JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 6697–6705 6697
DOI:10.1021/jf1007538

Characterization of Low Molecular Weight Chemical Fractions of Dry Bean (*Phaseolus vulgaris*) for Bioactivity Using *Caenorhabditis elegans* Longevity and Metabolite Fingerprinting

Meghan M. Mensack,[†] Vanessa K. Fitzgerald,[†] Matthew R. Lewis,[‡] and Henry J. Thompson*,[†]

[†]Cancer Prevention Laboratory, Department of Horticulture and [‡]Proteomics and Metabolomics Facility, Department of the Vice President for Research, Colorado State University, Fort Collins, Colorado 80523

Dry bean consumption has been reported to be associated with reduced risk for a number of chronic diseases including cancer. The extent to which these benefits are associated with primary versus secondary plant metabolites is not known. The work reported herein focuses on low molecular weight secondary metabolites and uses longevity extension of wild-type *Caenorhabditis elegans* nematodes as a surrogate marker for human health benefits. A modified Bligh and Dyer technique was used to extract freeze-dried bean, and the resulting fractions were evaluated for longevity extension and metabolite fingerprinting using ultra performance liquid chromatography−mass spectrometry (UPLC-MS). Dry bean extracts extended adult *C. elegans* lifespan by as much as 16%. Hydrophilic fractions increased lifespan, whereas the hydrophobic fraction induced longevity reduction. Metabolite fingerprinting revealed distinguishing spectral differences among the four chemical fractions evaluated and demonstrated that within each fraction chemical composition differed significantly based on dry bean genetic heritage.

KEYWORDS: Dry bean; *Phaseolus vulgaris*; *C. elegans* longevity; metabolomics

INTRODUCTION

Dry beans (*Phaseolus vulgaris*) are a good source of dietary fiber, minerals, and essential vitamins (*1*). A body of epidemiological evidence indicates that dry bean consumption is associated with reduced risk for a number of chronic diseases including cancer. In regions of the world where dry beans are considered a staple crop such as many Latin American, Eastern, and South African countries, the incidence of colorectal cancer has been shown to be inversely correlated to the amount of bean consumption (*2*), and results from the Nurses' Health Study II indicate an inverse relationship between dry bean and lentil intake and breast cancer risk with a reduction in risk of 24% (*3*). The Four-Corners Breast Cancer study also reported a relationship between dry bean consumption and reduced breast cancer incidence in which breast cancer incidence in Hispanic women who consumed a Native Mexican diet (characterized by high legume intake) was 2/3 that of non-Hispanic white population whose diet was characterized as high in red meat, sugar, and processed foods (*4*). Still others have published work on reduced risk of prostate cancer with increased dry bean consumption (*5*, *6*).

Preclinical studies conducted previously in our laboratory using diets to which various dry bean cultivars had been added showed inhibition of experimentally induced breast cancer by as much as 70% (*7*, *8*). Protective activity was not associated with isoflavone content, concentration of total phenolics, or presence of seed coat pigment (*8*). Rather, anticancer activity varied by dry bean heritage, suggesting a means by which to identify the genetic traits responsible for protection against cancer. Recently, Bobe et al. showed that fractions of navy beans, both soluble and insoluble, contribute to the cancer protective effect of cooked beans in a preclinical model for colon cancer (*9*). This was attributed to the fact that both the soluble and insoluble fractions contain several compounds known to prevent cancer including resistant starches, saponins, mono-, di-, oligo-, and polysaccharides, phenolics, and isoflavonoids.

Clinical and preclinical results demonstrating the positive health effects of phytochemical rich diets have prompted the *Caenorhabditis elegans* and metabolomics work presented here as it is likely that phytochemicals which appear to prevent the carcinogenic process in animals may also affect *C. elegans* lifespan (*10*, *11*). Evidence exists for evolutionarily conserved pathways that dictate aging and lifespan in several organisms including yeast, humans, and the nematode, *C. elegans* (*12*, *13*). In fact, two-thirds of all human disease genes have homologues in *C. elegans*, and many of these genes regulate lifespan of the nematode (*12*, *14*). Processes involved in aging are also influential in the progression of cancer including autophagy and apoptosis (*15*−*17*). The conservation of biological processes along with the short 18−21 day lifespan of the wildtype strain make *C. elegans* an attractive preclinical model for studying disease prevention and control via longevity extension (*18*, *19*).

*Corresponding author. E-mail: henry.thompson@colostate.edu.

Published on Web 05/10/2010

Several research areas including pharmacology widely apply metabolomics as a high-throughput screening tool for natural products and bioactive food components (*20*−*23*). Here, *C. elegans* longevity and nontargeted liquid chromatography-mass spectrometry (LC-MS) metabolic fingerprinting were used in concert to screen extracts of dry bean for bioactivity. In this case, a differential effect on longevity was a surrogate measure for health-related bioactivity. Germplasm from two genetically distinct dry bean market classes representing two centers of domestication were screened for longevity extension. A clear dependence on genetic heritage was observed, consistent with evidence that inhibitory activity of dry beans against experimentally induced breast cancer also varies by center of domestication (*8*). Moreover, differential effects on lifespan were observed when nematodes were fed hydrophobic versus hydrophilic extracts of white kidney or navy beans. The small molecule content of these bean fractions were assayed, and distinct metabolite fingerprints correlating with positive and negative life expectancy outcomes were identified.

MATERIALS AND METHODS

Plant Materials. Dry beans were domesticated in two geographically distinct regions of the world and the germplasm from each center of domestication has been shown to be genetically distinct (*24*). For these experiments, navy bean was selected as a representative of the Middle American center of domestication and white kidney bean was selected as a representative of the Andean center of domestication. Both dry bean market classes were subjected to *C. elegans* longevity assessment and metabolite fingerprinting. Beans used for initial survival analysis and metabolite fingerprinting were commercial bulk mixtures obtained from Archer Daniels Midland Company, Decatur, IL. This company purchases bean seed from several locations in the United States; therefore, the seed used initially was a mixture of several different cultivars from different fields produced under a broad range of environmental conditions. The seed lots obtained from Archer Daniels Midland were shipped to Bush Brothers & Company, Knoxville, TN, for canning using Bush Brothers' standard commercial canning process. The canned product was then drained, freeze-dried, and milled to a powder by Van Drunen Farms, Momence, IL.

The subsequent survival analysis and metabolomics analyses compared bean cultivars within the white kidney and navy market classes. White kidney (cv. Lassen) and navy (cv. Seahawk) beans were harvested from the research fields at the Agricultural Research, Development and Education Center, Colorado State University, Fort Collins, Colorado. For each dry bean cultivar approximately 500 ± 50 g of bean was soaked in distilled water for 3 h at room temperature (RT, 22 ± 2 °C), drained, rinsed with deionized water, and blanched for 5 min at 93 °C. The beans were cooked in a pressure cooker for 60 min in 1.5% KCl (w/v) and finally freeze-dried using a commercial freeze drier (Genesis SQ25LL, Virtis Company, Gardiner, NY) and ground into powdered form with a mortar and pestle. All freeze-dried bean powders were stored in airtight packaging at −20 °C.

Metabolite Extraction. The following chemicals and materials for metabolite extraction were used as received from Thermo Fisher (Pittsburgh, PA): chloroform, methanol, and HPLC-grade water. Metabolite extraction was carried out using a modified Bligh and Dyer liquid−liquid extraction method published previously by Sana et al. (*25*) To account for differential solubility of various compounds and to extract the largest number of compounds possible from the dried bean powders, extractions were carried out at pH 2, 7, and 9. Prior to extraction, three separate aqueous solutions were prepared. The first was acidified to pH 2 using concentrated formic acid to achieve a final formic acid concentration of 1%. The second was adjusted to pH 9 using 30% ammonium hydroxide for a final concentration of 2% ammonium hydroxide. The final flask was left at an uncontrolled neutral pH 7. These were added to methanol at 80:20 (methanol/water) for extraction. First, the freeze-dried bean powder (5.0 g) was added to each of the three pH solutions of methanol/water in a 1:4 w/v ratio and vortexed for 1 h at −20 °C in order to inactivate and remove all protein from the sample. Next, the insoluble components were separated from the solution using centrifugation (3220*g*, 10 min, 4 °C) and the extract was decanted from the solid pellet. Chloroform was then added to the methanol/water solution at a ratio of 3 parts chloroform to 5 parts methanol/water and the three component solution was vortexed. Following this step, pH adjusted water was added in the ratio of 1 part water to 6 parts chloroform/water/methanol mixture and vortexed to ensure complete mixing. To separate the phases, the solutions were again centrifuged (3220*g*, 10 min, 4 °C). The two layers were separated and stored at −20 °C overnight to ensure complete separation of the phases. The hydrophobic fractions for each bean were pooled. All extracts were dried under nitrogen at RT and resuspended in DMSO at a concentration of 50 $mg \cdot mL^{-1}$. All samples were stored at −80 °C until use (up to 1 month).

***C. elegans* Strain, Maintenance, and Longevity Assays.** The wild type N2 strain of *C. elegans* (*Caenorhabditis* Genetics Center) was used in longevity assays. Nematodes were maintained on nematode growth medium (NGM) plates seeded with 100 μL of cultured OP50 *Escherichia coli* (*Caenorhabditis* Genetics Center) as a food source at 20 °C as described previously (*26*).

Wells of a 24-well plate contained a cell culture insert with a 0.4 μm translucent polyethylene terephthalate (PET) membrane (BD Falcon) which served as a net to contain the nematodes but allowed diffusion of liquid culture components across the membrane as previously described (*27*). Liquid culture composed of 10^8 $cells \cdot mL^{-1}$ of concentrated OP50 bacteria, 10 $\mu g \cdot mL^{-1}$ cholesterol, 25 μM 5-fluoro-2′-deoxyuridine (FUDR) (Sigma-Aldrich), S basal medium, and 150 $\mu g \cdot mL^{-1}$ of bean extract or solvent (100% DMSO) was added, 750 μL to each well. To ensure that the nematodes were exposed to fresh extract containing active metabolites, the liquid culture in the treatment plates was changed 5 times per wk by aspirating the liquid beneath the insert and adding fresh liquid culture into the well. The liquid culture was made fresh and stored at 4 °C for no more than 7 days. Longevity assays were performed at room temperature (22 ± 2 °C) with gentle orbital shaking. The nematodes were monitored 5 times per week and the number of living worm scored. Nematodes were scored as dead that did not show spontaneous movement and failed to respond to forced movement of the liquid culture.

Feeding Rate Assays. Treatment plates were made by spreading bean extract (150 $\mu g \cdot mL^{-1}$) or control solvent (100% DMSO) on NGM agar plate surfaces. Once dry, 50 μL of OP50 *Escherichia coli* from an overnight culture was added and spread in the center of the plate. Synchronized young adult nematodes were transferred to treatment plates and allowed to eat for >5 h before feeding rate was monitored. Feeding rate was measured by counting the number of terminal pharyngeal bulb pumps during 30 s intervals using an Axioskop II microscope at 100× magnification. The rates of five nematodes per treatment were averaged and presented as means ± SD.

Metabolite Fingerprinting by LC-MS. Sample separation was performed using an Acquity UPLC controlled with MassLynx software, version 4.1 (Waters, Milford, MA, USA). Samples were held at 10 °C in a sample manager during the analysis. The complete sample set was randomized and profiled in three independent iterations. One microliter sample injections were made to a 1.0 × 100 mm Waters Acquity UPLC BEH C18 column with 1.7 μm particle size held at 40 °C. Separation was performed by reverse phase chromatography at a flow rate of 0.14 $mL \cdot min^{-1}$. The eluent consisted of water and methanol (Fisher, Optima LC-MS grade), isopropanol (Fisher, HPLC grade), and formic acid (Fluka, LC-MS grade) in the following proportions: Solvent A = 95:5 water/methanol +0.1% formic acid; solvent B = 2.5:90:7.5 water/methanol/isopropanol +0.1% formic acid. The separation method is described as follows (19 min total): 0.1 min hold at 100% A, 8.9 min linear gradient to 90% B, 3 min linear gradient to 95% B, 0.5 min linear gradient to 100% B, 5.5 min hold at 100% B, 0.1 min linear gradient to 100% A, and 0.9 min hold at 100% A. A blank injection of water and 10 min chromatographic run were preformed between samples to eliminate possible carryover of analytes and to re-equilibrate the column. This cleaning method is described as follows: 0.1 min hold at 100% A, 1.9 min linear gradient to 100% B, 2 min hold at 100% B, 0.1 min linear gradient to 100% A, and 5.9 min hold at 100% A for equilibration.

Eluate was directed to a Q-TOF Micro quadrupole orthogonal acceleration time-of-flight mass spectrometer (Waters/MicroMass) using positive mode electrospray ionization (ESI+). Mass data were collected between 50 and 1000 m/z at a rate of two scans per second. The voltage

and temperature parameters were tuned for general profiling as follows: capillary = 3000 V; sample cone = 30 V; extraction cone = 2.0 V; desolvation temperature = 300 °C; and source temperature = 130 °C. Mass spectral scans were centered in real time producing centroid data. Leucine Enkephalin was infused via a separate orthogonal ESI spray and baffle system (LockMass) which allowed ions to be detected for a single half-second scan every 10 s in an independent data collection channel. The standard mass was averaged across 10 scans providing a continuous reference for mass correction of analyte data.

Data Analysis. Nonparametric survival analysis of *C. elegans* longevity was carried out using Systat 12 (Systat Software, Inc., Chicago, IL) and graphically shown as Kaplan−Meier survival curves generated with Graphpad Prism (Graphpad Software, Inc., La Jolla, CA). *P* values were calculated using Mantel Log Rank test (LR) (*28*). Means and medians were calculated using bootstrap resampling with 10 000 samples and 95% confidence intervals were calculated by the standard percentile method. Results are reported as mean ± SE and medians (95% confidence intervals). Survival data were censored on the day when both bean treatment groups and the control group had >90% mortality. Right-hand censoring was found to decrease experiment time with negligible decrease in statistical power as reported in Fitzgerald et al. (*27*).

Chromatographic and spectral LC-MS peaks were detected, extracted, and aligned using MarkerLynx software (Waters). Chromatographic peaks were detected between 0 and 16 min with a retention time error window of 0.1 min. Apex track peak detection parameters were used, automatically detecting peak width and baseline noise. No smoothing was applied. To reduce the detection and inclusion of noise as data, an intensity threshold value of 40 and a noise elimination value of 6 were used. Mass spectral peaks were detected between 50 and 1000 m/z with a mass error window of 0.07 m/z. The deisotoping function was enabled to eliminate the inclusion of isotopic peaks. A matrix of features as defined by retention time and mass was generated, and the relative intensity of all features, as determined by area, was calculated for all individual samples. Potential effects of technical variability were minimized by normalizing the total ion current (TIC) intensity values among all samples such that the summation of all feature intensities in each individual sample was equal. Furthermore, the relative intensity of each feature was averaged over the three replicate injections preformed for each sample providing a reliable data matrix with minimal technical artifacts. The data matrix was then subjected to hierarchical cluster (HC) and principal components (PC) analyses using Partek Discovery Suite v6.0 (Partek Inc., St. Louis, MO) and Systat 12. Bar graphs throughout the manuscript were generated using Graphpad Prism (Graphpad Software, Inc.). Mass Profiler Professional software (MPP, Agilent Technologies, Inc., Santa Clara, CA) was used to determine differential abundances of metabolites using an unpaired t test with Benjamini-Hochberg FDR multiple-test correction ($p < 0.05$). The data were filtered prior to statistical analysis using the guided workflow provided in MPP. Results have been reported as percent of total metabolites detected and degree of overlap between the different treatments.

RESULTS

An initial study on *C. elegans* longevity was performed using a 65% methanol extraction (pH 7) of a commercially available mixture of cooked white kidney beans or navy beans. This extraction approach is commonly employed in the evaluation of plant derived foods for bioactivity. The Kaplan−Meier survival curves from this experiment are shown in **Figure 1**. For this exploratory analysis, pairwise comparisons were performed among all three treatment groups evaluated. While the survival of nematodes in either bean extract group was not different from that of the DMSO solvent control group, the survival of the white kidney group versus the navy group was statistically different ($p = 0.005$, LR test, **Figure 1**). The median lifespan of the white kidney bean treatment group (14.98 days, 95% CI: 14−18) was 7% greater than the navy bean treatment group (13.86 days, 95% CI: 12−14). A similar difference in mean lifespan was also observed, 15.82 ± 0.64 days for white kidney and 13.84 ± 0.45 days for navy-treated nematodes. The control group had a median lifespan of 14.47 days (CI: 14−15) and a mean lifespan

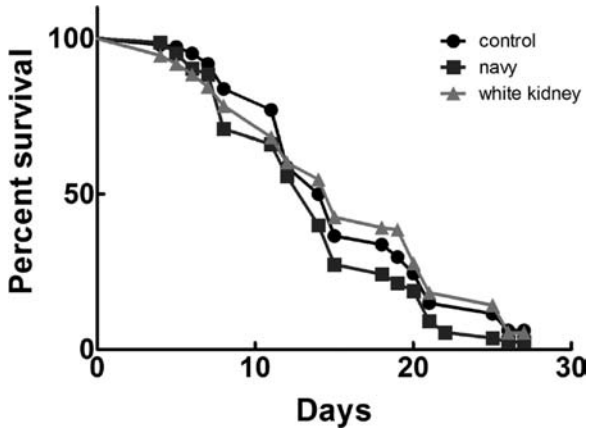

Figure 1. Kaplan−Meier survival curve of *C. elegans* exposed to methanol extracts of navy bean or white kidney bean or DMSO control solvent. A commercial mix of navy or white kidney beans was extracted with 65% methanol at neutral pH. *C. elegans* were treated with dry bean extract in liquid culture and supplemented with 5-fluoro-2′-deoxyuridine (FUDR) and OP50 *E. coli*.

of 15.53 ± 0.54 days. These values were calculated using the data from two separate longevity assays (control $n = 148$, navy $n = 165$, white kidney $n = 148$).

For the definitive analyses on nematode lifespan, cultivars of white kidney bean and navy bean were fractionated using the modified Bligh−Dyer technique described in the Methods section above. Three hydrophilic extracts (pH 2, 7, and 9) and a hydrophobic extract were evaluated. Survival analysis was conducted on the nematodes fed the four fractions of each bean cultivar and comparisons were made to the control group (**Figure 2A−D**). A summary of the results for mean and median lifespan and log rank p values for survival analysis are shown in **Table 1**. In the case of white kidney treatment groups, the log rank test for significance between the survival curves gives a statistically significant result in the group of nematodes fed hydrophilic acidic (pH 2) extract ($p = 0.013$) as well as in the nematodes fed basic (pH 9) extract ($p = 0.011$) when compared to control. These p values are paired with 10.88% and 13.66% increases in median longevity compared to control animals, respectively. No statistically significant effect on survival was seen in the white kidney bean pH 7 treatment group. In addition, the analysis showed no statistical difference between the survival curves of treatment groups fed pH 2, 7, or 9 extracts of the navy bean cultivar with respect to the control group. In both white kidney and navy treatment groups fed extracts containing hydrophobic compounds, a decrease in median longevity was seen when compared to the control (navy - 13.01 days, 95% CI: 13−13.5, white kidney - 13.00 days, 95% CI 12−14, control −13.98 days, 95% CI: 13−15). The log rank test for significance between the navy bean hydrophobic fraction treated nematodes compared to the control treated nematodes showed that the survival curves were statistically different ($p = 0.008$).

To address the possibility that bean extracts caused the nematodes to eat less food thereby inducing an indirect caloric restriction effect known to increase longevity, feeding rates of young adult nematodes eating bean extract or control solvent were measured. There was no significant difference in feeding rate among the treatment groups, suggesting that caloric restriction was not influencing the longer-lived treatment groups (**Figure 2E**).

Initial exploratory metabolite fingerprinting was carried out on methanol extracts of freeze-dried powders of white kidney and

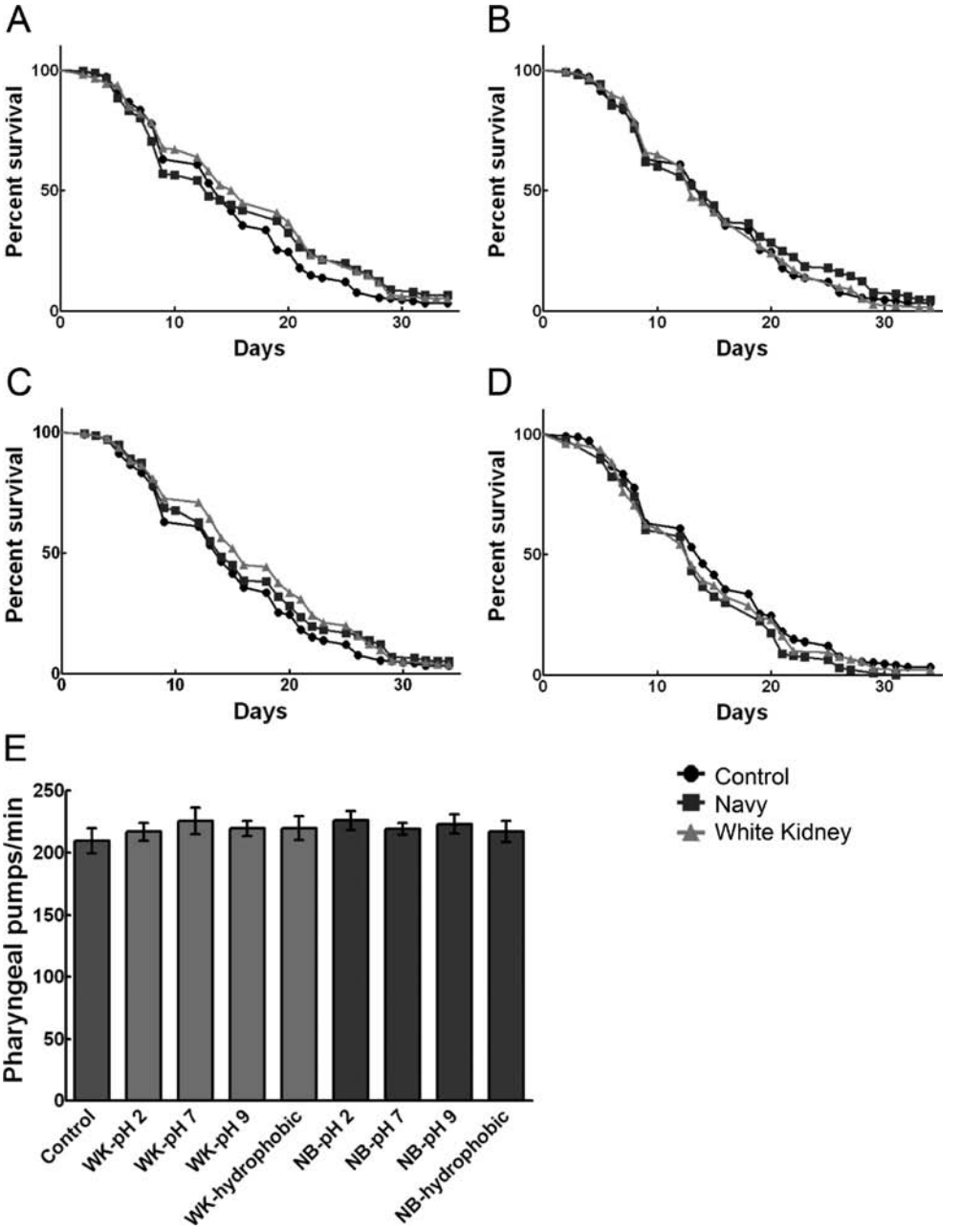

Figure 2. Kaplan−Meier survival curves of *C. elegans* exposed to (**A**) acidic, (**B**) neutral, (**C**) basic, and (**D**) chloroform extracts of navy bean (cv Seahawk), white kidney bean (cv Lassen), or control. Extracts were prepared using a modified Bligh and Dyer method. *C. elegans* treated with DMSO control solution or dry bean extract in liquid culture with 5-fluoro-2′-deoxyuridine (FUDR) and OP50 *E. coli.* (**E**) Feeding rates of *C. elegans* fed bean extracts used in survival analyses monitored on solid NGM-agar plates seeded with OP50 *E. coli.* Values are means ± SD.

Table 1. Lifespan Analysis of *C. elegans* Fed Fractional Extracts of Two Bean Varieties

treatment	*N* total	mean ± S.E.[a] (days)	median (95% CI)[a] (days)	% change[b] (mean)	% change[b] (median)	*P* (LR)[b]
control	233	14.95 ± 0.51	13.98 (13−15)			
WK-pH 9	241	16.93 ± 0.54	15.89 (14.5−19)	13.22	13.66	0.011
WK-pH 7	222	15.14 ± 0.51	13.32 (13−15)	1.26	−4.69	0.945
WK-pH 2	229	16.58 ± 0.59	15.50 (14−19)	10.93	10.88	0.013
WK-hydrophobic	258	14.05 ± 0.47	13.00 (12−14)	−6.01	−7.02	0.222
NB-pH 9	231	16.05 ± 0.56	14.34 (13−16)	7.37	2.56	0.115
NB-pH 7	233	15.62 ± 0.58	14.00 (12−16)	4.51	0.15	0.198
NB-pH 2	241	15.71 ± 0.61	13.26 (11−16)	5.06	−5.10	0.100
NB-hydrophobic	224	13.46 ± 0.45	13.01 (13−13.5)	−9.95	−6.92	0.008

[a] Medians and means estimated using bootstrap resampling with 10 000 samples and 95% confidence interval calculated by percentile method. [b] Values are relative to control.

navy beans prepared using the same method as that employed for the *C. elegans* experiment reported in **Figure 1**. As shown in **Figure 3**, the two bean germplasms are clearly separated along PC 1. In this analysis, 20.3% of the variance is explained by the

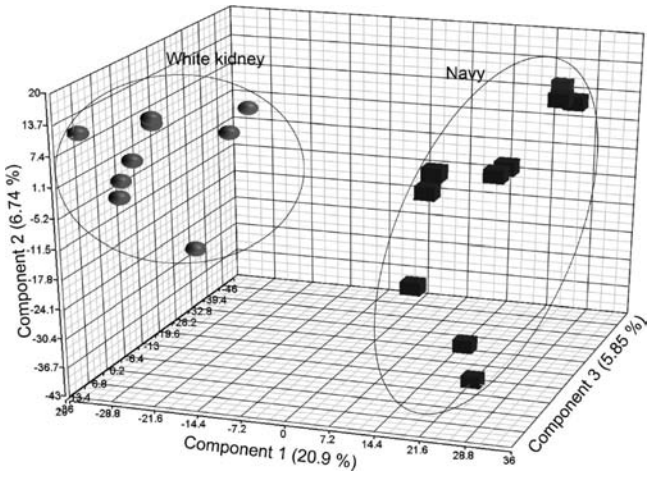

Figure 3. Metabolite fingerprinting of dry bean market classes. Comparison of 65% methanol extracts of white kidney bean and navy bean metabolite profiles based on positive ionization mode. Percentage of variability explained by each component is indicated on the axes. Each symbol represents an independent extraction and analysis of the same freeze-dried material. Ten replicate extractions were carried out. Two navy bean extracts were statistical outliers and were therefore excluded in PC analysis.

first component with 4365 features included in the PC analysis with no prior statistical filtering. The features may include metabolites and their isobars or fragments of larger metabolites.

To further refine the metabolite fingerprint affecting longevity in the nematode, hydrophilic (pH 2, 7, 9) and hydrophobic extracts of the two bean germplasm corresponding to the extracts administered to the worm treatment groups shown in **Figure 2** were analyzed using LC-MS. Normalized ESI-positive peak intensity values for the chromatographic features detected in these fractions were subjected to principal components analysis and hierarchical clustering. Initially, the data from the three hydrophilic fractions were combined and contrasted to the data from the hydrophobic fraction for each type of dry bean (**Figure 4A**). Unique metabolic clustering gave a clear separation based on center of domestication with the cumulative variance between hydrophilic and hydrophobic features being 57.1% (45.1% PC 1, 7.98% PC 2, and 4.07% PC 3). **Figure 4B** shows a dendrogram illustrating Euclidian distances resulting from agglomerative hierarchical cluster analysis using an average linkage clustering algorithm. The distances and pattern in the hierarchical clustering are similar to the clustering seen in the PC analysis. Furthermore, it was noted that the Euclidean distance separations of the hydrophilic fractions of white kidney bean and navy bean were greater than the distance between the hydrophobic fractions of these beans.

A detailed analysis of the ESI-positive mode data from **Figure 4A** showed that 37.8% of the total chromatographic features (421) were detected in the hydrophilic fraction and 62.2% of the features (694) were detected in the hydrophobic fraction. The number of features is irrespective of bean market class. **Table 2** details the breakdown of the hydrophobic and hydrophilic features by bean type following statistical analysis. Chromatographic features included in this table are those features which were determined to be at least 2-fold higher in either navy or white kidney bean extracts following unpaired t tests ($p < 0.05$) for each grouping (nonpolar, pH 2, pH 7, and pH 9). For the hydrophobic features, the number of unique features in white kidney and navy extracts represents only 1.87% (13 features) and 2.44% (17 features) of the total with 384 and 310 features found

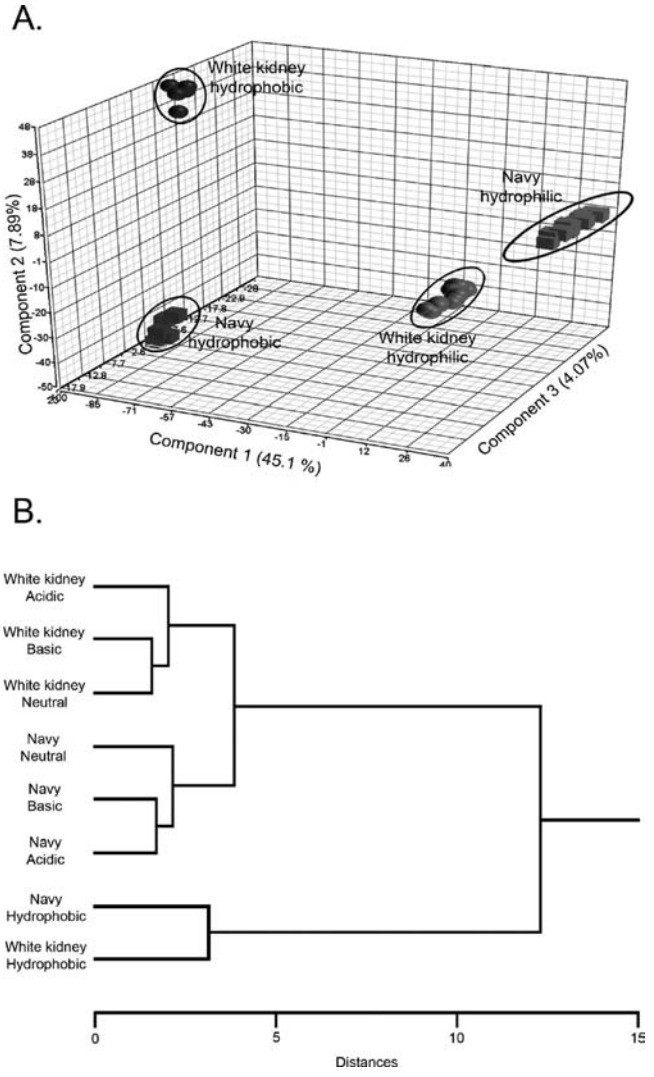

Figure 4. Variation between Bligh and Dyer extract fractions of dry bean as analyzed using LC-MS in positive ES mode. (**A**) Principal components analysis scores plots based on positive ionization mode for hydrophobic and hydrophilic extracts of white kidney and navy beans. (**B**) Dendrogram illustrating Euclidian distances resulting from agglomerative hierarchical cluster analysis using an average linkage clustering algorithm.

to be a minimum of 2-fold higher in navy and white kidney hydrophobic extracts, respectively. The 421 features detected in the hydrophilic fractions are further stratified by pH and bean type in **Table 2**. The number of features unique to each pH treatment is similar, and in each case, a larger number of features 2-fold greater in intensity were detected in navy bean compared to white kidney bean.

Analyses of the hydrophobic and hydrophilic extracts were also carried out independently due to the strikingly different effects on nematode longevity. Among the hydrophilic extracts, a marked separation was observed with 21.1% cumulative variance explained by the first three components (**Figure 5A**). The first component (10.1% of total variance) separates by bean market class, whereas the second PC (6.19% of total variance) and third PC (5.44% of total variance) account for the separation of the pH 2, 7, and 9 extracts within each bean market class. The hydrophobic extracts gave way to the highest cumulative variability (52.8%) and clearly resolved the two dry bean market classes with PC 1, PC 2, and PC 3 explaining 32.6%, 10.2%, and 9.94% of the total variance, respectively (**Figure 5B**).

Table 2. Features from Electrospray Data

LC-MS Hydrophobic Fraction

	unique features[a]	% of total features	features, FC > 2[b]	% of total features
white kidney	13	1.87	310	44.7
navy	17	2.44	384	55.3
total	694[c]			

LC-MS Hydrophilic Fraction

	unique features[a]	% of total features	features, FC > 2[d]	% of total features
pH 2	24	5.7		
white kidney	12	2.9	67	15.9
navy	12	2.9	142	33.7
pH 7	17	4.0		
white kidney	9	2.1	65	15.4
navy	8	1.9	84	19.95
pH 9	25	5.9		
white kidney	10	2.4	62	14.7
navy	15	3.6	149	35.4
total	421[c]			

[a] Number of unique features when comparing all pH levels irrespective of bean market class. Each pH stratified by 2-fold increases in either white kidney bean or navy bean. [b] Fold change comparison of white kidney and navy bean features at each individual pH. Number corresponds to features at least 2 fold higher in a given extract. [c] Number of chromatographic features statistically different between white kidney and navy (1-way ANOVA, $p < 0.05$, Benjamini Hochberg FDR multiple testing correction) detected in ESI+ mode. [d] Number of chromatographic features statistically different between white kidney and navy (1-way ANOVA, $p < 0.05$, Benjamini Hochberg FDR multiple testing correction) detected in ESI+ mode. Compounds may exist in more than one pH fraction due to differential solubility.

Bean germplasm fingerprints correlating to differences in *C. elegans* longevity were determined. Detected features were classified arbitrarily into 100 Da lists (assuming single charge). The classes of chemical compounds associated with each of these mass ranges are listed in **Table 3**. The unique features in each pH extract were divided according to a 2-fold increase in either navy bean or white kidney bean extracts. The number of chromatographic features in each mass group for navy and white kidney hydrophobic extracts is shown **Figure 6A** and for the hydrophilic extracts in **Figure 6B**. Both unique features and those statistically different between extracts are shown. Visual inspection of these figures reveals that a distinguishing characteristic of navy bean is the presence of more chromatographic features in the mass ranges from 200 to 500 and 600 to 700 in the hydrophobic fraction relative to the white kidney extract. A similar pattern was also identified in the hydrophilic fractions of navy bean versus white kidney bean.

DISCUSSION

The diet of an organism is generally comprised of a complex mixture of chemicals that provides (1) the nutrients required for that organism's normal cellular function, (2) non-nutrient chemicals that have bioactivity (frequently termed bioactive food components), and (3) chemicals without detectable biological activity (*29*). It is well documented that nutrients and bioactive food components exert a host of affects on metabolism and that different metabolic states are associated with effects on lifespan and on risk for chronic diseases (*30*−*32*). Model systems have been developed for screening for the effects of various dietary chemicals on longevity and disease risk, which have been hypothesized to be interrelated (*33*, *34*). One such model system involves the use of *C. elegans* in which an increase in longevity of

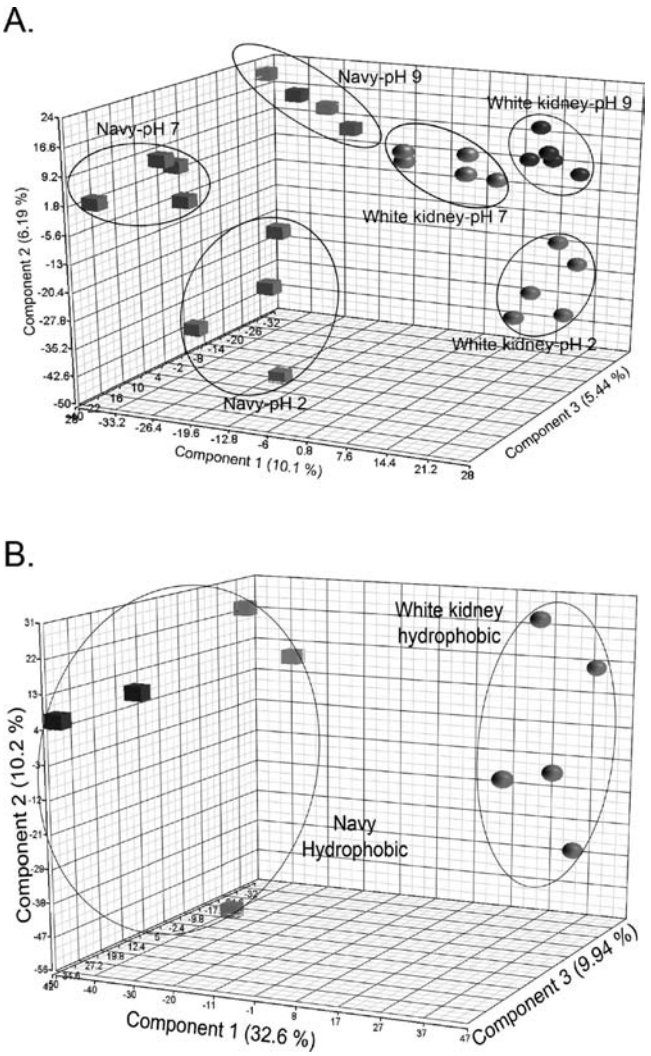

Figure 5. Comparison of features within hydrophobic and hydrophilic Bligh and Dyer dry bean extracts detected using LC-MS in positive ES mode. (**A**) Principal components analysis of acidic basic and neutral hydrophilic fractions of navy beans and white kidney beans. (**B**) Principal components analysis of hydrophobic features of navy beans and white kidney beans. Percentage of variability explained by each component is indicated on the axes.

Table 3. Chemical Classes Associated with Feature Mass Ranges

mass	possible compound classes
100−200	NPAAs, amines
200−300	flavonoids, sesquiterpenes, phenylpropanoids, alkaloids
300−400	flavonoids, alkaloids
400−500	triterpenes, steroids, saponins, alkaloids
500−600	tetraterpenes, alkaloids
600−700	alkaloids
700−800	alkaloids
800−900	alkaloids, phopholipids
900−1000	phopholipids

this nematode is used as a surrogate for age-related human diseases such as cancer and heart disease (*35*−*37*). There have been a number of papers published on the effect of individual phytochemical compounds on the longevity of *C. elegans*. Compound classes which have been shown to increased longevity include carboxylic acids (valproic acid) (*38*), flavonoids (quercetin) (*39*), alkaloids (catechins) (*40*, *41*), and polyphenols (resveratrol) (*42*). These reports have shown longevity extension

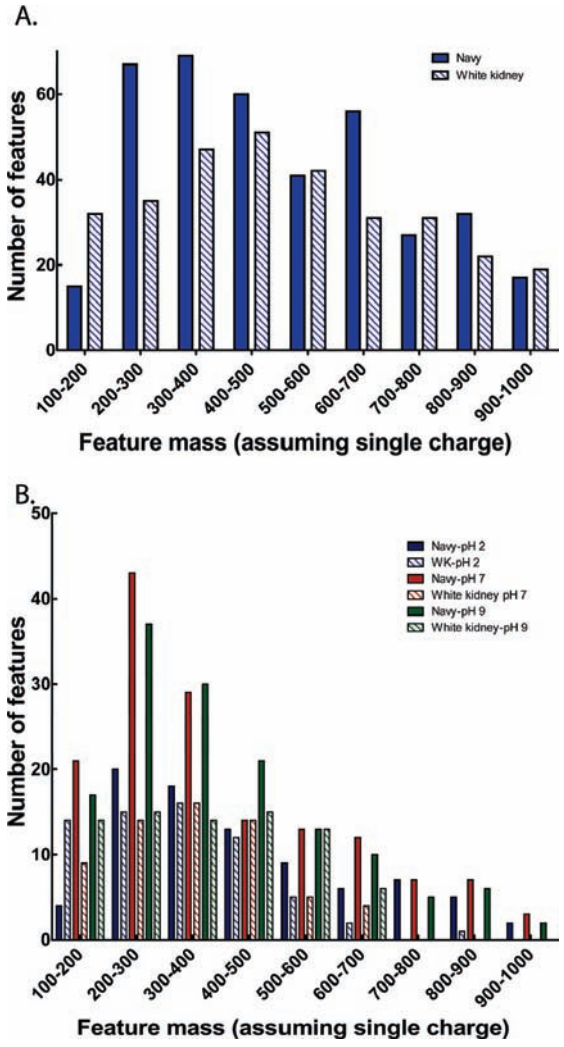

Figure 6. Classification of chromatographic features by mass. (**A**) Features in hydrophobic navy and white kidney bean extracts separated into 100 *m*/*z* increments. (**B**) Features in hydrophilic navy and white kidney bean extracts separated into 100 *m*/*z* increments.

ranging from 9% to 178% depending on administrated dose for single compounds. In addition, these same compounds have been reported to reduce cancer risk in various experimental models (*43*). It is thought, however, that a mixture of compounds will have the most impact on the development of cancer in humans (*44*, *45*), and in turn such mixtures would be expected to increase longevity in *C. elegans*. However, to date, little attention has been given to evaluating mixtures as complex as foods in *C. elegans*. Consequently, the approach described herein provides a framework for designing experiments that will ultimately lead to identification of health beneficial dietary patterns and the specific combinations of chemical constituents in the foods that comprise those dietary patterns (*46*). We argue that such information is essential for two reasons: to link chemical patterns in foods to the genetic traits in the plants from which those foods are derived that are associated with health benefits, and to identify chemical patterns and traits in the foods of those plants that suppress health benefits. Knowledge of health promoting and health suppressive traits is required by plant breeders engaged in crop improvement for human health benefit (*29*).

The alcohol soluble and insoluble fractions of dry beans have been reported to afford protection against the development of cancer in a preclinical for colon cancer (*9*), but it is not known if the anticancer activity is due to small molecules associated with and released from these fractions during digestive metabolism or if protective activity is due to secondary effects on gut microflora that in turn release compounds that exert health-related biological activity. Since a number of papers lend support to the role of small molecules (*43*, *47*), the focus of the work reported herein was on chromatographic features 50−1000 *m*/*z*. Exploratory analysis of the effects of a methanol extract of freeze-dried dry bean powder on *C. elegans* lifespan (**Figure 1**) showed a limited effect on longevity with only the navy bean versus white kidney bean being statistically significant. That a difference in longevity was induced by the methanol extract of white kidney versus navy bean was consistent with the chemical differences that exist between these beans as shown in the principal components analysis of the LC-MS data from the methanol extracts of these beans as shown in **Figure 3**.

It is well-known that neutral pH extractions fail to recover many chemical compounds due to differential solubility of ionizable groups. Despite this fact, it is common for plant foods to be extracted with a liquid−liquid extraction of either ethanol/water or methanol/water at neutral pH and the extracts evaluated for biological activity. For this reason, our definitive analyses were performed using extracts from the modified Bligh and Dyer technique described in the Methods section above and in ref *25*. This liquid−liquid extraction approach removes the proteins and other insoluble compounds including starches and polysaccharides that can interfere with chromatographic analyses therefore rendering enzymatic degradation of the extracted compounds very unlikely. In addition, hydrophilic metabolites are extracted at pH 2, 7, or 9 which considerably increases the number of distinct metabolites detected because plant metabolites that would otherwise be extracted into the nonpolar solvent (chloroform) at pH 7 are readily extracted into the aqueous phase due to the presence of ionizable groups on the molecules. This maximizes selectivity at a particular pH with minimal loss during metabolite recovery (*48*). For example, at pH 9 compounds with a carboxylic acid functional group (i.e., organic acids) rapidly form carboxylate anions and are in turn readily soluble. The converse is also true for weakly basic compounds which form soluble salts at acidic pH (pH 2) and are readily soluble in aqueous solutions. The nonpolar chloroform fractions from each pH are pooled to constitute a single hydrophobic fraction.

All four extracts of each cultivar were evaluated for their effects on nematode lifespan. As shown in **Figure 2A**−**D**, distinct effects on the longevity of *C. elegans* were observed. Consistent with the effects on longevity reported in **Figure 1**, the neutral hydrophilic fraction of either dry bean had no effect on lifespan in comparison to the control. On the other hand, both the acidic and basic white kidney bean fractions prolonged longevity in comparison to the control treatment, whereas these navy bean fractions had no effect. With this result in mind, it is considered noteworthy that **Figure 4** shows chemical differences of sufficient magnitude exist among the various hydrophilic fractions of dry bean that enable distinction of dry bean market classes based on center of domestication using unsupervised PC analyses. It is important to note that the chosen extract dose may be a significant contributor of the observed effects on *C. elegans* longevity. The dose was determined based on previously published *C. elegans* survival data investigating complex phytochemical mixtures (*49*).

While the vast majority of screening experiments using *C. elegans* focus on agents found to extend lifespan, it was notable to us that hydrophobic fractions of either white kidney bean or navy bean caused a statistically significant reduction in longevity, with the negative effect being the greatest for navy bean (**Figure 2D** and **Table 1**). Consistent with this observation,

chemical differences were detected not only between the hydrophobic and hydrophilic fractions of each dry bean type (**Figure 4**), but also between the hydrophobic fractions. As with the hydrophilic fractions, the differences between hydrophobic fractions were of sufficient magnitude such that unsupervised PC analyses distinguished between the dry bean market classes based on center of domestication.

In evaluating the lifespan data summarized in **Table 1**, we initially questioned whether the degree of longevity extension observed was likely to be biologically meaningful. A review of the literature revealed that dietary energy restriction, the most powerful form of intervention known to regulate aging and risk for chronic diseases, has been reported to extend *C. elegans* longevity 11.7−60% (*50*−*53*) and that resveratrol was identified as a longevity extension agent with a similar magnitude of effect (*11*). Similar magnitudes of lifespan extension are reported in **Table 1**. As shown in **Figure 2E**, the feeding rates of nematodes in all the treatment groups were comparable, a finding that indicates that the effects of dry bean extracts on longevity is not likely to be due to caloric restriction but is associated with the small molecules which have been left intact during the extraction process described in the Methods section. Varying the experimental design could alter the observed effects on longevity; therefore, the authors caution that this work is not sufficient in itself to state that white kidney bean is better than navy bean.

Secondary plant metabolites encompass a vast array of chemically diverse molecules. Identification of specific metabolites or classes of metabolites responsible for the differential effects shown here is beyond of the scope of this paper; however, a semiquantitative evaluation indicates there are indeed distinct groupings of metabolites that are likely accounting for the differences seen in *C. elegans* longevity. Of note is that the differences are not just based on presence/absence of the compounds but also on statistical differences ($p < 0.05$, fold change >2.0) in peak intensity across extracts. This is illustrated in **Table 2**. The largest profile difference is seen when comparing hydrophobic and hydrophilic fractions. A large number of these compounds are most likely phospholipids and glycophospholipids which are entirely absent in the hydrophilic extracts. The metabolite fingerprint differences are evident in both PC analysis and HC analysis where the greatest Euclidian distance metric is between the hydrophobic and hydrophilic fractions. Clustering patterns seen in PC analysis were solidified using hierarchical clustering. When molecular features were stratified by mass (**Figure 6**), the number of features is heavily weighted toward masses less than 700 Da (assuming single charge). Interestingly, the extracts that significantly extended *C. elegans* lifespan (white kidney pH 2 and pH 9) had the greatest number of features in the lower mass groupings, whereas the extracts that decreased lifespan (navy and white kidney hydrophobic extracts) contained higher molecular weight compounds. As a number of low molecular weight compound classes (**Table 3**) can be hypothesized to contain pro-longevity metabolites, while high molecular weight features may correspond to known antilongevity metabolites. However, the bioavailability of the hydrophilic and hydrophobic extracts is unknown and could also be affecting the results of the longevity assays, an issue that is currently being investigated.

In concluding, the utilization of the modified Bligh and Dyer method of chemical extraction in combination with *C. elegans* longevity extension assay permitted rapid screening of a food crop for potential human health benefits. The use of *C. elegans* provided an inexpensive tool that permitted the use of an animal in bioactivity guided fractionation of whole food extracts. However, we propose that attention should be directed not only to chemical fractions that extend lifespan but also to those that decrease longevity since compounds that suppress longevity may be important to select against in efforts to improve the human health characteristics of a food crop through plant breeding and selection. Moreover, use of a high throughput LC-MS platform for metabolite fingerprinting showed potential in focusing the second stage of discovery to specific chemical classes of bioactive phytochemicals on which to focus the rigors of LC-MS-MS techniques for compound identification associated with human health benefit.

ACKNOWLEDGMENT

Our sincerest thanks to Christopher Link and Michael Grusak for technical advice and helpful discussions related to *C. elegans* longevity experiments. We also thank Mark Brick for providing dry bean samples and *C. elegans* work space. We would like to thank Steven Fischer and Theodore Sana for their guidance and assistance regarding metabolomics analysis. The nematode strain used in this work was provided by the *Caenorhabditis* Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR).

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Received for review February 23, 2010. Revised manuscript received April 26, 2010. Accepted May 3, 2010. This work was supported by a grant from the American Institute for Cancer Research (Grant No. 08A032).