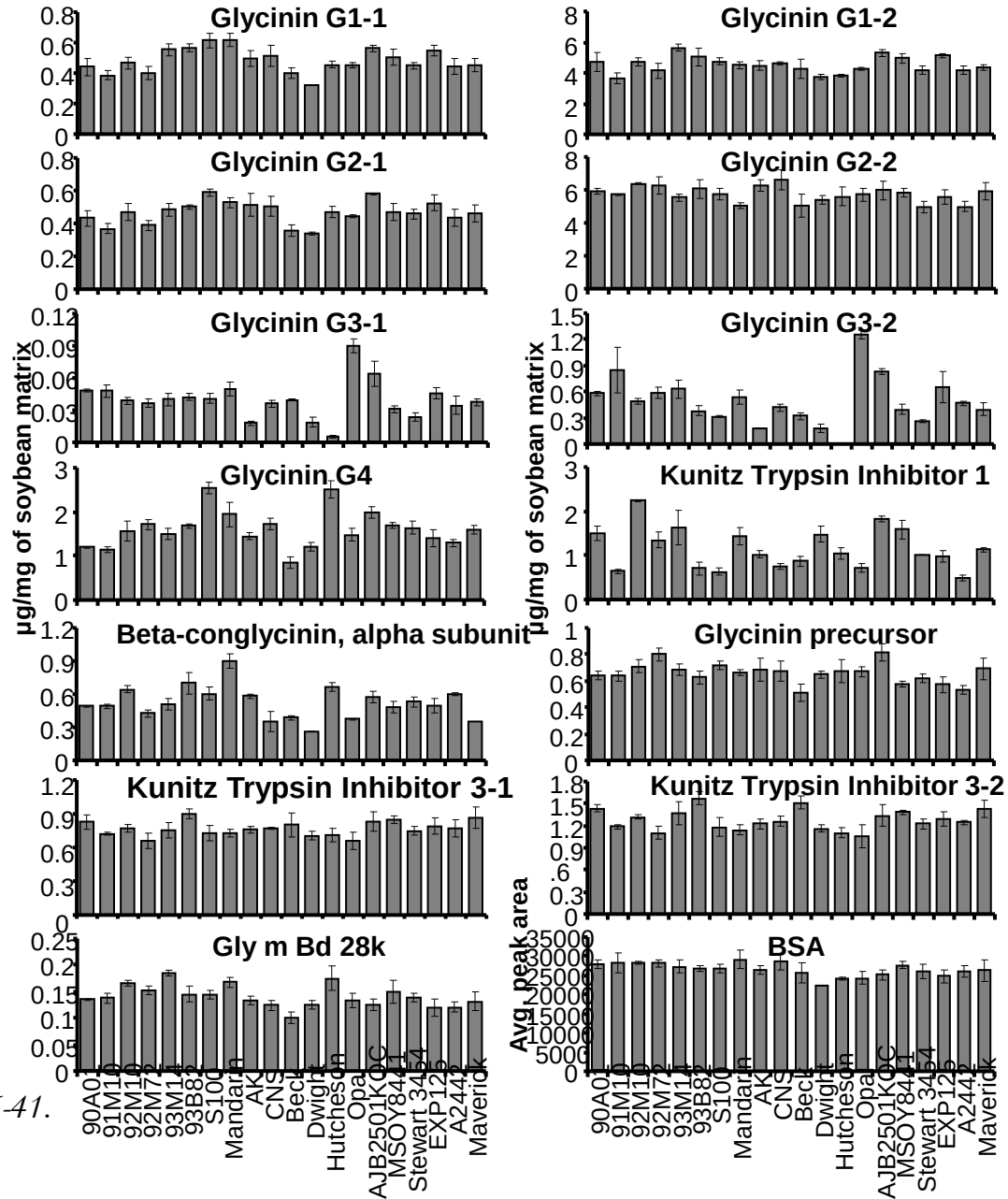
AQUA peptides were designed and synthesized for ten soy allergens

Allergen	Peptide name
Glycinin G1	GlyG1-1
	GlyG1-2
Glycinin G2	GlyG2-1
	GlyG2-2
Glycinin G3	GlyG3-1
	GlyG3-2
Glycinin G4	GlyG4
Beta-conglycinin, alpha subunit	beta-con
Kunitz trypsin inhibitor 3	KTI3-1
	KTI3-2
Kunitz trypsin inhibitor 1	KTI1
Gly m Bd 28K	AllGly28
Glycinin precursor	GlyPre-1
	GlyPre-2
Gly m Bd 30K	34MatureSeed-1

Evaluation of allergen levels in 20 different varieties of non-GM Soybean (HESI PATC)

Some allergen levels were variable in expression across varieties, others were constant

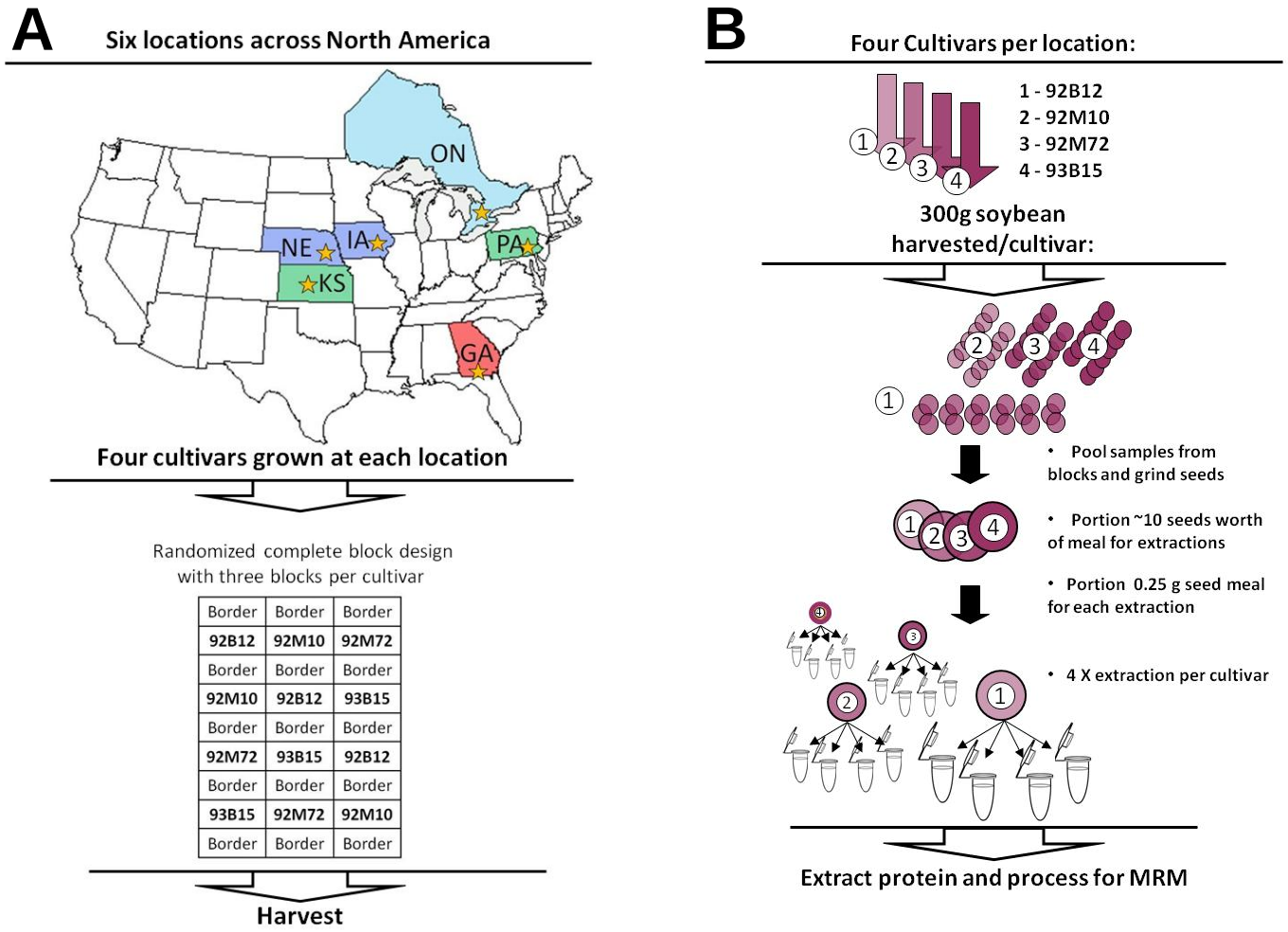
Absolute quantities spanned over a 10-fold range [glycinin G3 (a Gly m 6 isoform)]; however, total allergen levels showed little variation amongst varieties, suggesting individual protein variation.



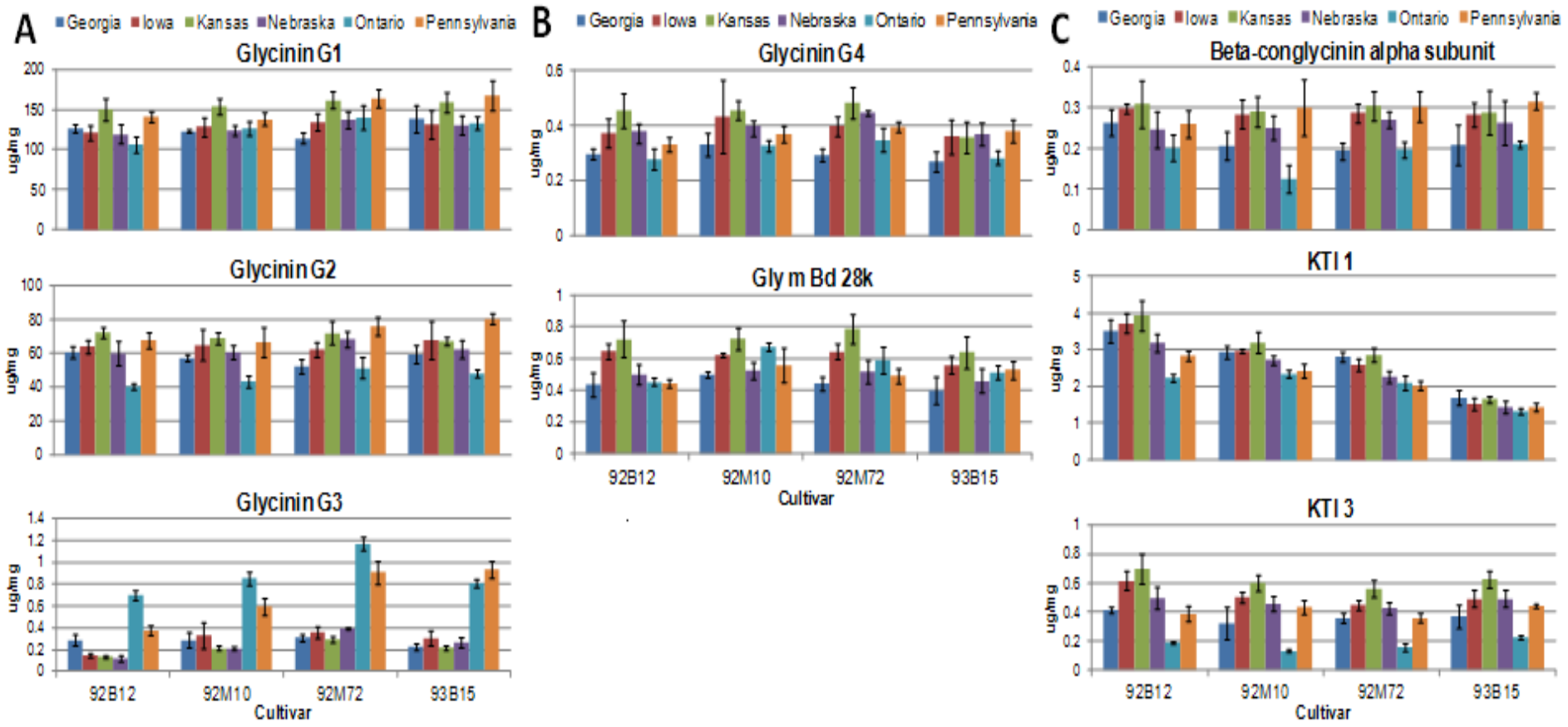
Severin E. Stevenson, Norma L. Houston, and Jay J. Thelen, 2010. *Regul. Toxicol. Pharmacol. 58, 3, S36-41.*

Experimental workflow (Genotype versus Environment) (DuPont/Pioneer)

S. Stevenson et. al., Frontiers in Plant Proteomics, 2012., *submitted*



Some allergens were variable in expression within the six regions studied, others were constant…



The results indicate a greater influence of environment on allergen variation, although this was dependent upon the allergen in question, suggesting that protein function is the determining factor

Gly m 4

Mittag et al. (2004) reported that the mean content of extractable and immune-reactive Gly m 4 was 256 ± 30 ppm over 10 soybean varieties. Unripe green soybeans contained approximately 8 ppm, ripe fresh soybeans contained 96 ppm, and ripe soybeans after 3 years of storage contained 224 ppm.

Apple

- Mal d 3 (LTP) content varied more than six-fold (μg/mg of dry material) across 10 cultivars of apples (apple peel extract) (Carnes et al., 2006).

- Mal d 3 content varied up to 100-fold, both in allergen quantification and IgE-inhibition assays across 88 apple cultivars (Zuidmeer, 2006).

- Mal d 1 levels among ten cultivars ranged between 3.8 and 72.5 μg/g pulp (~19-fold). Growing conditions and postharvest storage increase Mal d 1 expression at a translational and transcriptional level (Sancho et al., 2006).

- Mal d 1 levels ranged from 0.84 to 33.17 μg/g fresh weight (~39-fold) in 39 apple cultivars (Marzban et al., 2005).

- Apples grown at low altitude showed decreased (~20%) gene expression vs. those of higher elevation. Early harvest = highest allergenic potential vs. late ripening varieties (Botton et al., 2008).

Peach and Strawberry

- Pru p 3 levels varied over 6-fold (53 to 338 μg/g of fresh weight-peel) across 15 peach cultivars from Spain, U.S., and Italy (Ahrazem et al., 2007).

- Pru p 1 levels varied over 10-fold (0.07 to 0.68 μg/g- pulp) and over 12-fold (0.14 to 1.76 μg/g- peel). Most U.S. cultivars showed higher levels of both allergens than Spanish cultivars (Ahrazem et al., 2007).

- Enhancing the light radiation and decreasing the fruit load reduced transcription rate of most allergen-related genes in peach (Botton et al., 2009).

- Lower temperatures increased Fra a 1 (PR-10) levels in strawberry (Marzban et al., 2009).

Conclusions

- There appears to be marked variability in the levels of allergenic proteins in non-GM crops already in the food supply based on variety, abiotic and biotic stresses, and growing location.

- Measurement of endogenous allergens is not warranted for GM food safety assessment purposes-
  - Lack of clarity on the GM safety assessment question this information is supposed to answer
  - Interpretation of small differences is problematic because information on sensitisation is lacking
  - Potential adverse effects cannot be directly deduced from measuring the levels, irrespective of improvements in the LOD (cf. differences in levels of natural toxicants, anti-nutrients, etc)
  - Impractical to distinguish only between GM and conventional varieties in terms of allergenicity (i.e., consumption levels)