How Much Of A Hazardous Substance Would Have To Be Produced Before It Poses A Risk ?

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Echium Plantagineum http://www.dpi.nsw.gov.au/agriculture/pests-weeds/weeds/profiles/patersons-curse

How Much Of A Hazardous Substance Would Have To Be Produced Before It Poses A Risk

or

How Strong is a Piece of String ?

- Analogous to asking “How strong is a piece of String”
- To answer either question the context must be established.
  - Theoretical speculation or real world consideration
- The question reflects confusion or misunderstanding around;
  - The range of scientifically plausible unintended changes possible from simple insertion of a transgene
- To address either question boundaries must be placed around the subject
  - Exclude;
    - hypothetical, extreme or scientifically implausible circumstances
  - Identify
    - practical, scientifically plausible possibilities/probabilities.
    - What the data actually tells us rather than speculative excursions
  - Define
    - the nature of the risk (population health or individual)
    - the type of hazard, (toxicological, immunological, dietary)
    - Time frame (acute or chronic)

Exclude

- *De novo* High potency protein toxins
  - Very small amounts of an extreme toxin such as botulinum toxin in food presents an immediate, high risk if present,

  BUT
  - there is zero risk that such a compound would be formed accidentally just from transgene insertion.
    - **Systemic** toxicity of an ingested protein requires at least three highly specific, **and separate**, structural characteristics
      - Resistance to digestion
      - Ligand for specific, and species specific, gut uptake transporters
      - Ligand for site and species specific receptor mediating toxicity
    - Potential for random effects resulting from transgene insertion, to modify existing non toxic proteins to create a toxic protein is essentially zero
      - Eg. Millions of SNPs across corn and other food crop varieties have never produced a corn with a toxic protein

Exclude

- *De novo* generation of biochemical machinery to produce a novel toxic secondary metabolite unrelated to the parent plant or the source or function of the transgene itself
  - Because - as for protein toxins, multiple co-ordinated alterations would be required which is implausible at best
- Reactivation of dormant pathways
  - Because- Dormant pathways accumulate mutations (no evolutionary/survival advantage to preservation of remaining integrity)
  - Not scientifically plausible

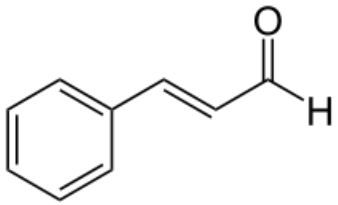
Identify Plausible Possibilities

- In common with conventional plant breeding these include
  - Up (& down) regulation of pre-existing endogenous plant toxins
  - Increased/decreased uptake of minerals (eg Cd, As, Se)
  - Increase in pre-existing endogenous nutrients or antinutrients associated with adverse population health outcomes
  - Increased/altered production of pesticide metabolites
  - Increased levels of toxic substrates (precursors) due to blocking of an enzyme pathway
  - Increased release/availability of endogenous toxin due to altered enzyme levels
    - Eg increased or altered location/storage of β- glucosidase in cassava may increase the rate of release of cyanide from cyanogenic glycosides
    - Cyanide is rapidly detoxified so rate of exposure is equally as important as level
    - ie it may not be the amount of the toxin itself which is the key determinant of toxicity.

Define

- the nature of the risk exposed group of interest
  - Population health
    - Much smaller levels of risk will be potentially identifiable / of concern on a population basis than on an individual basis
    - Population risk may not be relevant for specific individuals
  - Individual safety
    - Generally concerned with more immediate, direct and substantial risks likely to affect most individuals exposed.
- the type of hazard
  - toxicological,
    - Primarily a function of the nature of the chemical of interest
    - Individual variability only a minor factor
    - Generally a good level of cross species similarity of effect
    - Animal studies useful and generally predictive (with caveats)
  - Immunological, idiosyncratic (ie allergy, intolerance)
    - Primarily a function of the individual[1]
    - Chemical characteristics necessary but not sufficient
    - At best poor cross species correlation
    - Animal studies generally of marginal value
  - Dietary
    - Primarily a function of individual dietary patterns
    - Chemical characteristics necessary but not sufficient
    - Animal studies generally of very limited value

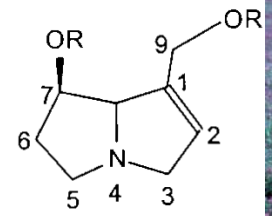
Cinnamomum verum, from Koehler's *Medicinal-Plants* (1887)



[1]Hikmat Hayder*, Utz Mueller and Andrew Bartholomaeus (2011) Review of Intolerance Reactions to Food and Food Additives. International Food Risk Analysis Journal . 2011, Vol. 1, No. 2, 23-32

Potential Candidates

- Secondary metabolites
  - Endogenous pesticides
  - Herbivore anti-feedants
  - Stress response
- Nutrients, trace elements
  - Macro
    - Lipid profiles
  - Micro
    - Minerals & vitamins
    - Heavy metals
- In humans these may be
  - Contact allergens
    - Psoralens in celery
  - Carcinogens
    - Aristolochic acid
  - Specific organ toxins
    - Pyrrolizidine liver toxins
  - Nutritional
    - Lipid profile



Pyrrolizidine alkaloid

http://www.dpi.nsw.gov.au/agriculture/pests-weeds/weeds/profiles/patersons-curse

Table 1.1 Number of known secondary metabolites from higher plants

Type of secondary metabolite	Number[a]
Nitrogen-containing	
Alkaloids	21 000
Non-protein amino acids (NPAAs)	700
Amines	100
Cyanogenic glycosides	60
Glucosinolates	100
Alkamides	150
Lectins, peptides, polypeptides	2000
Without nitrogen	
Monoterpenes (C10)[b]	2500
Sesquiterpenes C15)[b]	5000
Diterpenes (C20)[b]	2500
Triterpenes, steroids, saponins (C30, C27)[b]	5000
Tetraterpenes (C40)[b]	500
Flavonoids, tannins	5000
Phenylpropanoids, lignin, coumarins, lignans	2000
Polyacetylenes, fatty acids, waxes	1500
Polyketides	750
Carbohydrates, organic acids	200

Secondary metabolite toxicity not random

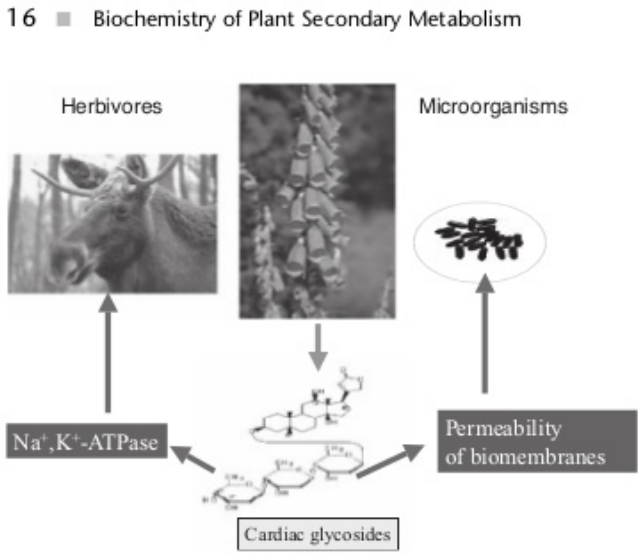


Figure 1.8 Schematic view of the ecological roles of plant SM. Foxglove (*Digitalis purpurea*) produces cardiac glycosides, which are very toxic to animals (vertebrates, insects) because they inhibit Na^+, K^+-ATPase, one of the most important transporters in animal cells. Cardiac glycosides are additionally toxic to microbes because the molecules have detergent properties and disturb membrane fluidity. (See Plate 7 in colour plate section.)

Biochemistry of plant secondary metabolism. 2nd edition, Annual Plant Reviews volume 40 Michael Wink ed. 2010. Wiley-Blackwell

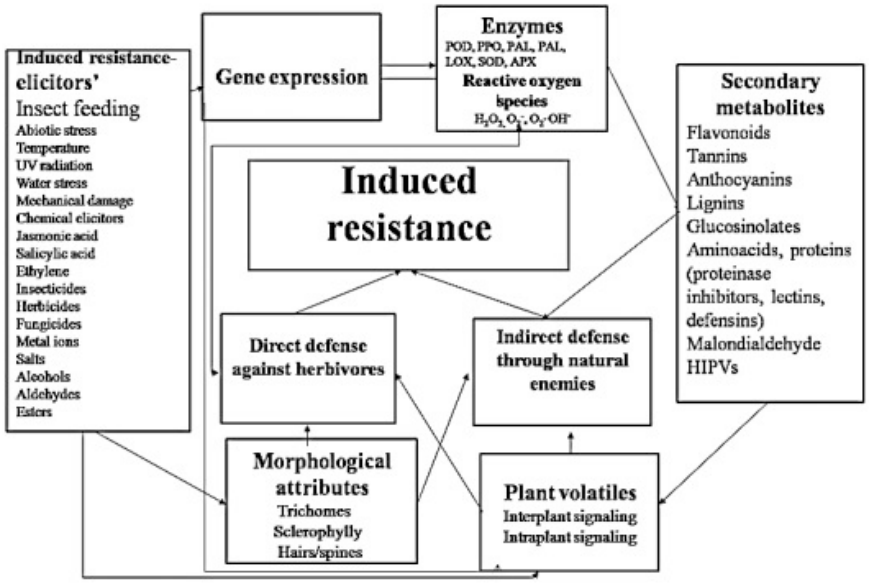


Figure 1. Mechanism of induced resistance in plants. POD, peroxidase; PPO, polyphenol oxidase; PAL, phenylalanine ammonia lyase; TAL, tyrosine alanine ammonia lyase; LOX, lipoxygenase; SOD, superoxide dismutase; APX, ascorbate peroxidase; HIPVs, Herbivore induced plant volatiles

Plant Signal Behav. 2012 October 1; 7(10): 1306–1320.

Determinants of Secondary Metabolite levels - Psoralens

Table 1
Effect of environmental conditions and elicitor treatments on furocoumarin content in higher plants

Environmental factor or elicitor	Plant species	Effect on furocoumarin content
Plant diseases		
Ceratocystis fimbriata	*Pastinaca sativa* (root apex)	×20 (8-MOP)
Sclerotinia sclerotiorum	*Apium graveolens* (stalks)	×235 (Psoralen) ×24 (Total furocoumarins)
Unknown	*Daucus carota* (roots)	×77 (8-MOP)
Erwinia carotovora	*Apium graveolens* (stalks)	×24 (Total furocoumarins)
Rhodotula rubra	*Ruta graveolens* (hydroponic)	No modification
Phoma companata	*Pastinaca sativa* (leaves)	×5 (Total furocoumarins)
Pseudomonas cichorii	*Glehnia littoralis* (roots)	×9 (psoralen)
Insect damages	*Pastinaca sativa* (leaves)	×2.2 (8-MOP) ×1.8 (Psoralen)
Effect of light		
UV	*Apium graveolens* (stalks)	×3.4 (Linear furocoumarin)
UV	*Ruta graveolens* (leaves)	×2.5–10 (Total furocoumarin)
UV	*Glehnia littoralis* (roots)	×2 (Psoralen)
Air quality		
Acidic fog	*Apium graveolens* (leaves)	×5.4 (Linear furocoumarins)
Ozone	*Petroselinum crispum* (leaves)	×2 (Total furocoumarins)
Temperatures		
Cold (−15° C, control 26° C)	*Apium graveolens* (leaves)	×8.8 (Linear furocoumarins)
Hot (32° C, 21° C) control 21° C)	*Psoralea cinerea* (leaves)	×11 (Psoralen)
Chemicals		
$CuSO_4$	*Apium graveolens* (leaves)	×2.2 (Linear furocoumarins)
$CuSO_4$	*Psoralea cinerea* (fruits)	×2.8 (Psoralen)
NaCl	*Ruta graveolens* (leaves)	Decrease but higher percentage on leaf surface
NaCl	*Psoralea cinerea* (fruits)	×2 (Psoralen)
H_2SO_4	*Ruta graveolens* (leaves)	Decrease but higher percentage on leaf surface
$Ca(OCl)_2$	*Psoralea cinerea* (fruits)	×1.5 (Psoralen)

Review

Production of plant secondary metabolites: a historical perspective

F. Bourgaud *, A. Gravot, S. Milesi, E. Gontier

Plant Science 161 (2001) 839–851

- A response to
  - Disease
  - Heat/cold
  - Light
  - Air quality
  - Insect damage
  - Agronomic practices
- Highly variable 2 orders of magnitude

Avoid Distractions from Faux Carcinogens (etc) Tumours in Rodents Neither Challenging nor necessarily predictive

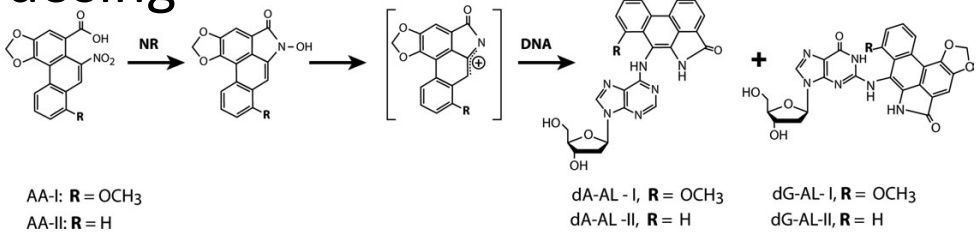
Table 2
Carcinogenicity of natural plant pesticides tested in rodents[a]

Carcinogens: $N = 37$	Acetaldehyde methylformylhydrazone, allyl isothiocyanate, arecoline · HCl, benzaldehyde, benzyl acetate, caffeic acid, capsaicin, catechol, clivorine, coumarin, crotonaldehyde, 3,4-dihydrocoumarin, estragole, ethyl acrylate, N^2-γ-glutamyl-*p*-hydrazinobenzoic acid, hexanal methylformylhydrazine, *p*-hydrazinobenzoic acid · HCl, hydroquinone, 1-hydroxyanthraquinone, lasiocarpine, *d*-limonene, 3-methoxycatechol, 8-methoxypsoralen, *N*-methyl-*N*-formylhydrazine, α-methylbenzyl alcohol, 3-methylbutanal methylformylhydrazone, 4-methylcatechol, methylhydrazine, monocrotaline, pentanal methylformylhydrazone, petasitenine, quercetin, reserpine, safrole, senkirkine, sesamol, symphytine
Noncarcinogens: $N = 34$	Atropine, benzyl alcohol, benzyl isothiocyanate, benzyl thiocyanate, biphenyl, *d*-carvone, codeine, deserpidine, disodium glycyrrhizinate, ephedrine sulphate, epigallocatechin eucalyptol, eugenol, gallic acid, geranyl acetate, β-*N*-[γ-*l*(+)-glutamyl]-4-hydroxy-methylphenylhydrazine, glycyrrhetinic acid, *p*-hydrazinobenzoic acid, isosafrole, kaempferol, *d*-menthol, nicotine, norharman, phenethyl isothiocyanate, pilocarpine, piperidine, protocatechuic acid, rotenone, rutin sulfate, sodium benzoate, tannic acid, 1-*trans*-δ^9-tetrahydrocannabinol, turmeric oleoresin, vinblastine
These rodent carcinogens occur in:	Absinthe, allspice, anise, apple, apricot, banana, basil, beet, broccoli, Brussels sprouts, cabbage, cantaloupe, caraway, cardamom, carrot, cauliflower, celery, cherries, chili pepper, chocolate, cinnamon, cloves, coffee, collard greens, comfrey herb tea, corn, coriander, currants, dill, eggplant, endive, fennel, garlic, grapefruit, grapes, guava, honey, honeydew melon, horseradish, kale, lemon, lentils, lettuce, licorice, lime, mace, mango, marjoram, mint, mushrooms, mustard, nutmeg, onion, orange, paprika, parsley, parsnip, peach, pear, peas, black pepper, pineapple, plum, potato, radish, raspberries, rhubarb, rosemary, rutabaga, sage, savory, sesame seeds, soybean, star anise, tarragon, tea, thyme, tomato, turmeric, and turnip.

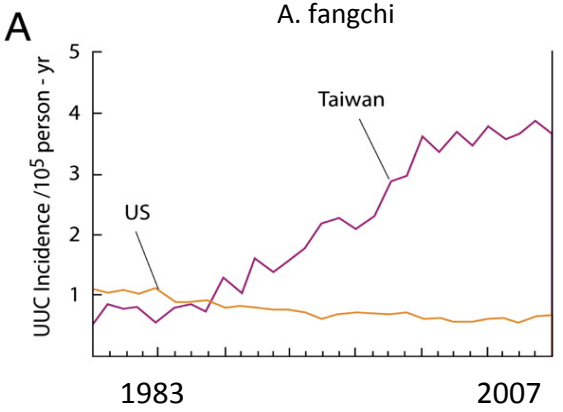
Ames & Gold Paracelsus to parascience: the environmental cancer distraction. Mutation Research 44, 7, 2000.3–13

Case Study - Aristolochic acid an Endogenous "True" carcinogens

- Aristolochia spp widely used as herbal remedies
- Aristolochic acid responsible for
  - Balkans endemic nephropathy (50% end up with cancer), due to seeds mixing with wheat for bread
  - Chinese herbal nephropathy in Belgium (A.fangchi), and
  - Kidney cancer in Taiwan (worlds highest incidence)
- Produces tumors in both mice and rats at low doses at multiple sites after a few weeks of dosing



A. fangchi



The age-adjusted incidence rate of UUC in Taiwan and the United States. **UUC Upper urothelial cancer**. Chen 2012 PNAS 2012, 109, 21 8241-8246

A. clematitis

http://www.thepoisongarden.co.uk/atoz/aristolochia_clematitis.htm

Chung-Hsin Chen, Kathleen G. Dickman, Masaaki Moriya, Jiri Zavadil, Viktoriya S. Sidorenko, Karen L. Edwards, Dmitri V. Gnatenko, Lin Wu, Robert J. Turesky, Xue-Ru Wu, Yeong-Shiau Pu, and Arthur P. Grollman **Aristolochic acid-associated urothelial cancer in Taiwan** PNAS 2012 109: 8241-8246

Aristolochic acid

Table 1. Tumour incidence within 56 weeks in female mice following oral administration of 5.0 mg AA/kg over a period of 3 weeks

Time of sacrifice (weeks after beginning of treatment)	*n*	Forestomach		Gl. stomach Carcinoma	Kidneys Adenoma	Lungs Carcinoma	Uteri Haemangioma	Malignant lymphoma
		Papilloma	Carcinoma					
3[a]	10[b]	0[c]	0	0	0	0	0	0
9	4	0	0	0	0	0	0	0
18	4	4	0	0	0	0	0	0
26	3	3	0	0	1	0	0	1
37	5	4	1	1	1	2	0	2
48	5	4	1	0	3	3	0	3
56	8	0	8	0	6	8	3	4

No tumours were detected in the control mice ($n = 11$) after 56 weeks

	♀	8/9	7/10	10/11				25/30
10.0	♂	10/10	18/18					28/28
	♀	10/10	13/13	4/4				27/27

Mengs U (1988). Tumour induction in mice following exposure to aristolochic acid. *Arch Toxicol*, 61: 504–505.
Mengs U, Lang W, Poch JA (1982). The carcinogenic action of aristolochic acid in rats. *Arch Toxicol*, 51: 107–119.

Aristolochic acid

- Oral dosing of rats causes a dose- and time-dependent tumour response.
  - 50 mg/kg bw for 3 days
  - kidney tumors after 6 months
- 1–10 mg/kg bw for 3–6 months or 0.1 mg/kg bw for 12 months
  - Various benign or malignant tumours, including
    - forestomach,
    - kidney,
    - renal pelvis,
    - urinary bladder,
    - ear duct,
    - thymus,
    - small intestine, and
    - pancreas.

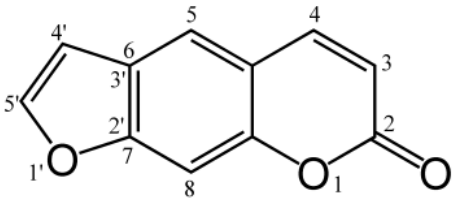
Aristolochia macrophylla at Aarhus Botanical Garden, taken August 2003 by User:Stan Shebs

Case Study Aristolochic acid

- So how much is too much
- A potent genotoxic carcinogen
- Short term exposures carcinogenic
- On a population basis even micrograms per kg bw/day over protracted periods likely to increase cancer incidence

PLANTS CONTAINING ARISTOLOCHIC ACID. IARC monograph
http://monographs.iarc.fr/ENG/Monographs/vol100A/mono100A-23.pdf

Case Studies
Psoralens in Celery



Phytophotodermatitis From Celery Among Grocery Store Workers

Paul J. Seligman, MD; C. G. Toby Mathias, MD; Michael A. O'Malley, MD, MPH; Ross C. Beier, PhD; Laura J. Fehrs, MD; W. Scott Serrill, MD; William E. Halperin, MD, MPH

Arch Dermatol 1987;123:1478-1482)

- 19 cases of phytophotodermatitis in 2 Oregon grocery stores part of the same supermarket chain.
- Outdoor sunlight exposure during the workshift and tanning salon use identified as risk factors
  - most severe cutaneous reactions among tanning salon users.
- all 19 cases were from one store
  - held a celery sale resulting in a quadrupling of celery sold.
- psoralen levels as high as 25 µg per cm^2 of trimmed surface of celery
- confirmed by cutaneous provocation tests with trimmed surfaces of these celery
- So;
  - clinical phytophotodermatitis caused by healthy celery
  - results from a complex interaction of exposure variables, including
    - ultraviolet radiation from tanning salon use,
    - frequency of handling celery,
    - Nature of presentation for sale (cut surfaces)
    - celery origin, and
    - sporadic elevations of psoralen content from environmental stresses.

http://www.dermis.net/dermisroot/en/13121/image.htm

Celery Case Study

- Celery infected with *Sclerotinia sclerotiorum* (pink rot fungus) a common cause of phytophotodermatitis in celery harvesters
  - due to increased synthesis of furanocoumarins in response to infection.
- withering celery, sold as "soup celery" infected with small colonies of *S sclerotiorum*, also reported to cause phytophotodermatitis.
- Determinants of Psoralen levels in celery include
  - amount of UV light,
  - temperature, and other climatic stresses to which the growing celery is subjected
  - treatment of field celery with the copper salts, herbicides, or other fungicides; and
  - inadequate or prolonged storage of the celery

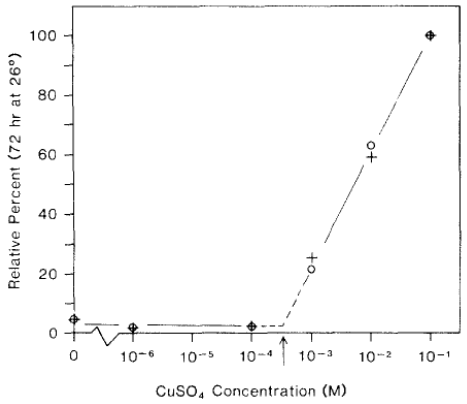


Fig. 2. Relative percentage of bergapten (+) and xanthotoxin (○) measured after 72 hr in celery cv 5270-R treated with different

Phytochemistry, Vol. 22, No. 11, pp. 2595–2597, 1983.

Celery Case Study

- The case illustrates
  - Many influences on the level of secondary metabolites in a plant
  - Consequent high natural variability
  - Human exposure may depend on many factors
    - Practices during harvesting, sale, preparation
- An example of individual risk as dominant rather than population risk
- So how much would need to be present to be a risk
  - It all depends…… !

Case Study
Conjugated Linoleic Acid

- CLA is a naturally occurring trans fat,
  - especially in ruminant meat and dairy products
  - also at low levels in some vegetable oils esp after processing
- CLA increases LDL-C and decreases HDL-C as do industrial trans fats
  - This pattern of changes is associated with an increased risk of cardiovascular disease
- Mozaffarian et al. calculated that increasing trans fatty intake by 1% of energy increases the risk of cardiovascular disease by 3 to 12%.
- average dietary intake of 0.3 g of cis-9, trans-11 CLA a day, or 0.1% of energy, would increase the risk of cardiovascular disease by 0.3 to 1.2%.
- If manipulation of fatty acid composition of a GM oil crop increased levels of CLA – "How much would have to be produced before it was a risk?"

Wanders AJ, Brouwer IA, Siebelink E, Katan MB (2010) Effect of a High Intake of Conjugated Linoleic Acid on Lipoprotein Levels in Healthy Human Subjects. PLoS ONE, 5, 2, e900
Mozaffarian D, Katan MB, Ascherio A, Stampfer MJ, Willett WC (2006) Trans fatty acids and cardiovascular disease. N Engl J Med 354: 1601–1613.

CLA

- This is a population health issue
- Individual risk from individual dietary practices is unquantifiable without clinical assessment
  - Multiple individual variables impact on risk
    - Genetics
    - Life style – exercise, alcohol, smoking
    - Broader dietary pattern
    - Co-existing disease
    - Age, sex, etc
- No rat study can inform this risk assessment
- Specific experimental assessment of alteration of human risk requires human clinical/nutrition studies
  - Changes to blood lipid composition can be determined over 2 weeks
  - Estimation of risk is then based on these biomarkers and *Assumed* correlations with the primary disease endpoint(s)
  - Beware the niacin paradox (increased HDL did not decrease heart disease in the AIM-HIGH trial)
- Clinical nutrition trials have been conducted and submitted as components of regulatory submissions for GM food crops[1]
- How much increased risk is too much is largely a policy issue
- Dietary context a key consideration
  - Eg Comparison with levels of TF intake from other sources

[1]Lemke, S.L., Vicini, J.L., Su, H., Goldstein, D.A., Nemeth, M.A. *et al.* (2010) Dietary intake of stearidonic acid-enriched soybean oil increases the omega-3 index: randomized, double-blind clinical study of efficacy and safety. *Amer J Clin Nutr*, **92**: 766-775.

Conclusions

How Much Of A Hazardous Substance Would Have To Be Produced Before It Poses A Risk ?

It All Depends…….

PLANTS CONTAINING ARISTOLOCHIC ACID. IARC monograph
http://monographs.iarc.fr/ENG/Monographs/vol100A/mono100A-23.pdf

Conclusions

- Plausible toxicants of concern in plant breeding, whether “conventional” or biotechnology, are;
  - Endogenous to the parent plant, or
  - Endogenous to the source of the transgene, or
  - Arise directly from the function of the transgene, or
  - Related to endogenous pathways up or down regulated due to the operation of the transgene
  - May involve plant/transgene/environment interactions
- These effects are largely predictable – even if unintended - & therefore testable using analytical chemistry
- Levels of concern will depend on the risk focus – individual food safety versus population health but must be compatible with high natural background variability within and across species
  - For a high consumption staple food, modest levels of a low toxicity constituent, such as a trans-fatty-acid, may have significant population health implications without presenting a food safety issue in the traditional sense.
  - Scientifically plausible substances resulting from transgene insertion are those known to be produced by the recipient or donor organisms or related to existing pathways within the recipient organism that are potentially perturbed by the inserted gene or its products. The potential hazards are dependent on the quantities of the substance(s) present in the edible portion of the food, the quantity of that food consumed and the background levels of intake of the substance(s).