FINAL PROJECT REPORT
WTFRC Project Number: 3055-7938

Project Title: Identifying disease prevention benefits of apple consumption

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Other funding Sources

Agency Name: No other funding sources for this project
Amount awarded:
Notes:

Total Project Funding: $94,617

Budget History:

Item	**Year 1:** 2005-06	**Year 2:** 2006-07
Salaries	19,320	20,286
Benefits	1,789	1,862
Wages	8,000	8,000
Benefits	880	880
Equipment	0	0
Supplies	16,000	16,000
Travel	800	800
Miscellaneous	0	0
Total	46,789	47,828

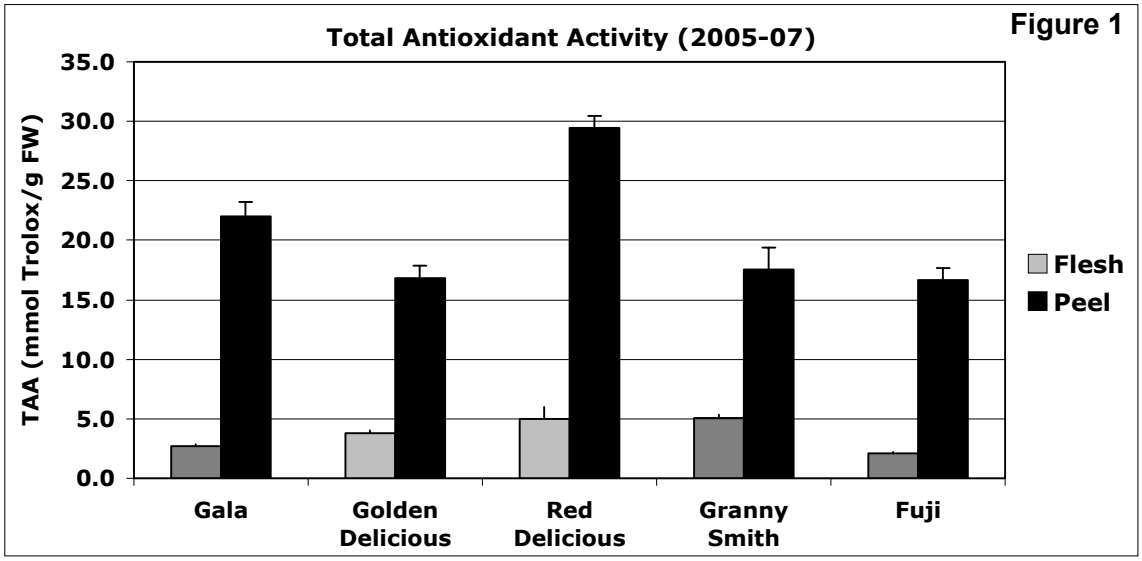
SIGNIFICANT FINDINGS:

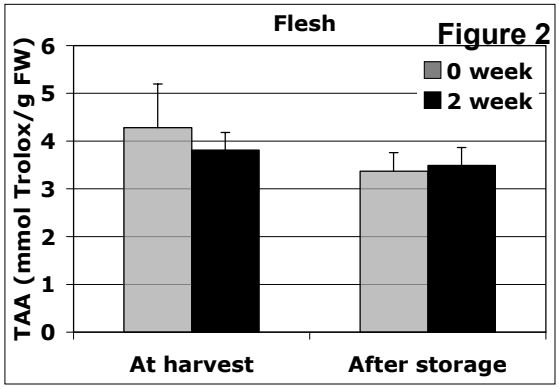
- We determined that **anti-oxidant activity** was consistently and many fold higher in peel tissue than flesh tissue of Gala, Golden Delicious, Red Delicious, Granny Smith, and Fuji apples, with the highest activity in Red Delicious peel. There was little loss in anti-oxidant activity after two-week shelf-life periods and CA storage.
- We determined that **vitamin C** (ascorbic acid) concentrations were also many fold higher in peel than flesh tissue of these varieties, however, they decreased significantly after two-week shelf-life periods at harvest and after CA storage.
- We determined that the concentrations of **phenolic compounds** were many fold higher in peel than flesh tissues, and showed no decline after two-week shelf-life periods either at harvest or after CA storage. Therefore, certain phenolic compounds (i.e. flavonoids) are robust and contribute significantly to the long-lasting anti-oxidant activity of apples that are un-refrigerated or are stored for significant periods of time.
- To quantify **flavonoids** in apples we developed a novel high-performance liquid chromatography (HPLC) method to analyze the glycosides and aglycones of quercetin, kaempferol, phoretin, naringenin enantiomers, and ellagic acid.
- Using this HPLC method, we determined that Gala, Golden Delicious, Red Delicious, Granny Smith, and Fuji apples contained all of the **flavonoids** measured, and that generally there were higher concentrations of them in peel than in flesh tissues, with little loss after two-week shelf-life periods at harvest and after CA storage.
- We recently acquired the instrumentation to separate, identify, and quantify 26 different phenolic phytochemicals using a modified liquid chromatography–mass spectrometry–electrospray ionization (LC/MS/ESI) method.
- We determined that extracts from the peel and flesh tissues of Gala and Red Delicious apples were active, in a dose-dependent manner against *in vitro* cells lines of colorectal, breast, and prostate **cancers**.
- We developed and validated an *in vitro* **anti-inflammatory** assay in canine chondrocytes (i.e. cartilage) using biomarkers for nitric oxide (NO), sulphated glycosaminoglycans (sGAG), and (prostaglandin E3) PGE_2 to quantify the anti-inflammatory activity of Gala and Red Delicious extracts.
- We developed and validated an *in vitro* **anti-adipogenesis** assay to quantify the inhibitory activity of Gala and Red Delicious extracts to the accumulation of triglycerides in a pre-adipocyte cell line.

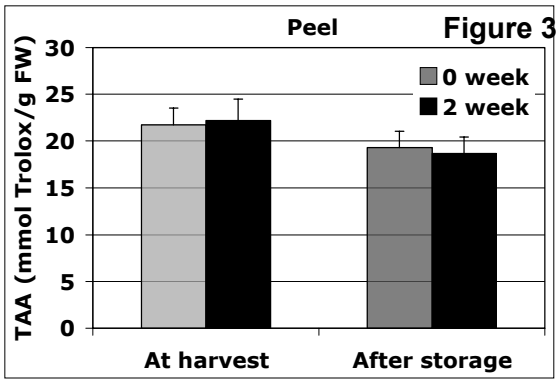
RESULTS AND DISCUSSION:

Fruit sampling and maturity. During 2005 and 2006 we received freshly harvested Gala, Golden Delicious, Red Delicious, Granny Smith, and Fuji apples from orchards in north-central Washington, with sub-samples stored in commercial CA storage until 2006 and 2007, respectively. Upon receipt of either freshly harvested or CA-stored fruit, some of the apples were separated into peel and flesh tissues, which were immediately frozen and powdered in liquid nitrogen and stored in an ultralow temperature (-80^{o}C) freezer for later analysis. Another sub-sample of fruit was left out in the laboratory at room temperature for two weeks before freezing. The average fruit weight and maturity (firmness, soluble solids, and starch index) of each sample was determined. The maturity data will be correlated with biochemical assays and disease inhibition models in scientific publications.

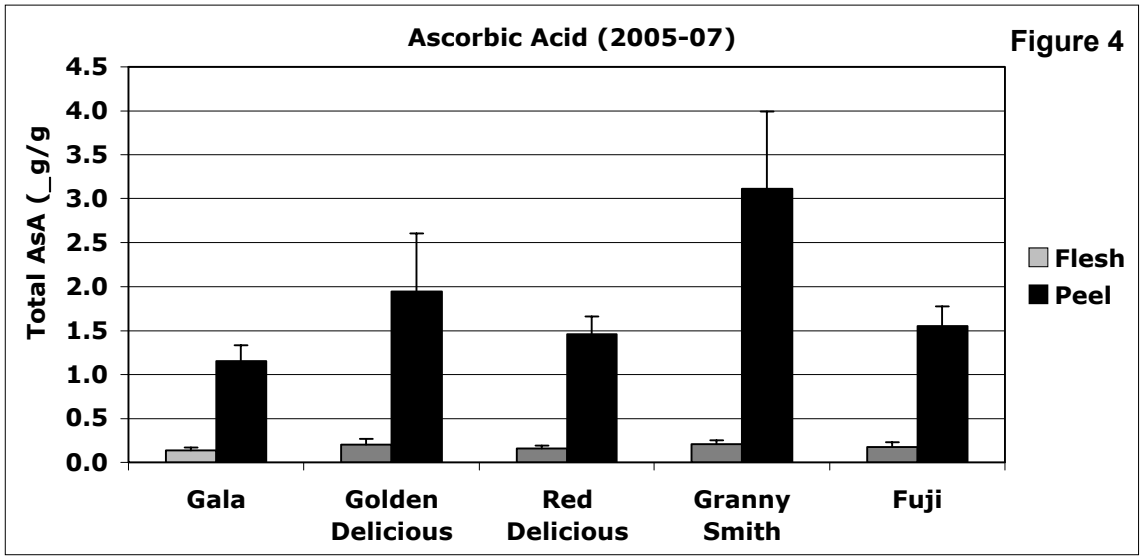
Anti-oxidant activity. We tested peel and flesh tissues of all varieties for anti-oxidant activity using an established ABTS method (1, 2). Peel tissue had approximately 5X higher total anti-oxidant activity (TAA) than flesh tissue, and Red Delicious peel had the highest activity (Fig. 1). There were no significant losses in activity during CA storage or after a two-week, non-refrigerated shelf life (Figs. 2-3). (Error bars in all graphs indicate standard error of the mean.)

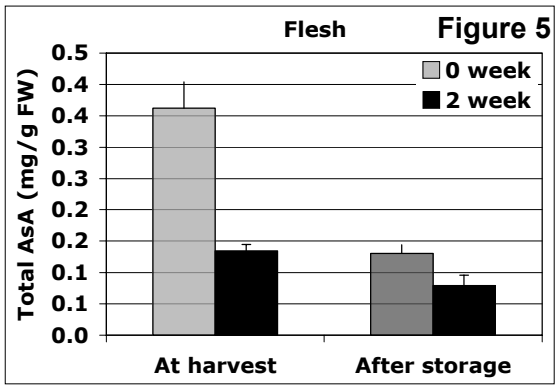


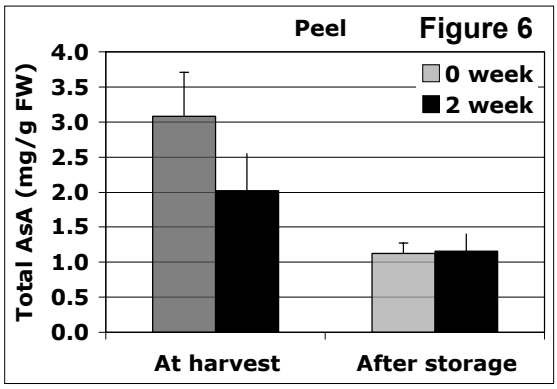




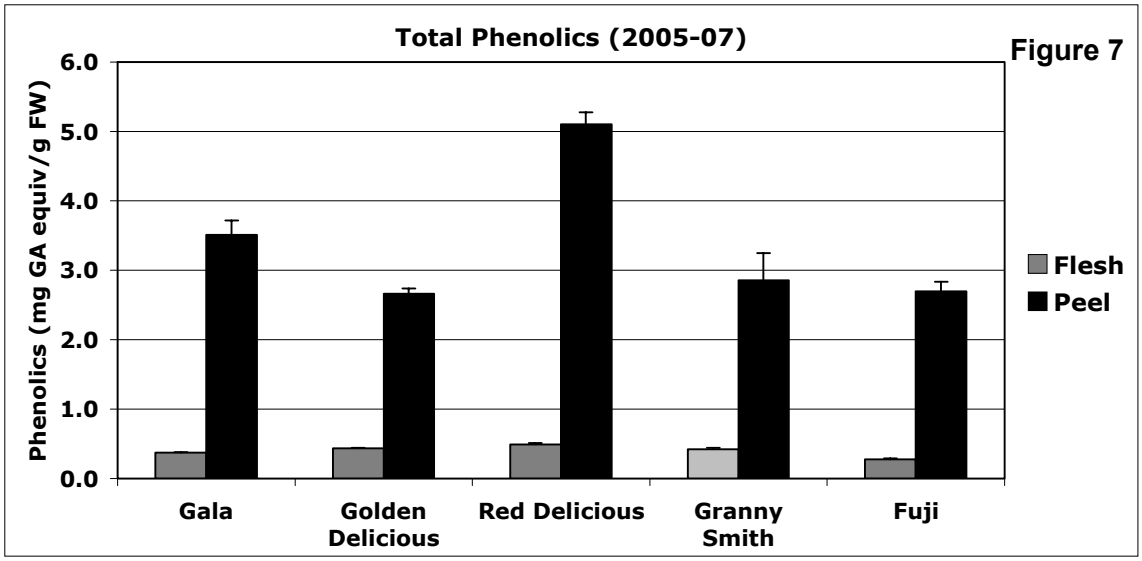
Vitamin C. Concentrations of total ascorbic acid (AsA), measured with a method adapted for apples (3), were up to 15X higher in peel than in flesh tissue of these varieties (Fig. 4), however, they decreased significantly after two-week shelf-life periods both at harvest and after CA storage (Fig. 5-6). Granny Smith peel had the highest concentration of vitamin C (Fig. 4).

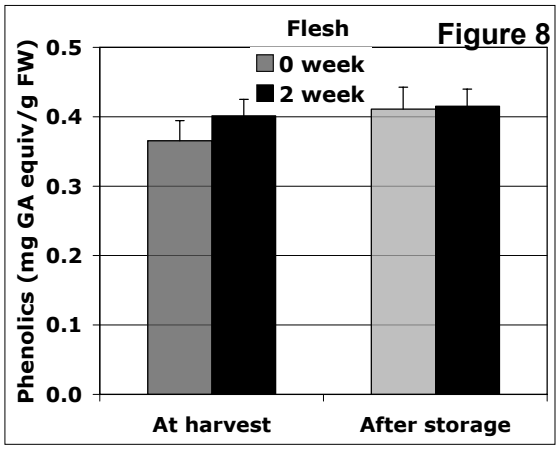


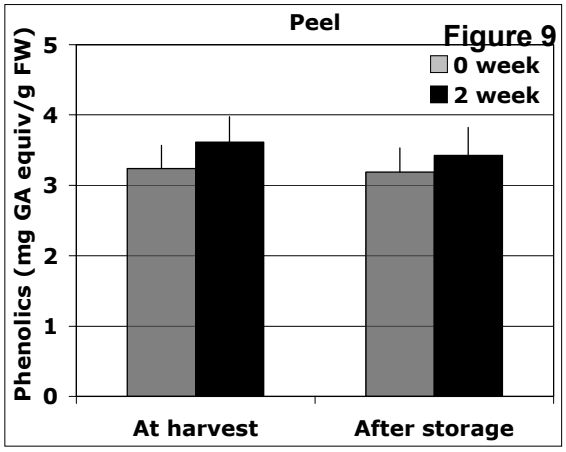




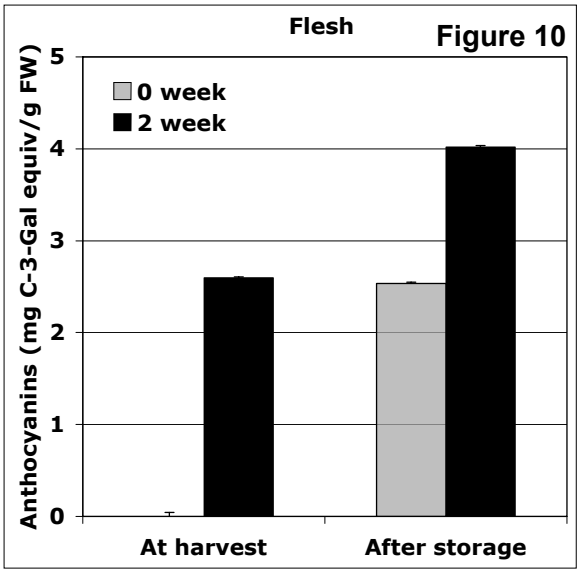
Phenolics. Concentrations of total phenolic compounds, measured as gallic acid (GA) equivalents using Folin-Ciocalteau reagent (4), were nearly 10X higher in peel than flesh tissue, and showed no decline after two-week shelf-life periods either at harvest or after CA storage (Figs 7-9). Phenolic phytochemicals, probably flavonoids, are robust and contribute significantly to the long-lasting antioxidant activity of apples that are either left un-refrigerated or are stored for months in CA storage.



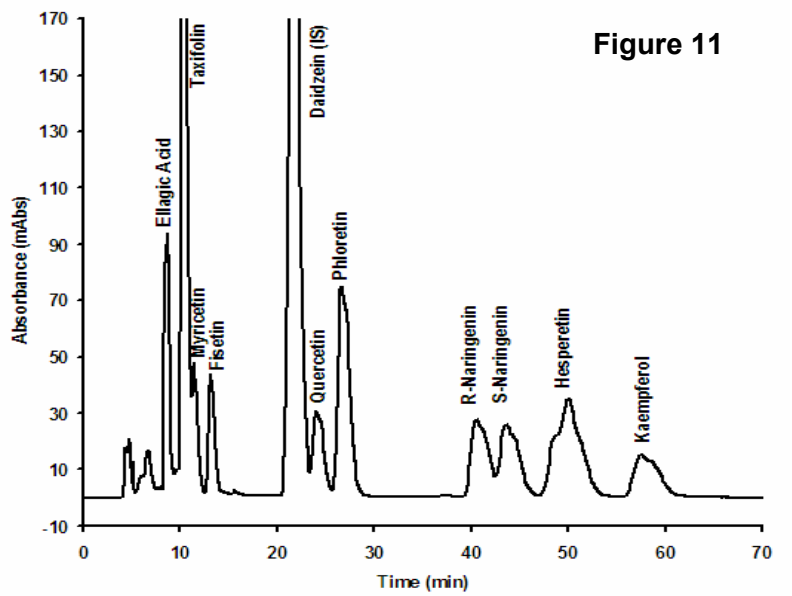




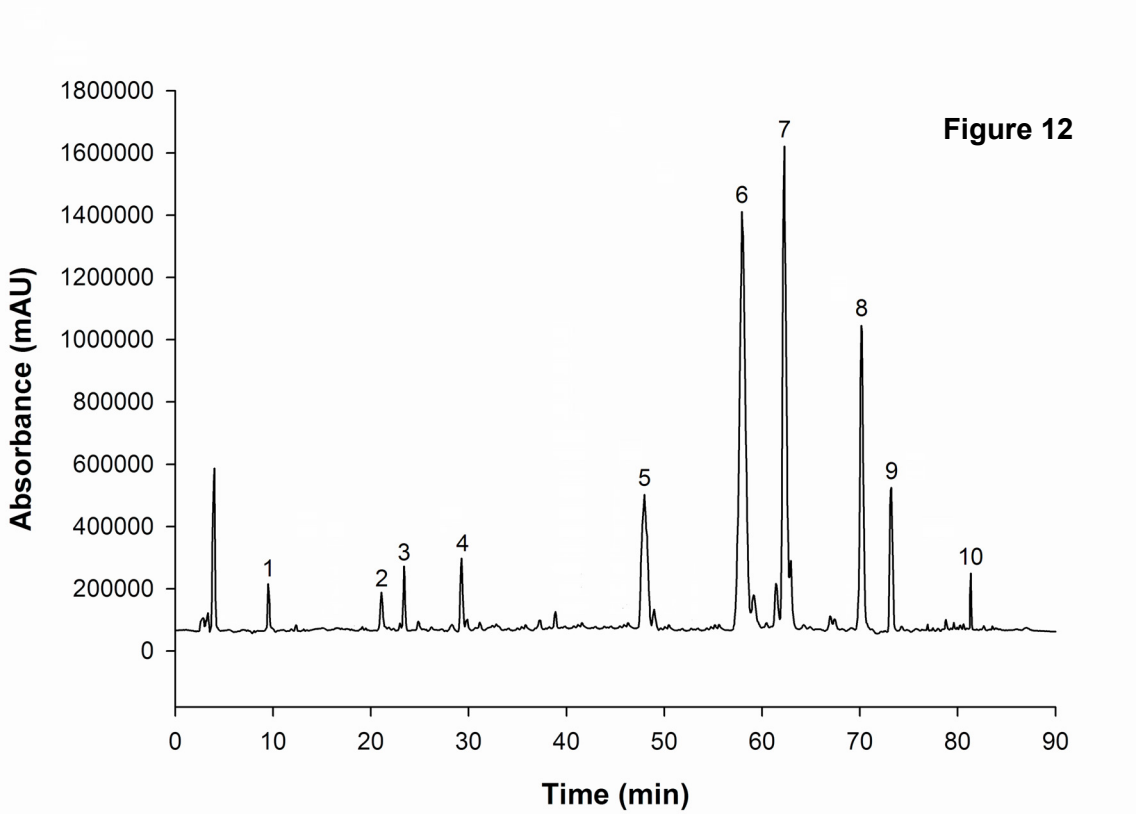
Anthocyanins. Total anthocyanins (a class of flavonoids), extracted with 1% HCl in methanol, were expectedly found in the peel tissue of Gala, Fuji, and especially Red Delicious apples (not shown). There was only a slight loss in anthocyanins (cyanidin-3-galactoside equivalents) in peel tissue after CA storage, and no loss after the two-week shelf-life period (not shown). Despite the small quantities of anthocyanins in flesh tissue, there was surprisingly an increase after the shelf-life period both at harvest and after CA storage (Fig. 10).



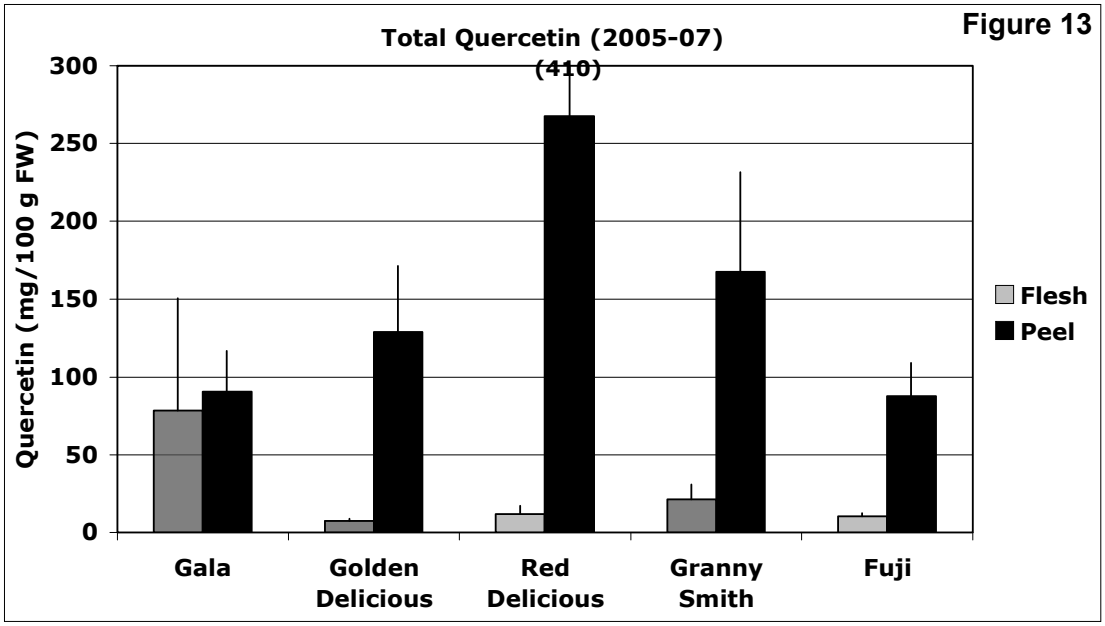
Flavonoids. To quantify flavonoids in apples we developed a novel high-performance liquid chromatography (HPLC) method to simultaneously analyze several of these important phytochemicals, including the glycosides (i.e. with attached sugars) and aglycones (i.e. without sugars) of quercetin, kaempferol, phoretin, naringenin enantiomers, and ellagic acid (Fig. 11).

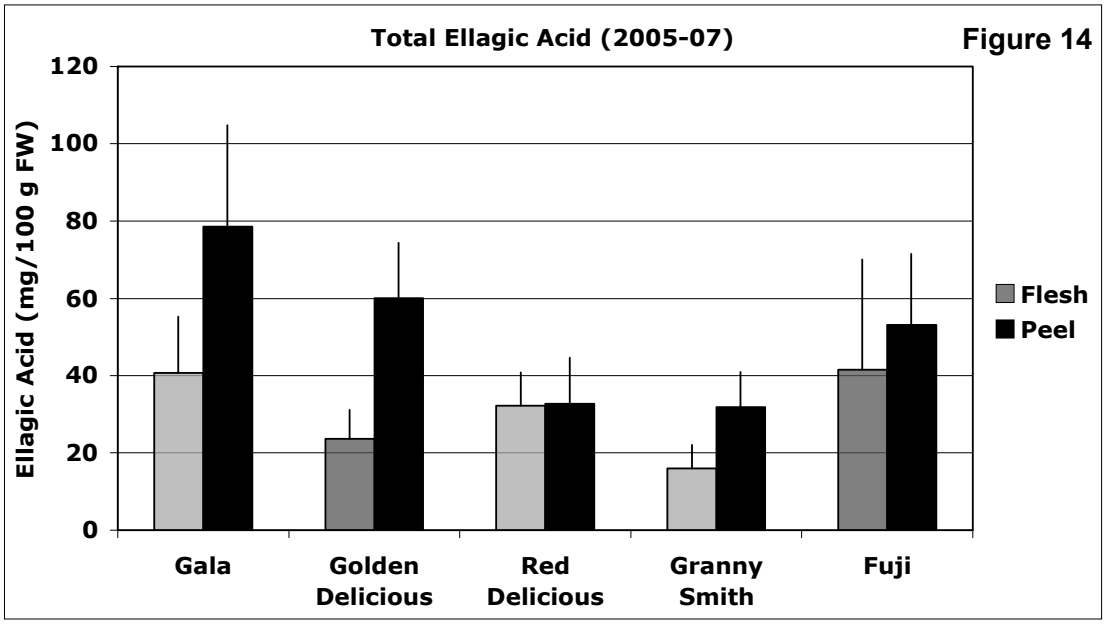


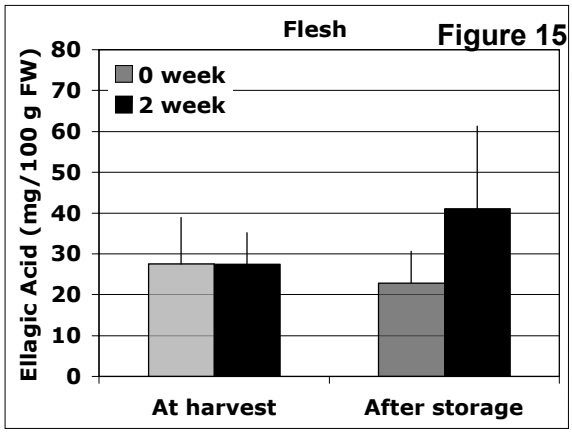
We recently acquired the instrumentation to separate, identify, and quantify 26 different phenolic phytochemicals using a modified liquid chromatography–mass spectrometry–electrospray ionization (LC/MS/ESI) method. We adapted a previously published methodology (5) with this instrument in order to detect nine different phenolic compounds in apple tissue (Fig. 12). [Graph legend: 1=gallic acid, 2=catechin, 3=chlorogenic acid, 4=epicatechin, 5=quercetin-3-rutinoside (rutin), 6=quercetin-3-rhamnoside (quercitrin), 7=phloridzin, 8=daidzein (internal standard), 9=quercetin, and 10=phloretin]

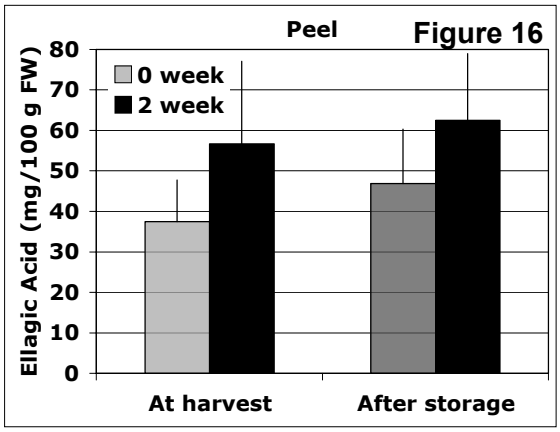


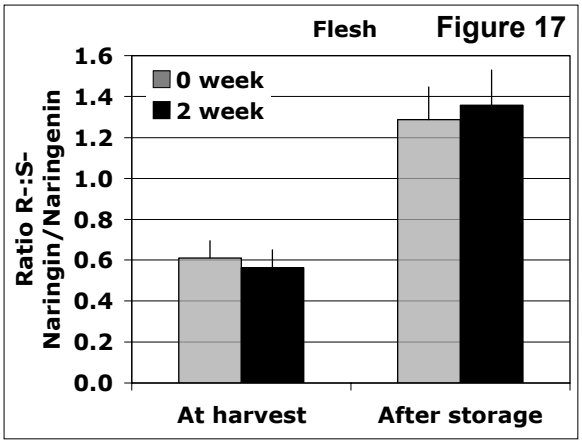
Using the HPLC method of analysis, we generally found higher concentrations of specific flavonoids in peel tissue than in flesh tissue, although in some cases variability was high resulting in large error bars. Quercetin, a predominant apple flavonol (6), was greater in peel tissue of most varieties, with highest concentrations in Red Delicious peel despite the large variability (number in parenthesis indicates maximum value of error bar) (Fig 13). Ellagic acid, primarily found in raspberries and strawberries (7), was measured in significant concentrations in both flesh and peel tissues of these apple varieties (Fig. 14), and may even increase following a two-week shelf-life period (Figs 15-16). Naringenin, known as a citrus flavonone found primarily in grapefruit (8, 9), is unique among the measured flavonoids because it exists naturally as a chiral compound with mirror image enantiomers designated R and S. Both the glycoside and aglycone forms of R- and S-naringenin were found in small concentrations in both the flesh and peel tissues of all varieties, and after a two-week shelf-life period and CA storage (not shown). Their occurrence in apple has not been previously reported. Interestingly, there was inter-conversion between R- and S-enantiomers from harvest to storage, with freshly harvested fruit having relatively more S-naringin/naringenin and fruit from CA storage having relatively more R-naringin/naringenin (Fig. 17). These differences in enantiomer composition could be important to the bioactivity of these enantiomers in human tissue because differences in pharmacokinetics have been shown for them (10). There were also differences in the relative amount of glycosides (with sugars) and aglycones (with sugars) among these flavonoids, with glycosides usually decreasing relative to aglycones following CA storage (Fig 18). These flavonoids are variously known to possess anti-oxidant, anti-cancer, and anti-inflammatory properties, which may aid in the prevention of heart, respiratory, and neurological diseases, diabetes, allergies and infections, and generally promote immune system responses (11, 12).

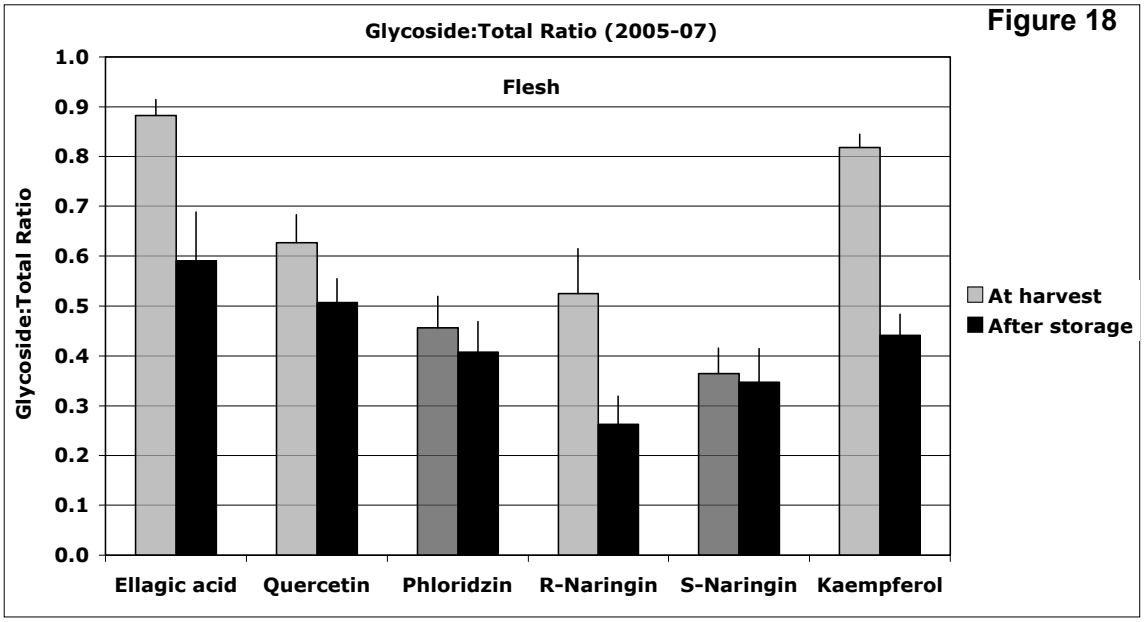

Total Quercetin (2005-07)
(410)
Figure 13
Quercetin (mg/100 g FW)
300
250
200
150
100
50
0
Gala
Golden Delicious
Red Delicious
Granny Smith
Fuji
Flesh
Peel


Total Ellagic Acid (2005-07)
Figure 14
Ellagic Acid (mg/100 g FW)
120
100
80
60
40
20
0
Gala
Golden Delicious
Red Delicious
Granny Smith
Fuji
Flesh
Peel


Flesh
Figure 15
Ellagic Acid (mg/100 g FW)
80
70
60
50
40
30
20
10
0
0 week
2 week
At harvest
After storage


Peel
Figure 16
Ellagic Acid (mg/100 g FW)
80
70
60
50
40
30
20
10
0
0 week
2 week
At harvest
After storage





Anti-cancer activity. Extracts of flesh and peel tissues of Gala and Red Delicious apples were active, in a dose-dependent manner against colorectal (HCT-116), breast (MDA-MB-231), and prostate (PC-3) adenocarcinoma cancer cell lines using an *in vitro* Alamar Blue (resazurin) fluorescent dye assay to determine cytotoxicity. Both peel and flesh extracts of Gala and Red Delicious reduced colorectal cancer cell viability, especially when sugars were cleaved from the flavonoids by enzymatic hydrolysis, although Red Delicious was more active than Gala (Fig. 19). Against prostate cancer cells, after cleavage of sugars from the flavonoids, the Gala flesh extract was most inhibitory followed by Red Delicious flesh extract (Fig. 20). Again, the aglycone Gala flesh extract was most inhibitory against breast cancer cells, followed by the peel extracts of both Red Delicious and Gala apples (Fig. 21).

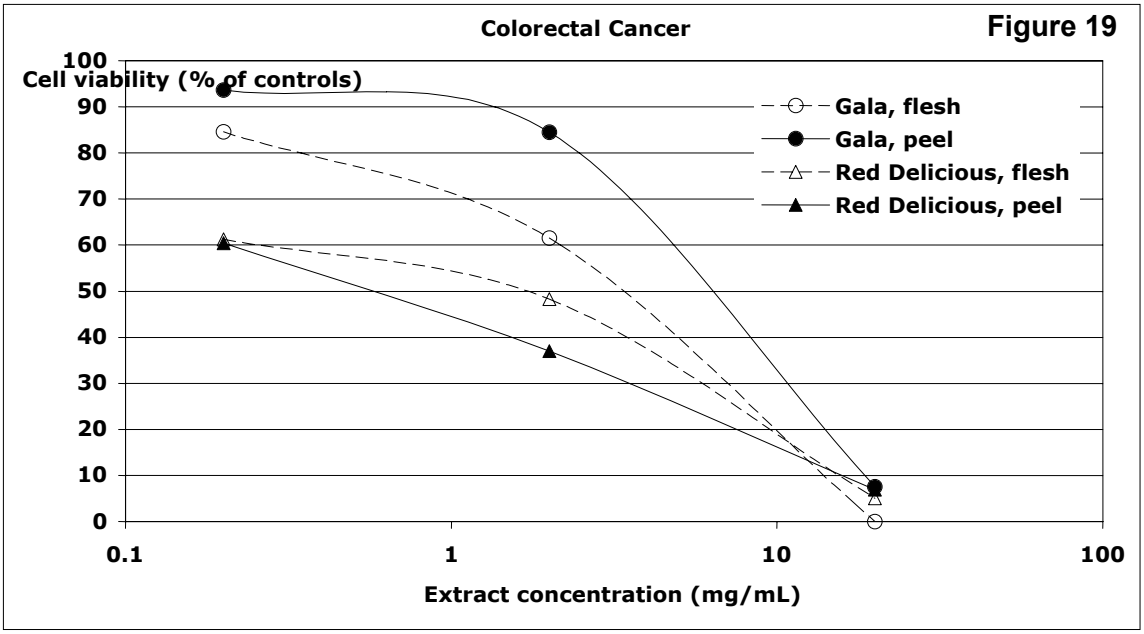


Figure 19

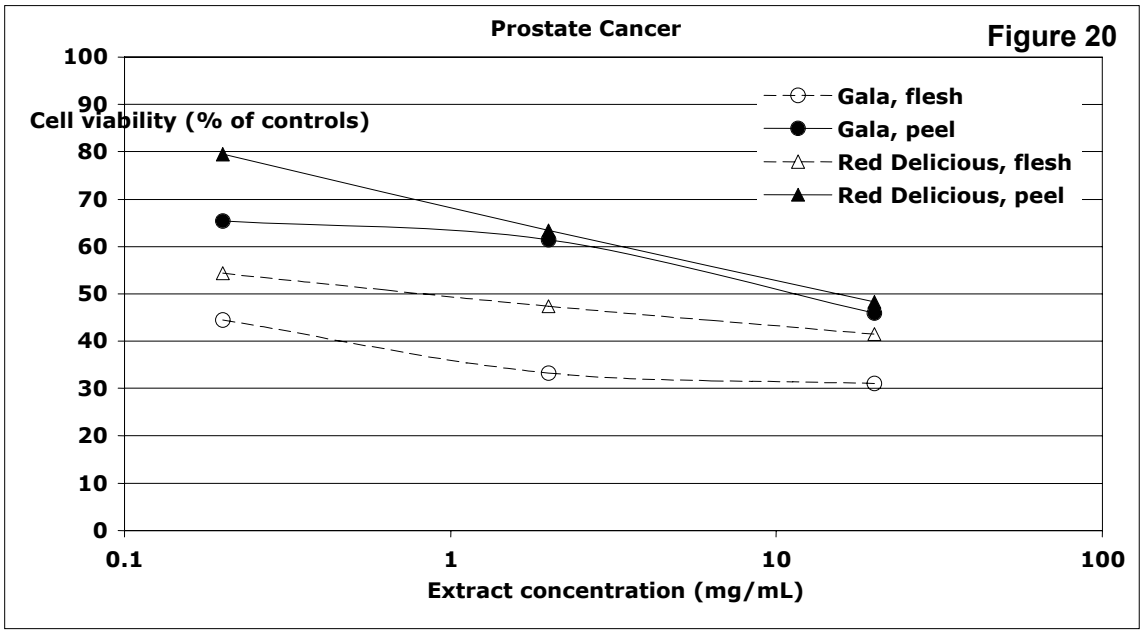
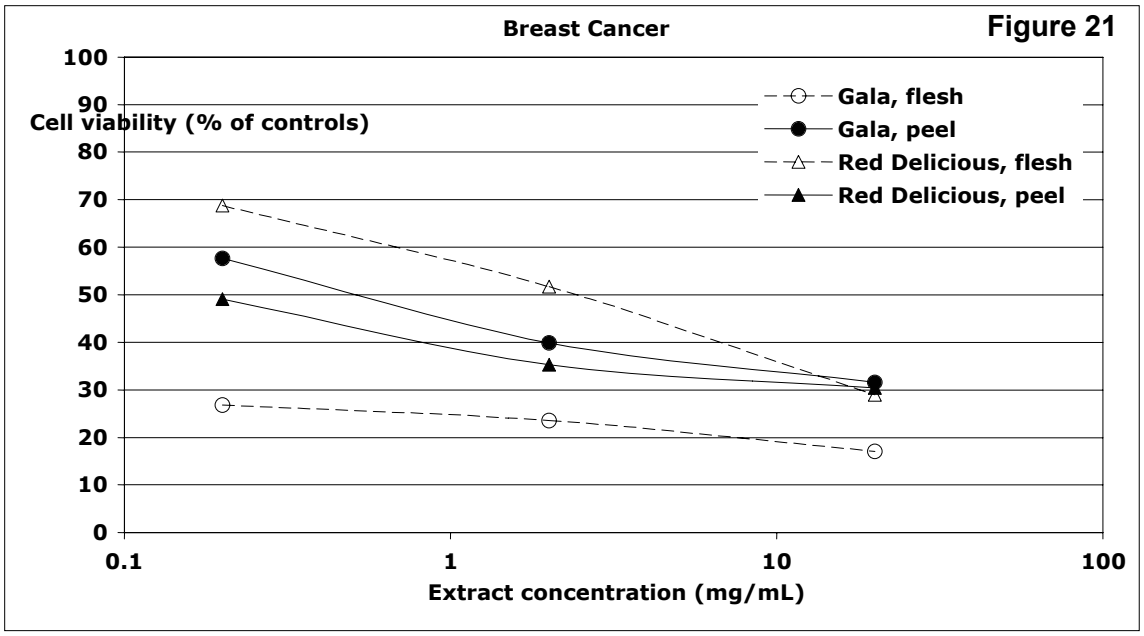


Figure 20



Anti-inflammatory activity. Inflammatory bowel activity was measured using an *in vitro* colitis model in colorectal adenocarcinoma (HT-29) cancer cells after inflammatory insult with tumor necrosis alpha (TNF-α) and measuring prostaglandin E_2 (PGE_2) levels. Quantification of the anti-inflammatory activity of flesh and peel tissues of Gala and Red Delicious apples is still underway, and so, the results of this disease model will be presented at the research review meeting.

Anti-adipogenic activity. This method replaced the proposed anti-hyperlipidemia disease model, because of its direct relevance to obesity. Pre-adipocyte (3T3-L1) cells were chemically induced to accumulate triglycerides, and then challenged with apple extracts from Gala and Red Delicious flesh and peel tissues. Adipogenesis was evaluated using a commercially available adipogenesis assay kit that stains triglycerides in the adipocytes. Lipid droplets are visualized microscopically and quantified by measuring absorbance at 492 nm. Quantification is still underway, and so, the results of this disease model will be presented at the research review meeting.

Significance and impact. The presence in five major Washington apple varieties of both known and novel phytochemicals that are active in various disease models is a very positive finding of this research. These phytochemicals, which represent different classes of flavonoids, appear to be durable to both extensive refrigerated CA storage and shelf-life periods at room temperature. These flavonoids have powerful anti-oxidant properties, which may contribute in large measure to the long-lasting anti-oxidant activities of Washington apples.

Two of the major apple varieties grown in Washington, Gala and Red Delicious, exhibited pronounced anti-cancer activities against three major cancers – colorectal, breast, and prostate. The expected anti-inflammatory and anti-adipogenic properties of these apple varieties will also provide strong evidence for the health benefits of consuming Washington apples. It is known that inflammation plays a role in gastrointestinal and other disease states. Increased consumption of apples, as well as other fruits and vegetables, could help counter various inflammatory disease states and the growing U.S. obesity epidemic (14).

This research provides a strong basis for claims regarding the health benefits of consuming Washington apples, which could be utilized by Washington apple growers to enhance their marketing efforts. However, more detail studies should be undertaken to identify other beneficial phytochemicals in Washington apples, to determine how the environment and crop management

practices influence their contents in apples, and the role that these phytochemicals in apples play in preventing various diseases. These efforts would provide additional evidence for the presumed health benefits of apple consumption.

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