A Metabolomics Study of a Breast Cancer Rat Model with Compound Identification Using an Accurate Mass Retention Time Database

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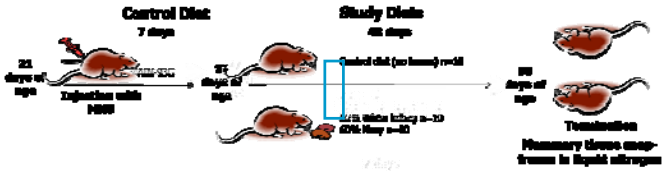
Introduction

It is widely recognized that an increased intake of vegetables, fruits, and whole grains reduces chronic disease risk. The complete profile of bioactive chemicals that account for the health benefits of increased plant food consumption, however, is currently unknown. The most recent evidence points to the fact that it is most likely groups of compounds working in synergy which account for health benefits, contrary to a single chemical or "magic bullet". To better understand the method of cancer prevention of staple crops, we have chosen to study *Phaseolus vulgaris* L. (dry bean). Studies conducted in the Thompson laboratory have demonstrated that dry bean extracts significantly extend the lifespan of the nematode, *Caenorhabditis elegans*. Longevity extension in *C. elegans* has been proposed as a phenotype for chronic disease prevention in humans. Additional pre-clinical studies demonstrated a 70% inhibition of experimentally-induced breast cancer in a well characterized rat model not attributable to isoflavone content (which is low), total phenolic content, or seed coat pigment (the bean which showed the largest positive effect on cancer endpoints was colorless). Results from these pre-clinical studies also indicated that cancer inhibitory activity differs markedly across cultivars of *Phaseolus vulgaris*. Two cultivars have been selected for metabolomic analysis: white kidney (WK, high cancer inhibitory activity) and navy (NB, low cancer inhibitory activity). Clear differences in the metabolite profile of dry bean market classes were detected. The relationship between the metabolite profile of dry bean and that found in mammary gland tissue of animals which have consumed diets containing either navy or white kidney beans was also investigated to determine the effect of dry bean diets on the mammalian metabolite profile. Mammary tissues of animals fed control diets versus those fed diets containing dry beans were clearly distinguishable based on 6% of the total number of metabolites.

Experimental

Figure 1: Mouse / bean experimental protocol



Sample extraction:

•Diet 50 mg, mammary gland tissue 50 mg
•Modified Bligh and Dyer method (water:methanol:chloroform) at pH 2 and pH 9

Experimental

Sample preparation for LC/MS:
•Speed-Vac to dryness
•Protein precipitate with 70% ACN
•Reconstitute with 50 µL methanol, vortex then 50 µL water, vortex
•Blend pH 2 and pH 9 extracts
•Inject 5 µL into LC/MS system

LC/MS method:
Reverse phase separation method
Solvent A: 0.2% acetic acid in water
Solvent B: 0.2% acetic acid in methanol
Guard column: Zorbax SB-C8, 2.1 x 30 mm, 3.5 µm
Analytical column: Zorbax SB-Aq, 2.1 x 50 mm, 1.8 µm
Flow rate: 0.6 mL/min
Simple linear gradient: 24 min cycle time
• 2% methanol to 98% methanol in 13 minutes
• 6 min hold at 98% methanol

LC/MS instrument
•Agilent 1200 SL system include binary pump, autosampler with chiller and thermostatted column compartment
•Agilent 6520 Accurate Mass Q-TOF with dual ESI source

Results and Discussion

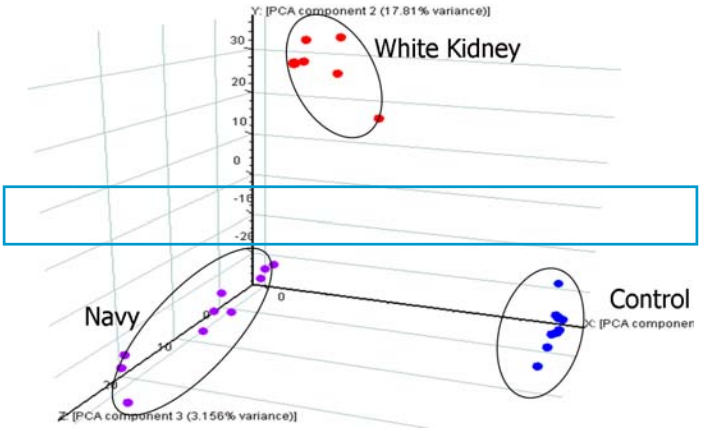
Table 1: Mouse experiment - Tumor observations

Treatment	Sample Number	Incidence %	average number of tumors	Tumor burden
Control	10	93.3	3.2	0.48
NavyBean	10	70.0	1.9	0.34
White Bean	10	66.7	1.0	0.05

Table 1 tumor biopsy results show the bean diets have a significant impact on the tumor burden in mice. Figure 2 demonstrates that a multivariate analysis can distinguish the bean diets on the basis of their chemical fingerprint.

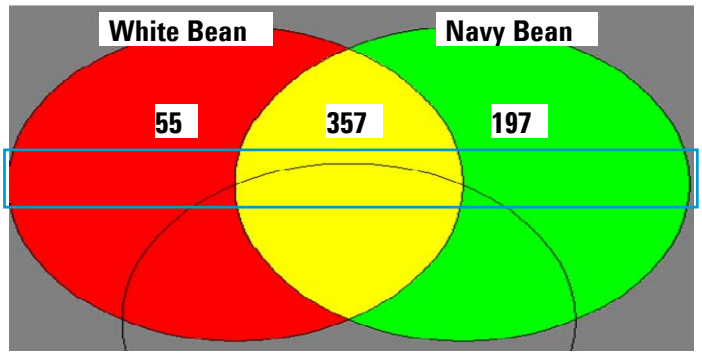
Analysis of dry bean diet extracts

Figure 2: 3D PCA representation of bean diet results



Results and Discussion

Figure 3: Compound analysis of bean diets



Bean metabolite overview (Control as baseline):
Present in both bean diets: 357
Unique to White Bean: 55
Unique to Navy Bean: 197
Bean diet compounds greater than control:

- 255 ≥10X higher
- 28 ≥ 2x higher in white bean
- 58 ≥ 2x higher in navy bean

Compound Identification:
Metlin search using mass and retention time
•357 masses (± 10 ppm, ± 0.2 min)

- 131 mass only matches
- 5 mass with RT matches

Table 2: Compounds matched from database search against Accurate Mass retention Time Database (AMRT)1

Compounds	Formula	Mass	RT (min)
3-Hydroxy-2-methyl-4-pyrone	C6H6O3	126.03169	2.015
Tryptophan	C11H12N2O2	204.08988	1.254
Niacinamide	C6H6N2O	122.04801	0.935
Phenylalanine	C9H11NO2	165.07898	0.833
Adenylic acid (adenosine monophosphate)	C10H14N5O7P	347.06308	0.773

Analysis of mammary gland tissue extracts

Metabolite overview (Control as baseline):
Present in both bean diet groups: 74
Unique to White Bean fed: 7
Unique to Navy Bean fed: 14
Bean diets greater than control

- 28 ≥ 2x higher
- 23 higher in white bean fed animals
- 5 higher in navy bean fed animals

Control diets greater than bean diets

- 60 ≥ 2x higher in control fed animals

Figure 4: 3D PCA representation of mammary gland results

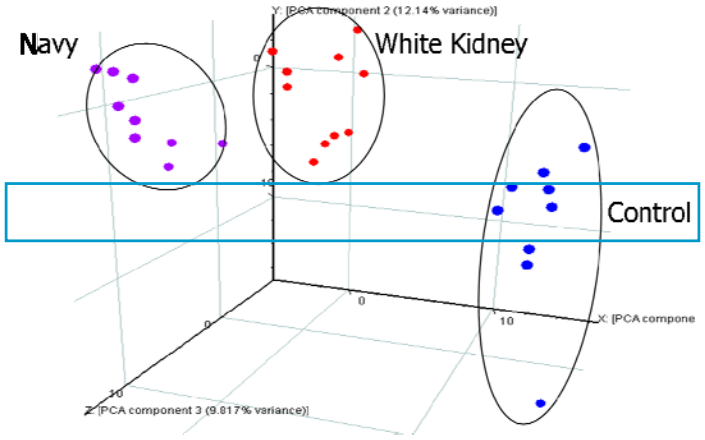


Table 3: Compounds matched from database search against Accurate Mass retention Time Database (AMRT)1

Possible Compounds (**Bean Fed Animals**)	Formula	Mass
Phenylalanine	C9H11NO2	165.07898
19-Norandrosterone	C18H28O2	276.20893
Linolenic acid	C18H30O2	278.22458
Violaxanthin	C40H56O4	600.41786
Acitretin Ro 23-4293	C20H28O3	316.20385
(-)-8-hydroxy-11E,17-octadecadien-9-ynoic acid	C18H28O3	292.20385
Artemether	C16H26O5	298.17802
Perindoprilatlactam A	C17H26N2O4	322.18926

Compound Identification:
Metlin search using mass and retention time
•28 compounds higher in bean fed animals

- 10 mass only matches
- 1 mass with RT match

Table 4: Compounds matched from database search against Accurate Mass retention Time Database (AMRT)1

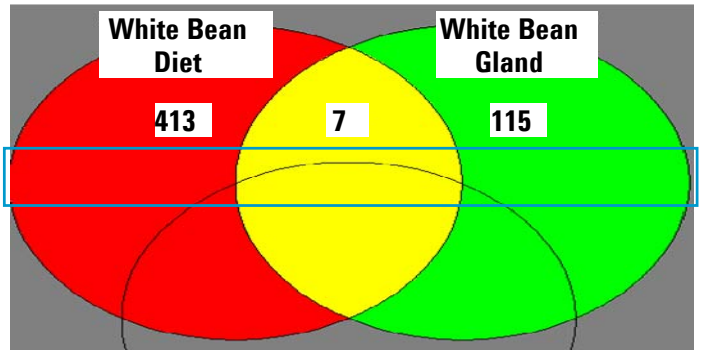
Possible Compounds (**Control Fed Animals**)	Formula	Mass
Elaidic acid	C18H34O2	282.25588
PheArg Pro	C20H30N6O4	418.23285
GPCho(O-12:0/O-2:0)	C22H49NO6P	454.32975
GPCho(7:0/0:0)	C15H33NO7P	370.19946
Flupenthixol	C23H25F3N2OS	434.16397
Artemether	C16H26O5	298.17802
1-(10-methyl-hexadecanyl-2-(8-[3]-ladderane-octanyl)-sn-glycerol	C40H74O3	602.56380
Crocetin	C20H24O4	328.16746
2,3-Dinor-6,15-diketo-13,14-dihydro-PGF1a	C18H26O6	338.17294
O-Desacetylcephalothin	C14H14N2O5S2	354.03441
Acitretin Ro 23-4750	C21H26O4	342.18311
Citalopram	C20H21FN2O	324.16379
Sulfadoxine	C12H14N4O4S	310.07358
cis-3-(6-Hydroxy-7-methoxy-5-benzofuranyl)acrylic acid glucuronide	C18H18O11	410.08491

Metlin search using mass and retention time of the 60 compounds higher in control fed animals

- 14 mass only matches
- 0 mass with RT match

Results and Discussion

Figure 5: White bean compounds found in mammary gland



Comparison of White Bean to mammary gland compounds

•Present in both: 7
•Unique to tissue: 115
•Unique to bean: 413

Compound Identifications

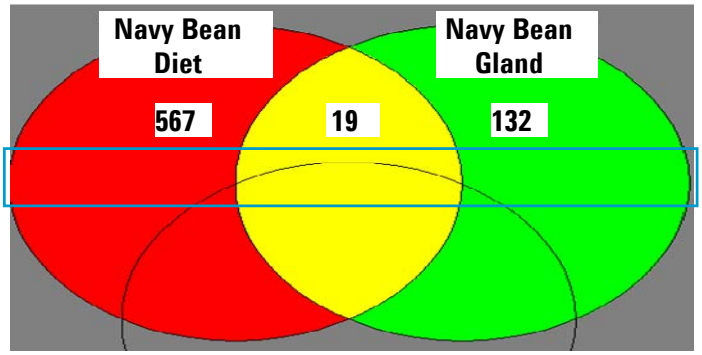
Metlin Search using mass and retention time

- 1 mass only match
- 4 mass with RT matches

Table 5: Compounds matched from database search against Accurate Mass retention Time Database (AMRT)1

Possible Compounds (**Common to white bean diet and mammary gland**)	Formula	Mass	RT (min)
15-Keto-protaglandin F2-alpha	C20H32O5	352.22497	8.55600
Phenylalanine	C9H11NO2	165.07898	0.83300
Guanine	C5H5N5O	151.04941	N/A
8-methoxy-13-hydroxy-9,11-octadecadienoic acid	C19H34O4	326.24571	N/A
Uracil	C4H4N2O2	112.02728	N/A

Figure 6: Navy bean compounds found in mammary gland



Comparison of Navy Bean to mammary gland compounds

•Present in both: 19
•Unique to tissue: 132
•Unique to bean: 567

Compound Identifications

Metlin Search using mass and retention time

- 5 mass only matches
- 1 mass with RT match

Table 6: Compounds matched from database search against Accurate Mass retention Time Database (AMRT)1

Possible Compounds (**Common to navy bean diet and mammary gland**)	Formula	Mass	RT (min)
15-Keto-protaglandin F2-alpha	C20H32O5	352.22500	8.556
15-keto-Prostaglandin E2	C20H30O5	350.20930	N/A
16-Hydroxy-4-carboxyretinoic acid derivative	C19H24O3	300.17250	N/A
Succinoadenosine	C14H17N5O8	383.10770	N/A
Enalapril	C20H28N2O5	376.19980	N/A

Conclusions

- Metabolite differences were detected between different bean diets with differing cancer prevention activity levels
- Differences were observed in metabolite fingerprints between mammary gland tissue in:
  - Animals fed both bean diets compared to control
  - Animals fed white bean versus navy bean
- Database searching provided potential identification for mass lists from:
  - differentiate dry bean diets
  - mammary gland tissue

References

[1] Molecular Formula and METLIN Personal Metabolite Database Matching Applied to the Identification of Compounds Generated by LC/TOF-MS. Sana, T.R., et. al., Journal of Biomolecular Techniques 19:258–266, 2008.