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Antioxidant Response Mechanism in Apples during Post-Harvest Storage and Implications for Human Health Benefits

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ANTIOXIDANT RESPONSE MECHANISM IN APPLES DURING POST-HARVEST STORAGE AND IMPLICATIONS FOR HUMAN HEALTH BENEFITS

A Thesis Presented

by

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CHAPTER 1

INTRODUCTION

Phenolic phytochemicals are secondary metabolites synthesized by plants, which constitute an important part of the diet in both humans and animals (1-3). In plants these phenolics exhibit protective functions against environmental and biological stress such as high energy radiation exposure, bacterial infection or fungal attacks (4). In addition phenolics are also important for cell structure, signaling and pigmentation. Due to diversity of functions, phenolic phytochemicals are expressed in diverse cell and tissue types (4-6). Since phenolics have been linked to many protective functions in plants it is likely they play similar protective roles in fruits; making them a key factor in post-harvest preservation of fruits.

In addition growing evidence suggests that the intake of phenolics via fruits and vegetables is linked to reduced risk of chronic diseases like diabetes, cardiovascular disease and cancer (7-11). A study with many popular fruits found apples to contain the second highest total phenolics content after cranberries, but the highest soluble phenolics (12). Apples also had the second highest free radical scavenging-linked antioxidant activity among the fruits investigated (12). Apples are a popular fruit all over the world and have the potential to contribute as a significant source of phenolic antioxidants. In the United States, 22% of phenolic intake via fruit comes from apples (13). In Finland the main source of dietary phenolics are onions and apples and in the Netherlands tea, onions and apples are the biggest sources of phenolics (7, 9). Since the emerging oxidation-linked diseases can benefit from high intake of fruits and vegetables, high phenolic

antioxidant-containing apples have promise for enhancing human health through cellular protective functions.

There are more than 180 million people worldwide who have diabetes and 90% of these suffer from Non-insulin-dependant Type II diabetes (14). The World Health Organization (WHO) estimates that the number of people with diabetes will double by 2030 (14). The WHO states that Type II diabetes can be prevented by physical activity, healthy eating and prevention of obesity (14). In addition WHO figures show that about 80% of people with Type II diabetes are from low and middle income countries (14). Therefore there is a need for low cost and easily available methods for management of diabetes in order to improve the standard of living of most diabetes patients.

A number of previous studies have found phenolics from many common foods like capsicum, cinnamon and fenugreek to have the relevant phytochemical profile of, α-glucosidase inhibition and low α-amylase inhibition coupled with free radical scavenging-linked antioxidant activity, for potential diabetes management (15-17). This offers the potential for good postprandial blood glucose management via α-glucosidase inhibition without the common side-effects associated with high α-amylase inhibition (15-17). In addition these same foods have free radical scavenging-linked antioxidant activity which can help maintain the redox balance in susceptible cells (15-17). Since apple is a common fruit with no known side effects, any α-glucosidase inhibiting effects, if found, are promising for Type II diabetes management.

Based on the above background this study explored the potential duel benefit of apple phenolics for better post-harvest preservation and health benefits. Specifically phenolic-linked changes were investigated during post-harvest storage of four varieties of

apple over three months. The objective was to determine the changes in phenolic content over the storage period and its relevance to post-harvest preservation and concurrently determine any anti-diabetes-linked health benefits that could be attributed to the phenolic content. The health relevant parameters investigated were *in vitro* antioxidant activity and inhibition of α-glucosidase and α-amylase relevant for glycemic index modulation. In addition understanding of how inducible phenolics and related antioxidant activity (both free radical and enzyme-linked) are coupled to the pentose phosphate pathway with positive consequences for post-harvest preservation of apples was investigated. This was done by evaluating antioxidant enzyme activity, proline content, phenolic content, free radical scavenging antioxidant activity and activity of key enzymes over a 3 month post-harvest storage period. The enzymes evaluated were glucose-6-phosphate dehydrogenase (G6PDH), succinate dehydrogenase (SDH), proline dehydrogenase (PDH), guaiacol peroxidase (GPX), superoxide dismutase (SOD) and catalase (CAT).

CHAPTER 2

LITERATURE REVIEW

2.1. Phenolics: Synthesis, Functions and Health Benefits

Phenolic phytochemicals are synthesized by a common biosynthetic pathway which incorporates precursors from both the shikimate and/or the acetate-malonate pathways (1, 18). The first step in the synthesis of phenolic phytochemicals is the commitment of glucose to the pentose phosphate pathway (PPP), converting glucose-6-phosphate irreversibly to ribulose-5-phosphate. This first committed step in the conversion to ribulose-5-phosphate is carried out by glucose-6-phosphate dehydrogenase (G6PDH). The conversion to ribulose-5-phosphate also produces reducing equivalents (NADPH) for cellular anabolic reactions (Fig. 1). PPP also generates erythrose-4-phosphate which along with phosphoenolpyruvate, from glycolysis, is channeled to the shikimate pathway to produce phenylalanine, which is directed through the phenylpropanoid pathway to produce phenolic phytochemicals (1, 6, 19-21); (Fig.2).

These phenolic phytochemicals have been shown to have a wide array of functions in plants. In plants biotic and abiotic stress has been shown to stimulate secondary metabolite synthesis which results in the production of phenolics (22, 23). Ozone exposure has been shown to increase transcript levels of enzymes involved in the phenolic synthesis and lignin pathways (24). Studies have linked increase in thermo-tolerance during hyperthermia to accumulation of heat shock proteins and phenolic metabolites (25). Many phenolics are induced in response to infection, wounding, nutritional stress, cold stress and UV irradiation (22, 23, 26-30). Phenolics can function as effective antioxidants by scavenging singlet oxygen and free radicals via their ability

to donate hydrogens from hydroxyl groups positioned around the aromatic ring (10, 31-34). A recent study of antioxidant activity in apples by Lee *et al.* 2003 (35) found that flavonoids like quercetin, epicatechin and procyanidin B_2 rather than vitamin C contributed significantly to total antioxidant activity (35).

In light of the vast array of cellular protective functions phenolics have in plants it is not surprising therefore that phenolics have diverse medicinal properties for human health applications. For example curcumin from *Curcuma longa* and *Curcuma mannga* and rosmarinic acid from *Rosmarinus officinalis* are used as antioxidants and anti-inflammatory compounds (36-41). Also lithospermic acid from *Lithospermum sp.* is used as antigonadotropic agent, proanthocyanidins from cranberry can be used to treat urinary tract infections and anethole from *Pimpinella anisum* is used as an antifungal agent (42-45).

Phenolic phytochemicals have also been found to have potential in the management of oxidative stress linked chronic diseases like diabetes, cancer and cardiovascular disease (5, 20, 46-48). In a study involving Empire apples found phenolics from the apples to have potential cancer chemopreventive activity due to their combined antioxidant and anti-tumor promoting activities (49). Similarly an epidemiological study by Knekt *et al.* 1997 (7) found that intake of dietary flavanoids showed an inverse association with lung cancer incidence (7). Another epidemiological study which examined the relationship between fruit and vegetable intake and the incidence of different cancers found that for most cancers the increase in risk for people with low fruit intake in the study was about twice as high as people with high intake (50). *In vitro* anti-proliferation studies with HepG2, human liver-cancer cells have shown phenolics from

many common fruits like cranberry, lemon, apples and strawberries to inhibit proliferation (12).

An epidemiological study by Knekt *et al* in 2001 (8) of more than 10,000 Finnish men and women showed intake of foods containing flavanoids was associated with lower risk of Type II diabetes. The study indicated the lower risk was associated with high intake of quercetin. The same study also states the strongest association between high flavonoid intake with lower risk for Type II diabetes was seen when the source of flavonoids was from apples and berries (8). Therefore a diet containing apples has the potential to reduce risk of Type II diabetes. Since apple is a common fruit with no known side effects, any α-glucosidase inhibiting effects, if found, are promising for Type II diabetes management.

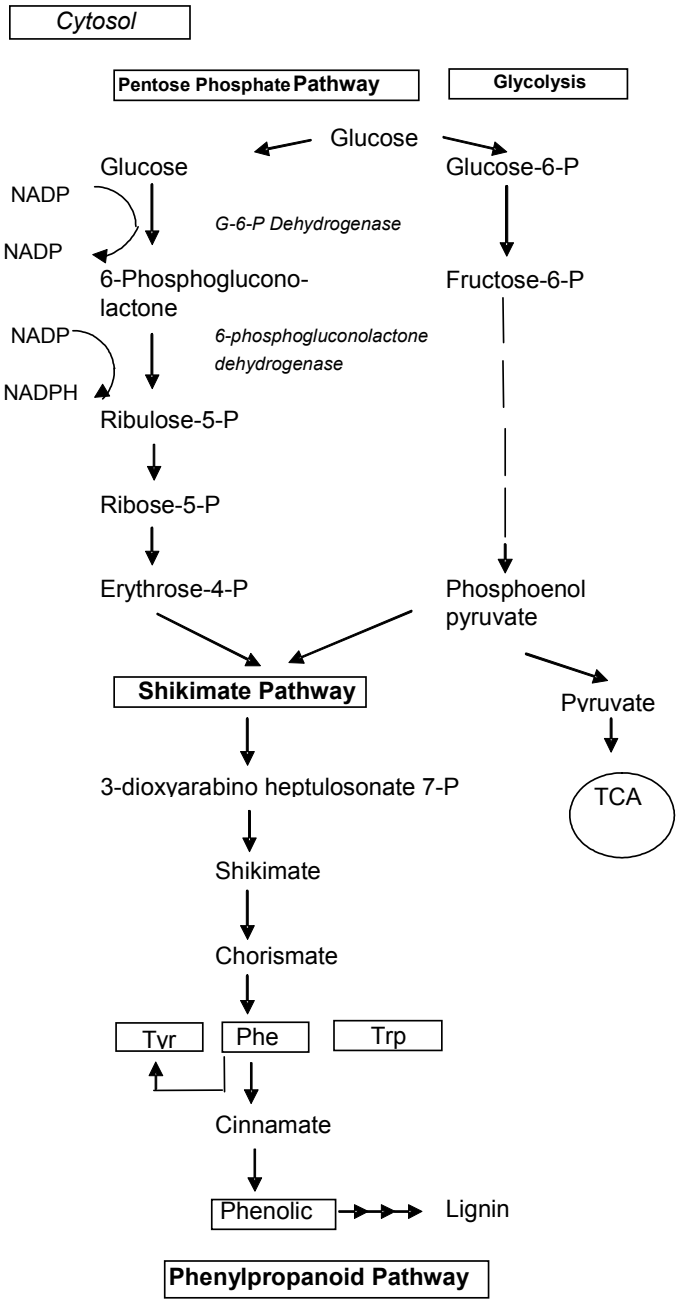


Figure 1. Biosynthesis of phenolic phytochemicals.

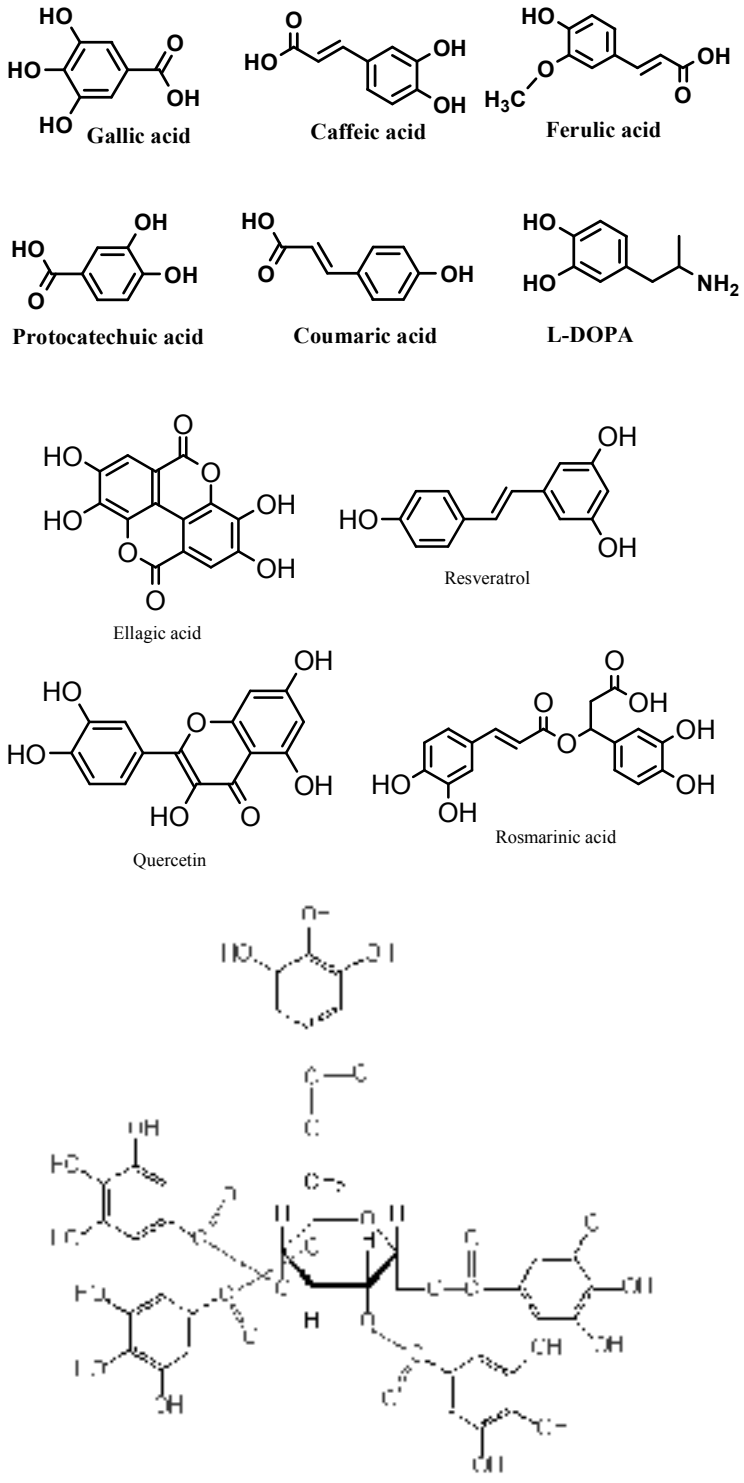


Figure 2. Common simple phenol, biphenyls, flavanoids and tannins in plants.

2.2. Diabetes

Hyperglycemia has been linked to the onset of insulin independent Type II diabetes (51). A good strategy for management of Type II diabetes is inhibition of enzymes that hydrolyze dietary polysaccharides in the gut which can significantly reduce the rise in blood sugar levels after a meal by reducing the absorption of monosaccharides by the entrocytes of the small intestine (52, 53). Enzymes that hydrolyze dietary polysaccharides and modulate gut absorption are pancreatic α-amylase and α-glucosidases (54, 55). However a common side effect of these enzyme inhibitory drugs like acarbose is the excessive inhibition of pancreatic α-amylase which can result in abdominal distention, flatulence and diarrhea (56, 57). These side effects are caused by abnormal fermentation of unhydrolyzed polysaccharides by gut bacteria (56, 58). Therefore in order to reduce these side effects but still manage hyperglycemia a high α-glucosidase inhibition and low α-amylase inhibition is beneficial.

Acute complications in patients suffering from Type II diabetes are generally hyperglycemia-induced metabolic problems and infection. Long term effects of hyperglycemia are microvascular complications like nephropathy, diabetic neuropathy, sexual dysfunction and retinopathy and the macrovascular complication of hypertension (59); (Fig. 3). Recent studies have shown that hyperglycemia triggers generation of free radicals in mesangial cells in the renal glomerulus, neuron cells in peripheral nerves and capillary endothelial cells in the retina (60). The generation of free radicals in all these cells types causes oxidative stress which can be the cause of microvascular complications generally linked with hyperglycemia (60). Most cells are capable of reducing glucose transport inside the cell in hyperglycemic conditions so as to maintain a constant internal

glucose level, however the cells usually damaged by hyperglycemia were found to be inefficient in keeping their internal glucose levels constant (61, 62). Therefore it is not only important to control postprandial hyperglycemia but also keep in check any cellular redox imbalances to prevent diabetic complications.

In addition another study has shown quercetin, which is a phenolic abundant in apples, to be an aldose reductase (alditol: NADP+ oxidoreductase) inhibitor (63). Aldose reductase is the first enzyme of the polyol pathway. Glucose metabolism through the polyol pathway has been linked long-term diabetic complication like cataract, nephropathy, neuropathy and retinopathy (63).

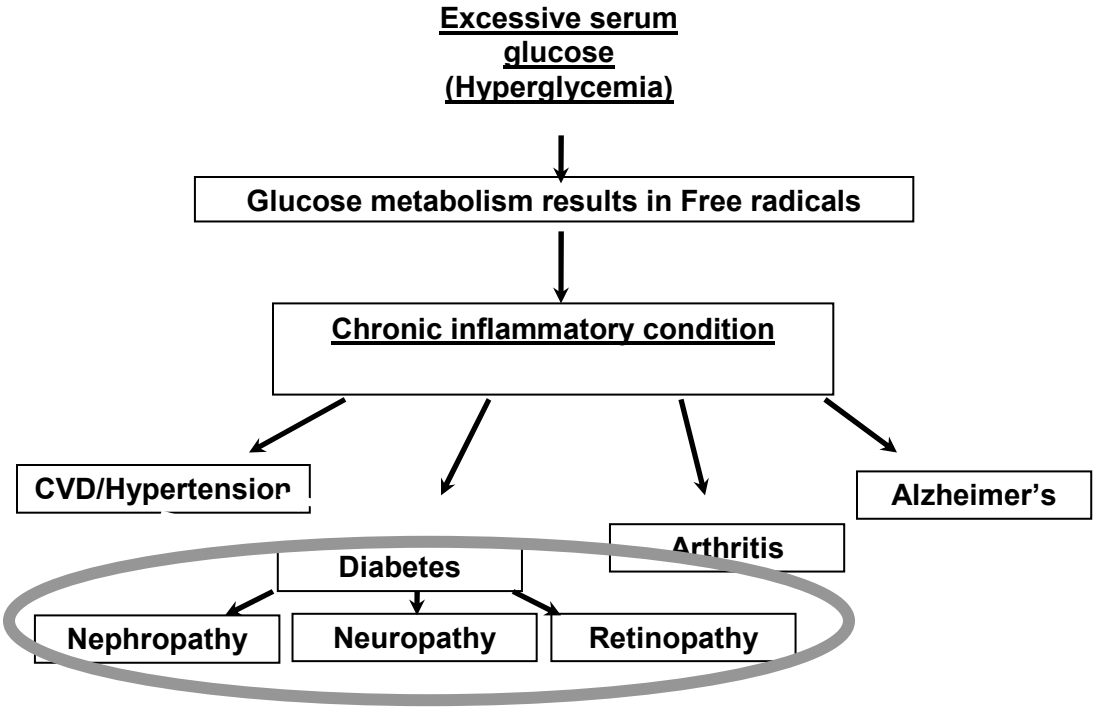


Figure 3. Oxidative stress related hyperglycemia and complications.

2.3. Role of Free Radical Scavenging and Enzyme Antioxidant Activity in Post-Harvest Preservation of Fruits

Reactive oxygen species (ROS) like superoxide radicals ($O_2^{\cdot -}$), hydroxyl radicals ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2) are the products of oxidative dysfunctional biochemical reactions within cells. These ROS when left unchecked cause oxidative damage resulting in lipid peroxidation, protein denaturation and mutagenesis. An increase in ROS is linked to different types of stress such as drought, heat stress, metal toxicity, radiation exposure, pathogens and salinity (64-70); (Fig. 4). Further ROS is also involved in natural and induced senescence and cell death in plants (71-74). For example, studies have indicated increase in hydroperoxides during pepper, banana, pear and tomato ripening during which senescence is induced (75-77).

Plants counter harmful effects of ROS with antioxidants metabolites and enzymes. Antioxidants metabolites include; water soluble compounds like ascorbate, glutathione and flavonoids and lipid soluble compounds like carotenoids and tocopherols. Enzymes linked to antioxidant response include superoxide dismutase (SOD), catalase (CAT), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), ascorbate peroxidase (ASPX) and glutathione reductase (GR). SOD converts $O_2^{\cdot -}$ to H_2O_2 which is then reduced to H_2O by CAT or ASPX depending cellular localization (78-80); (Fig. 5). Both ascorbate and glutathione can also interact directly with and scavenge ROS (79). Oxidized ascorbate is reduced by DHAR, MDHAR and glutathione and oxidized glutathione is reduced by GR. Reduction of both oxidized glutathione and ascorbate by their respective enzymes is NADPH dependant (20). Studies have shown many phenolic compounds especially flavonoids e.g., quercetin, rutin and

catechin have free radical scavenging antioxidant activity (49, 81). In addition, studies in fava beans and peas have shown that even exogenous phenolics can stimulate antioxidant enzyme activity (6, 82).

Therefore, since ROS is involved in plant development, including fruit ripening and senescence, antioxidant response coupling phenolic synthesis and antioxidant enzyme response may be recruited to counter ROS and senescence (70, 80, 83-85). Previous studies with muskmelon fruits and sunflower seeds indicated that delayed senescence in specific tissue types correlated to high antioxidant enzyme response (86, 87). In order to couple cellular antioxidants like ascorbate, glutathione and phenolic phytochemicals with antioxidant enzymes for effective antioxidant response cellular reducing equivalents such as $FADH_2$ and NADPH are required. Studies have found that reduced reductant levels increased the rate of senescence in a number of herbaceous species (73, 88).

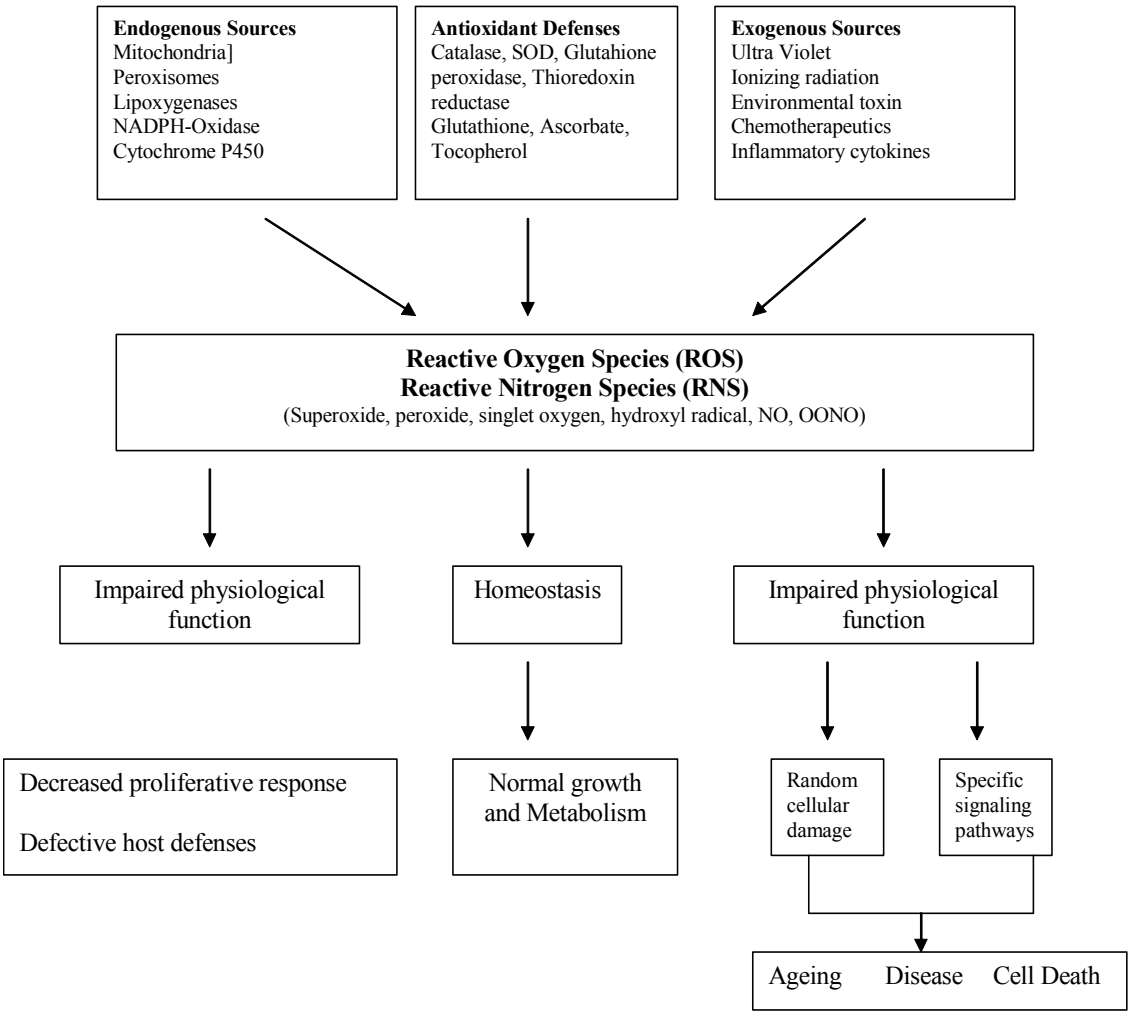


Figure 4. Reactive oxygen species and cellular homeostasis.

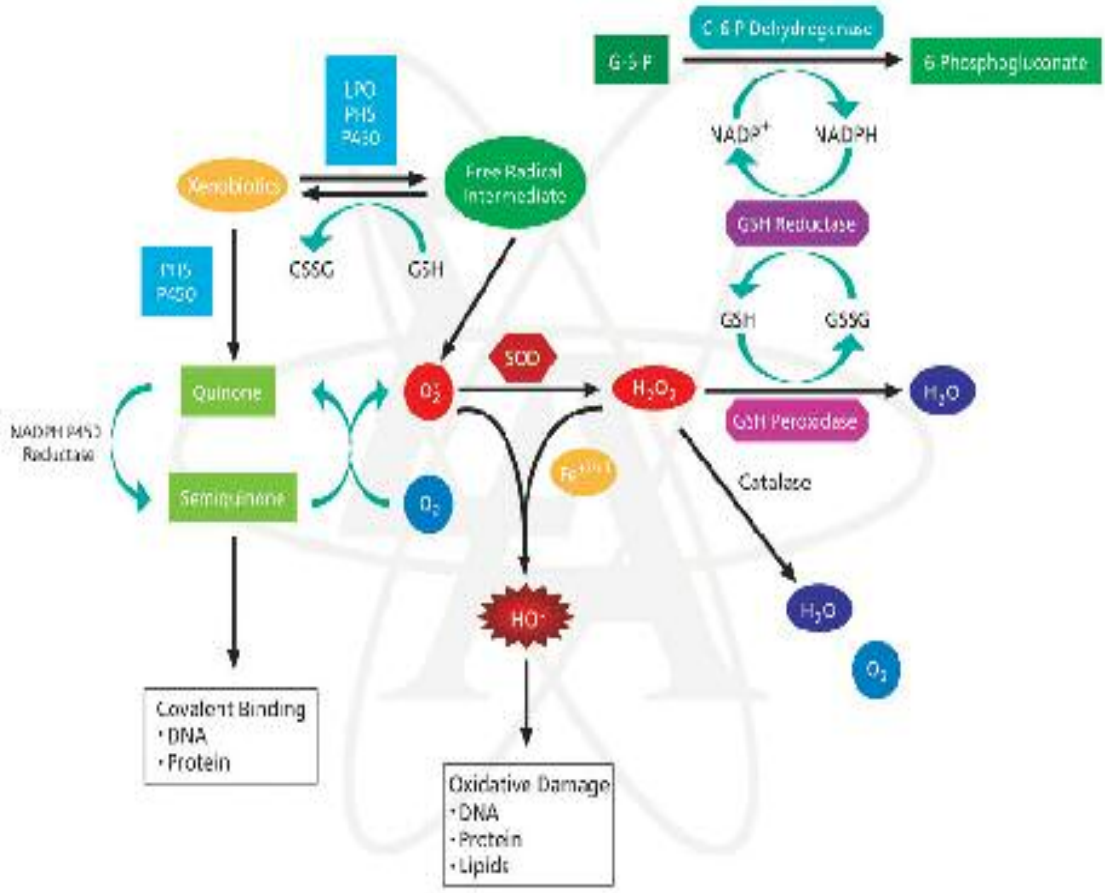


Figure 5. Biological mechanisms of ROS formation and detoxification reactions (From Sigma-Aldrich website).

2.4. Proline Linked Pentose Phosphate Pathway for Effective Antioxidant Activity and Phenolic Synthesis Enhancing Post-Harvest Preservation

Another commonly seen stress response in plants is an increase in the synthesis of proline. Studies have shown proline biosynthesis to be stimulated in cases of water stress and salinity stress (89-93). Proline synthesis and accumulation is also seen during senescence, freezing tolerance, water stress, salt stress and dehydration in plant cells (94-99). It has been suggested that proline protects membranes in times of stress like those mentioned above (100, 101).

An alternative model for coupling proline synthesis with the PPP has been proposed where proline biosynthesis in response to stress can manage energy and reductant needs of anabolic pathways (20). This active metabolic role of proline could have implications for plant senescence where proline can act as an antioxidant or stimulate phenolic-linked antioxidant response (20, 97, 102). Proline is synthesized via the reduction of glutamate to Δ^1-pyrroline-5-carboxylate (P5C) which is further reduced to proline, with both reactions using NADPH as a reductant. (20, 103, 104). Since the reduction of P5C in the cytosol requires NADPH an increase in the proline synthesis would result in a reduction in the NADPH/$NADP^+$ ratio which has been shown to activate G6PDH (105, 106). G6PDH catalyzes the first rate limiting step in the PPP, therefore it is possible that during the post-harvest storage period different stress factors induce proline synthesis which in turn stimulates the PPP (20, 107).

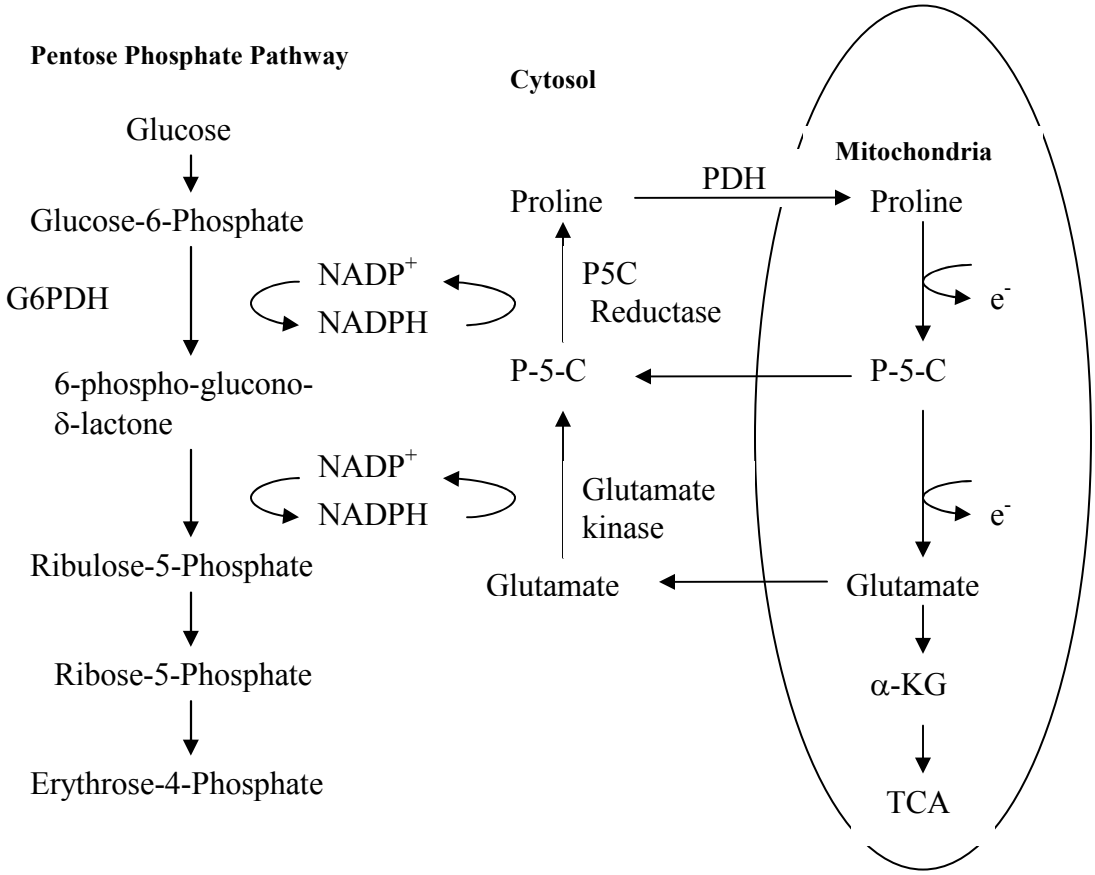


Figure 6. Proline synthesis coupled to the pentose phosphate pathway.
G6PDH=Glucose-6-phosphate dehydrogenase, PDH=Proline dehydrogenase, P-5-C= Δ^1-pyrroline-5-carboxylate, α-KG = α-ketoglutarate, TCA = tricarboxylic acid cycle

This stimulation of the PPP would result in more essential reducing equivalents in the form of NADPH for efficient antioxidant enzyme response. The PPP stimulation would also make more sugar phosphate precursors which along with the NADPH produced can support pathways for the synthesis of antioxidants, phenolic phytochemicals and other protective compounds (20, 107). The proline synthesized can function as an alternative reductant (in place of NADH) in mitochondrial oxidative phosphorylation to generate ATP (20, 104, 107) where proline dehydrogenase (PDH) catalyzes the first reaction of proline oxidation to P5C and linking the electron transport

chain (ETC) in the mitochondria with oxygen as the terminal electron acceptor. P5C is then hydrolyzed nonenzymatically and oxidized to glutamate by the NAD-dependent P5C dehydrogenase. Following oxidative deamination by glutamate dehydrogenase it flows into the TCA cycle through α-ketoglutarate to generate NADH for oxidative phosphorylation or recycled back into the cytosol (20, 93, 108) (Fig. 6, 7).

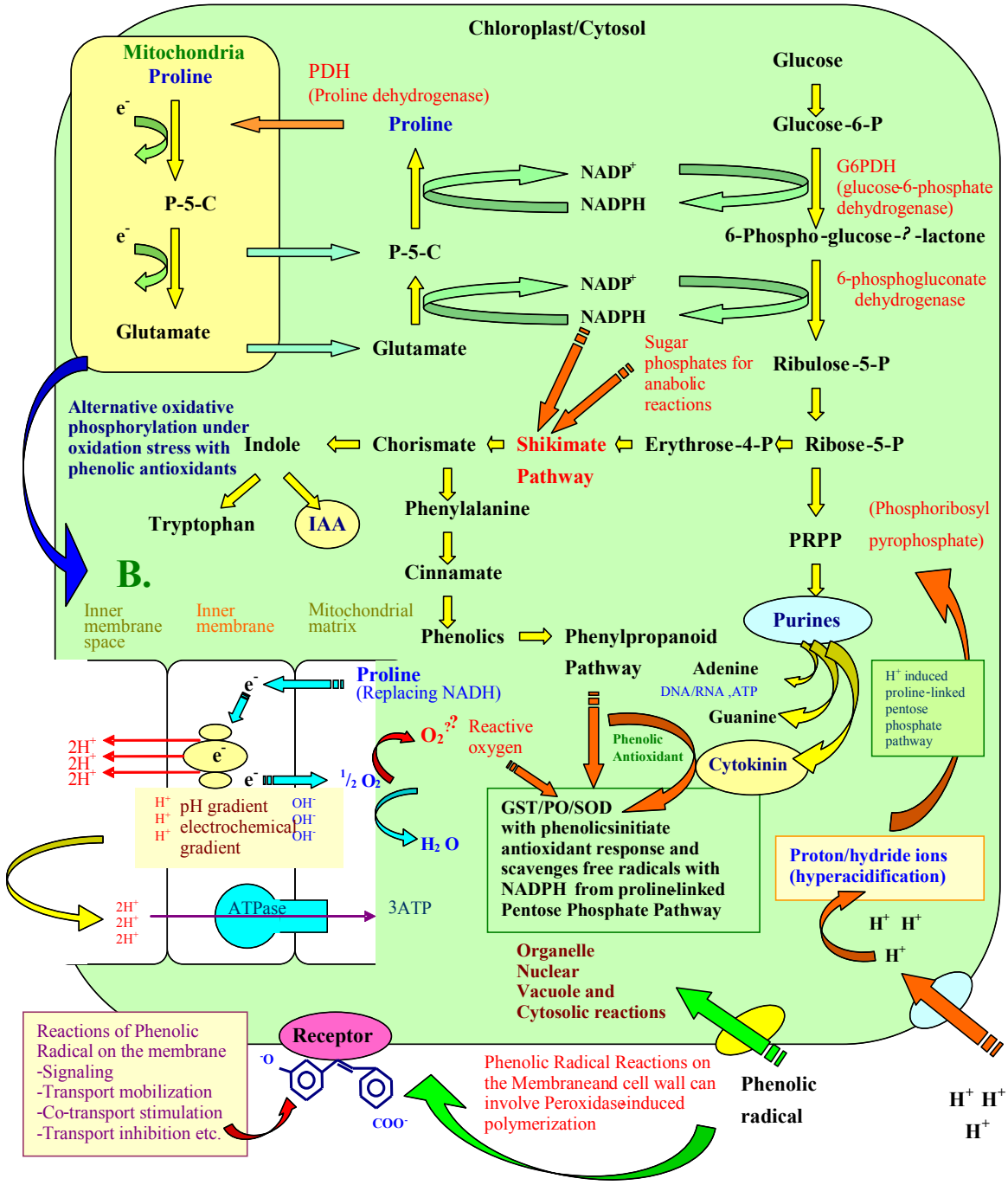


Figure 7. Proline-linked pentose phosphate pathway for phenolic synthesis and efficient antioxidant response.

CHAPTER 3

OBJECTIVES

1) To investigate the role of phenolic-linked antioxidant response in post-harvest preservation of apples.
2) To investigate the health benefits of phenolic-linked free radical scavenging activity of apple extracts for potential management of glycemic index related to Type II diabetes.

CHAPTER 4

APPLE POST-HARVEST PRESERVATION LINKED TO PHENOLICS AND SOD ACTIVITY

4.1. Abstract

The biochemical factors affecting post-harvest preservation in apples indicated that well-preserved varieties of apples had increased superoxide dismutase (SOD) activity initially and the activity declined during later storage as apples deteriorated. The SOD link to better preservation correlated with higher phenolic content and free-radical scavenging linked antioxidant activity. Well-preserved varieties were able to maintain a more stable pentose phosphate pathway (PPP) (measured by the activity of glucose-6-phosphate dehydrogenase, G6PDH) throughout the storage period. Proline content increased in all varieties with an increase in proline dehydrogenase (PDH) activity in the initial period indicating proline catabolism supporting potential ATP synthesis. During later storage succinate dehydrogenase (SDH) activity increased while PDH activity declined indicating a shift to tricarboxylic acid cycle and likely NADH generation for ATP synthesis. This shift coupled with the declining SOD activity coincides with rapid deterioration. The guaiacol peroxidase activity (GPX) activity generally declined in late stages indicating post-harvest deterioration.

Key words:

Apple post-harvest preservation, phenolic, antioxidant activity, superoxide dismutase, glucose-6-phosphate dehydrogenase, succinate dehydrogenase, proline dehydrogenase, guaiacol peroxidase.

4.2. Introduction

Phenolic enriched fruits and vegetables are important for both post-harvest preservation and human health benefits linked to the phenolic-associated antioxidant activity (109). A previous investigation with different varieties of apples indicated a positive link between phenolic content and better post-harvest preservation over 3 months. In the same study phenolic levels directly correlated with free radical scavenging antioxidant activity. Phenolic phytochemicals are secondary metabolites synthesized by plants, which constitute an important part of the diet in both humans and animals with potential health benefits (1-3). Phenolic phytochemicals are synthesized by a common biosynthetic pathway which incorporates precursors from both the shikimate and/or the acetate-malonate pathways (1, 18). The frist step in the synthesis of phenolic phytochemicals is the commitment of glucose to the pentose phosphate pathway (PPP), conveting glucose-6-phosphate irreversibly to ribulose-5-phosphate. This two step process also produces reducing equivalents (NADPH) for cellular anabolic reactions. PPP also generates erythrose-4-phosphate which is channeled to the shikimate pathways to produce phenylalanine, which is directed through the Phenylpropanoid pathway to produce phenolic phytochemicals (1, 6, 19-21).

Reactive oxygen species (ROS) like superoxide radicals ($O_2^{\cdot -}$), hydroxyl radicals ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2) are the products of oxidative dysfunctional biochemical reactions within cells. These ROS when left unchecked cause oxidative damage resulting in lipid peroxidation, protein denaturation and mutagenesis. An increase in ROS is linked to different types of stress such as drought, heat stress, metal toxicity, radiation exposure, pathogens and salinity (64-70). Further ROS is also involved

in natural and induced senescence and cell death in plants (71-74). For example, studies have indicated increase in hydroperoxides during pepper, banana, pear and tomato ripening during which senescence is induced (75-77).

Plants counter harmful effects of ROS with antioxidant metabolites and enzymes. Studies have showed many phenolic compounds especially flavonoids e.g., quercetin, rutin and catechin have free radical scavenging antioxidant activity to counter ROS (49, 81). In addition, studies in fava beans and peas have shown that exogenous phenolics can stimulate antioxidant enzyme activity to potentially counter ROS (6, 82).

Therefore, since ROS is involved in plant development including fruit ripening and senescence antioxidant response coupling phenolics and antioxidant enzyme response may be recruited to counter ROS and senescence (70, 80, 83-85). Previous studies with muskmelon fruits and sunflower seeds indicated that delayed senescence in specific tissue types correlated to high antioxidant enzyme response (86, 87). In order to couple cellular antioxidants like ascorbate, glutathione and phenolic phytochemicals with antioxidant enzymes for effective antioxidant response cellular reducing equivalents such as $FADH_2$ and NADPH are required. Studies have found that reduced reductant levels increased the rate of senescence in a number of herbaceous species (73, 88). NADPH is regenerated by the PPP along with while synthesizing sugar phosphates, supplying precursors for phenolic biosynthesis. Therefore the enzymes of PPP play an important role in preservation of plant tissue.

An alternative model for coupling proline synthesis with the PPP has been proposed where proline biosynthesis in response to stress can manage energy and reductant needs of anabolic pathways (20). This active metabolic role of proline could

have implications for plant senescence where proline can act as an antioxidant or stimulate phenolic linked antioxidant response (20, 97, 102). Proline is synthesized via the reduction of glutamate to Δ^1-pyrroline-5-carboxylate (P5C) which is further reduced to proline, with both reactions using NADPH as a reductant. (20, 103, 104). Since the reduction of P5C in the cytosol requires NADPH an increase in the proline synthesis would result in a reduction in the NADPH/NADP+ ratio which has been shown to activate G6PDH (105, 106). G6PDH catalyzes the first rate limiting step in the PPP, therefore it is possible that during the post-harvest storage period different stress factors induce proline synthesis which in turn stimulates the PPP (20, 107). This stimulation of the PPP would result in more essential reductant equivalents in the form of NADPH for efficient antioxidant enzyme response. The PPP stimulation would also make more sugar phosphate precursors which along with the NADPH produced can support pathways for the synthesis of antioxidants, phenolic phytochemicals and other protective compounds (20, 107). The proline synthesized can function as an alternative reductant (in place of NADH) in mitochondrial oxidative phosphorylation to generate ATP (20, 104, 107) where proline dehydrogenase (PDH) catalyzes the first reaction of proline oxidation to P5C and linking the electron transport chain (ETC) in the mitochondria with oxygen as the terminal electron acceptor. P5C is then hydrolyzed nonenzymatically and oxidized to glutamate by the NAD-dependent P5C dehydrogenase. Following oxidative deamination by glutamate dehydrogenase it flows into the TCA cycle through α-ketoglutarate to generate NADH for oxidative phosphorylation (20, 93, 108).

Based on the above rationale the objective of this investigation was to understand how inducible phenolics and related antioxidant activity (both free radical and enzyme-linked) are coupled to proline-linked PPP with positive consequences for post-harvest preservation of apples. This was done by evaluating antioxidant enzyme activity, proline content, phenolic content, free radical scavenging antioxidant activity and activity of key enzymes over a 3 month post-harvest storage period. The enzymes evaluated were glucose-6-phosphate dehydrogenase (G6PDH), succinate dehydrogenase (SDH), proline dehydrogenase (PDH), guaiacol peroxidase (GPX), superoxide dismutase (SOD) and catalase (CAT).

4.3. Materials and Methods

The freshly picked apples were purchased from a local orchard (Clarkdale Orchards Deerfield, MA, US). Four of the popular varieties of apples were selected to be evaluated for this study; Cortland, Macintosh, Empire and Mutsu. These were stored in a cold room (5°C) for 3 months. Unless noted otherwise, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

4.3.1. Phenolic Extraction

Soluble phenolics were extracted from the peel and pulp of each variety separately. Phenolics were extracted using distilled water and 12% ethanol. The water extractions were done with 20 g of peel in 50 ml of water and 100 g of pulp in 50 ml of water. The ethanol extractions were done with 5 g of peel in 15 ml of 12% ethanol and 10 g of pulp in 20 ml of 12% ethanol. Apples were first peeled then weighed and mixed with either distilled water or 12% ethanol. This was then homogenized for 2 min using a blender. Resulting mix was collected and centrifuged for 5 min. Supernatant was collected and stored at -20°C. This was done every month until the 3rd month of storage.

4.3.2 Total Phenolics Assay

The total phenolic content was determined by an assay modified from Shetty *et al*. (110). Briefly, one milliliter of extract was transferred into a test tube and mixed with 1 ml of 95% ethanol and 5 ml of distilled water. To each sample 0.5 ml of 50% (v/v) Folin-Ciocalteu reagent was added and mixed. After 5 min, 1 ml of 5% Na_2CO_3 was added to the reaction mixture and allowed to stand for 60 min. The absorbance was read at 725

nm. The absorbance values were converted to total phenolics and were expressed in micrograms equivalents of gallic acid per grams fresh weight (FW) of the sample. Standard curves were established using various concentrations of gallic acid in 95% ethanol.

4.3.3. Antioxidant Activity by 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) Inhibition Assay

To 3 ml of 60 μM DPPH in ethanol, 250 μl of each extract was added, the decrease in absorbance was monitored at 517 nm until a constant reading was obtained. The readings were compared with the controls, which contained 250 μl of 95% ethanol instead of the extract. The % inhibition was calculated by:

$$\%\ \text{inhibition} = \left(\left[\frac{A_{517}^{Control} - A_{517}^{Extract}}{\left[A_{517}^{Control} \right]} \right] \right) x100$$

4.3.4. Enzyme Extraction

For each variety of apple 2 g of pulp was ground in a mortar and pestle along with 2 ml enzyme extraction buffer. The mortar and pestle was kept cool in an ice bath during the extraction process. The sample was centrifuged at 12000xg for 15 min at 5 ºC and stored on ice. The supernatant was used for further analysis.

4.3.5. Total Protein Assay

Protein content was measured by the method of Bradford assay (111). The dye reagent concentrate (Bio-Rad protein assay kit II, Bio-Rad Laboratory, Hercules, CA) was diluted 1:4 with distilled water. A volume of 5 mL of diluted dye reagent was added to 100 µl cell extract. After vortexing and incubating for 5 min, the absorbance was measured at 595 nm against a 5 mL reagent blank and 100 µl buffers using a UV-VIS Genesys spectrophotometer (Milton Roy, Inc., Rochester, NY).

4.3.6. Superoxide Dismutase (SOD) Assay

A competitive inhibition assay was performed that used xanthine-xanthine oxidase-generated superoxide to reduce nitroblue tetrazolium (NBT) to blue formazan. Spectrophotometric assay of SOD activity was carried out by monitoring the reduction of NBT at 560 nm (112). The reaction mixture contained 13.8 ml of 50 mM potassium phosphate buffer (pH 7.8) containing 1.33mM DETAPAC; 0.5 ml of 2.45 mM NBT; 1.7 ml of 1.8 mM xanthine and 40 IU/ml catalase. To 0.8 ml of reagent mixture 100 µl of phosphate buffer and 100 µl of xanthine oxidase was added. The change in absorbance at 560 nm was measured every 20 sec for 2 min and the concentration of xanthine oxidase was adjusted to obtain a linear curve with a slope of 0.025 absorbance per min. The phosphate buffer was then replaced by the enzyme extract and the change in absorbance was monitored every 20 sec for 2 min. One unit of SOD was defined as the amount of protein that inhibits NBT reduction to 50% of the maximum.

4.3.7. HPLC Analysis of Proline

High Performance Liquid Chromatography (HPLC) was performed using an Agilent 1100 liquid chromatograph equipped with a diode array detector (DAD 1100). The analytical column was a reverse phase Nucleosil C18, 250 mm x 4.6 mm with a packing material of 5 μm particle size. The samples were eluted out in an isocratic manner with a mobile phase consisting of 20 mM potassium phosphate (pH 2.5 by phosphoric acid) at a flow rate of 1 ml/min and detected at 210 nm. L-Proline (Sigma chemicals, St. Louis, MO) dissolved in the 20 mM Potassium phosphate solution was used to calibrate the standard curve. The amount of proline in the sample was reported as μmol of proline per milligram of sample protein.

4.3.8. Proline Dehydrogenase (PDH) Assay

Modified method described by Costilow and Cooper (113) was used to assay the activity of proline dehydrogenase. Briefly, the enzyme reaction mixture contained 100 mM sodium carbonate buffer (pH 10.3), 20 mM L-proline solution and 10 mM NAD. To 1 ml of this reaction mixture, 200 μl of enzyme extract was added. The increase in absorbance was followed at 340 nm for 3 min, at 32^{o}C. The absorbance was noted at zero time and then after 3 min. In the spectrophotometric assay, one unit of enzyme activity is that amount causing an increase in absorbance of 0.01 per min at 340 nm (1.0 cm light path).

4.3.9. Succinate Dehydrogenase (SDH) Assay

Modified method described by Bregman (114) was used to assay the activity of succinate dehydrogenase. The cell suspension was diluted with 2.0 ml of cell extraction buffer. The sample was then assayed at room temperature for succinate dehydrogenase activity. The assay mixture consists of the following: 1.01 ml of 0.4 M potassium phosphate buffer (pH 7.2); 40 µl of 0.15 M sodium succinate (pH 7.0); 40 µl of 0.2 M sodium azide; and 10 µl of 6.0 mg/ml DCPIP. This mixture was used to obtain baseline (zero) of the spectrophotometer reading at 600 nm wavelength. To 1.1 ml of this mixture, 200 µl of the sample was added. The rate of change in absorbance per minute was used to quantify the enzyme in the mixture using the extinction co-efficient of DCPIP ($19.1\ mM^{-1}cm^{-1}$).

4.3.10. Glucose-6-Phosphate Dehydrogenase (G6PDH) Assay

A modified version of the assay described by Deutsch (115) was followed. The enzyme reaction mixture containing 5.88 µmol ß-NADP, 88.5 µmol $MgCl_2$, 53.7 µmol glucose-6-phosphate, and 0.77 mmol malemide was prepared. This mixture was used to obtain baseline (zero) of the spectrophotometer reading at 340 nm wavelength. To 1 ml of this mixture, 50 µl of the sample was added. The rate of change in absorbance per minute was used to quantify the enzyme in the mixture using the extinction co-efficient of NADPH ($6.22\ mM^{-1}cm^{-1}$).

4.3.11. Guaiacol Peroxidase (GPX) Assay

Modified version of assay developed by Laloue *et al.* (116) was used. Briefly, the enzyme reaction mixture contained 0.1 M potassium phosphate buffer (pH 6.8), 50 mM guaiacol solution and 0.2 mM hydrogen peroxide. To 1 mL of this reaction mixture, 50 µL of enzyme extract was added. The absorbance was noted at zero time and then after 5 min. The rate of change in absorbance per minute was used to quantify the enzyme in the mixture using the extinction co-efficient of the oxidized product tetraguaiacol ($26.6\ mM^{-1}cm^{-1}$).

4.3.12. Statistical Analysis

Analysis at every time point was carried out in triplicates. Means, standard errors and standard deviations were calculated using Microsoft Excel 2003.

4.4. Results and Discussion

4.4.1. Phenolic-Linked Antioxidant Activity and Visual Observations

In all varieties, the phenolic content of peel extracts was much higher than pulp extracts in both water and 12% ethanol extraction (109). Similarly in most cases we see much higher free radical scavenging activity in peel extracts than pulp extracts was observed (Tab. 1). In general we see than Empire variety had consistently low phenolic levels during the storage period. Comparatively Macintosh and Cortland had higher phenolic content and Mutsu maintained consistent intermediate phenolic levels throughout the storage period. A clear link between phenolic content and antioxidant activity was observed. When phenolic content increased in antioxidant activity was also higher. For example Macintosh peel water extract at 2 months indicated a strong positive correlation between phenolic content and antioxidant activity (Fig. 8). This link is not surprising as phenolics are known to be free radical scavengers (49, 81). In addition the visual observations indicated that the varieties like Macintosh and Cortland which had high phenolic content also preserved better during the storage period (Fig. 9).

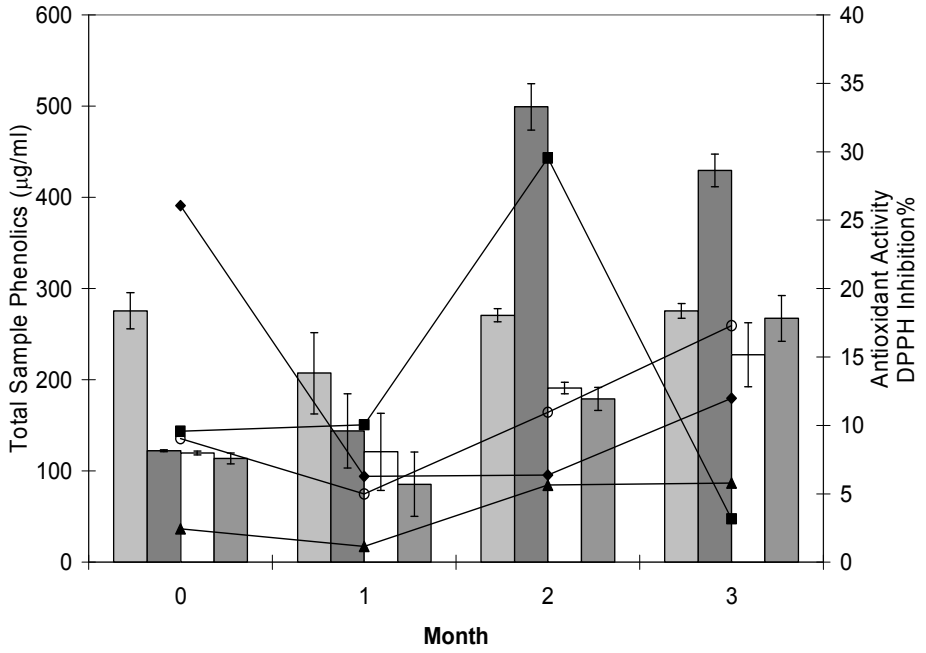
We also observed that Empire variety which had lower phenolic content deteriorated much faster than the other varieties. Empire variety began to deteriorate after 1 month of storage where as other varieties remain firm until 2 months of storage after which they too began to deteriorate. After 3 months of storage, all apples had completely deteriorated. In many fruits, increase in hydroperoxides due to increasing ROS levels has been observed during senescence (75-77). Since the antioxidant activity was directly linked to the phenolic content it is logical to assume varieties with higher phenolic

content preserved better due to their improved free radical scavenging-linked antioxidant response systems.

Table 1. Phenolic content and antioxidant activity of apples over 3 months.

Total Pehnolics Water Extraction	0 Month	1 Month	2 Month	3 Month
Cortland peel	689 μg/g	518 μg/g	677μg/g	689 μg/g
Macintosh peel	305 μg/g	360 μg/g	1248 μg/g	1074 μg/g
Mutsu peel	299 μg/g	303 μg/g	477 μg/g	569 μg/g
Empire peel	285 μg/g	214 μg/g	447 μg/g	668 μg/g
Cortland pulp	49 μg/g	27 μg/g	44 μg/g	61 μg/g
Macintosh pulp	30 μg/g	37 μg/g	53 μg/g	39μg/g
Mutsu pulp	49 μg/g	40 μg/g	12 μg/g	58 μg/g
Empire pulp	33 μg/g	22 μg/g	31 μg/g	38 μg/g
DPPH Inhibition% Water Extraction	0 Month	1 Month	2 Month	3 Month
Cortland peel	26%	6%	6%	12%
Macintosh peel	10%	10%	30%	3%
Mutsu peel	2%	1%	6%	6%
Empire peel	9%	5%	11%	17%
Cortland pulp	7%	5%	5%	6%
Macintosh pulp	7%	3%	5%	6%
Mutsu pulp	6%	6%	6%	5%
Empire pulp	7%	5%	6%	8%
Total Phenolics 12% Ethanol Extraction	0 Month	1 Month	2 Month	3 Month
Cortland peel	554 μg/g	235 μg/g	783 μg/g	739 μg/g
Macintosh peel	692 μg/g	301 μg/g	1039 μg/g	817 μg/g
Mutsu peel	588 μg/g	211 μg/g	622 μg/g	574 μg/g
Empire peel	466 μg/g	242 μg/g	525 μg/g	657 μg/g
Cortland pulp	123 μg/g	133 μg/g	98 μg/g	146 μg/g
Macintosh pulp	60μg/g	103 μg/g	175 μg/g	134 μg/g
Mutsu pulp	55 μg/g	103 μg/g	131 μg/g	108 μg/g
Empire pulp	42 μg/g	66 μg/g	77μg/g	74 μg/g
DPPH Inhibition% 12% Ethanol Extraction	0 Month	1 Month	2 Month	3 Month
Cortland peel	32%	23%	23%	3%
Macintosh peel	44%	45%	38%	4%
Mutsu peel	24%	25%	17%	20%
Empire peel	27%	10%	23%	19%
Cortland pulp	15%	12%	10%	10%
Macintosh pulp	11%	10%	10%	12%
Mutsu pulp	11%	12%	11%	11%
Empire pulp	8%	10%	9%	8%

A)



B)

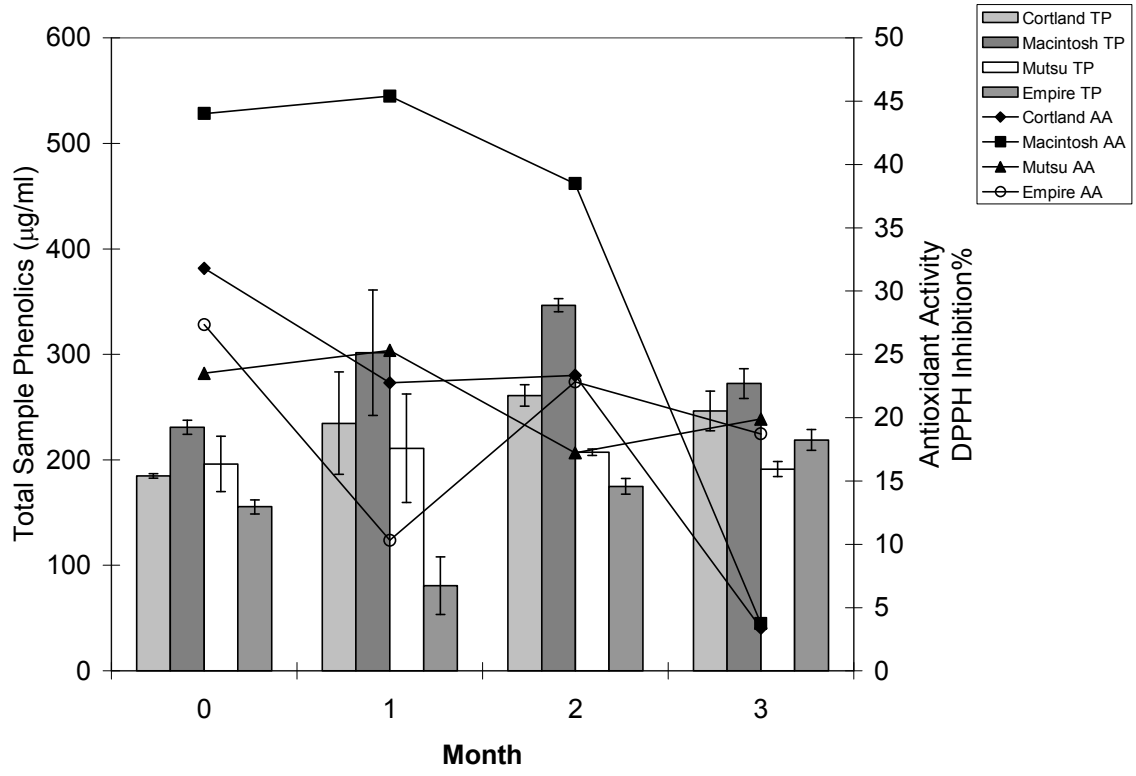
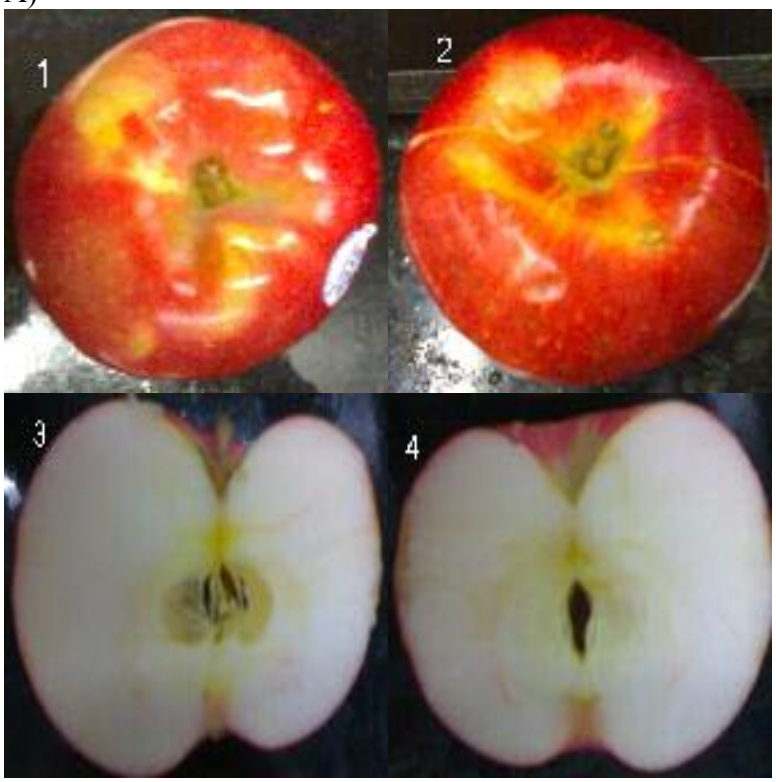


Figure 8. Sample phenolic concentration (µg/ml) and antioxidant activity. A) Peel water extract. B) Peel 12% ethanol extract.

A)



B)

Figure 9. Visual Observation of post-harvest changes in apples over 3 months. A) Macintosh B) Empire: 1) 0 Month 2-4) 3 Month.

4.4.2. Superoxide Dismutase (SOD) Activity

Previous studies in muskmelon and sunflower seeds indicated antioxidant enzyme response delayed senescence (86, 87). In Empire variety which had the worst post-harvest preservation of all varieties in this study deterioration began in the first month of storage itself where as all other varieties were well preserved until the second month of storage. Therefore it not surprising to observe that Empire variety had poor SOD activity compared to other varieties (Fig. 10). Over the storage period all varieties showed an increase in SOD activity after one month of storage followed by reduced activity. Empire variety, however, had a steady almost linear drop in SOD activity. As Phenolic levels have already been shown to boost antioxidant enzyme function in numerous plants (6, 82) the lower SOD activity in Empire variety could be attributed to its lower phenolic content. Conversely we also observe that apples with higher phenolic content like Macintosh had high SOD response. In general there is a positive correlation between phenolic content and SOD activity. However in Macintosh, a well preserved variety in later stages of storage (2-3 months) SOD activity was reduced, while phenolic content is still high. This indicates that combination of both phenolic metabolite and antioxidant enzyme response are essential for better post-harvest preservation.

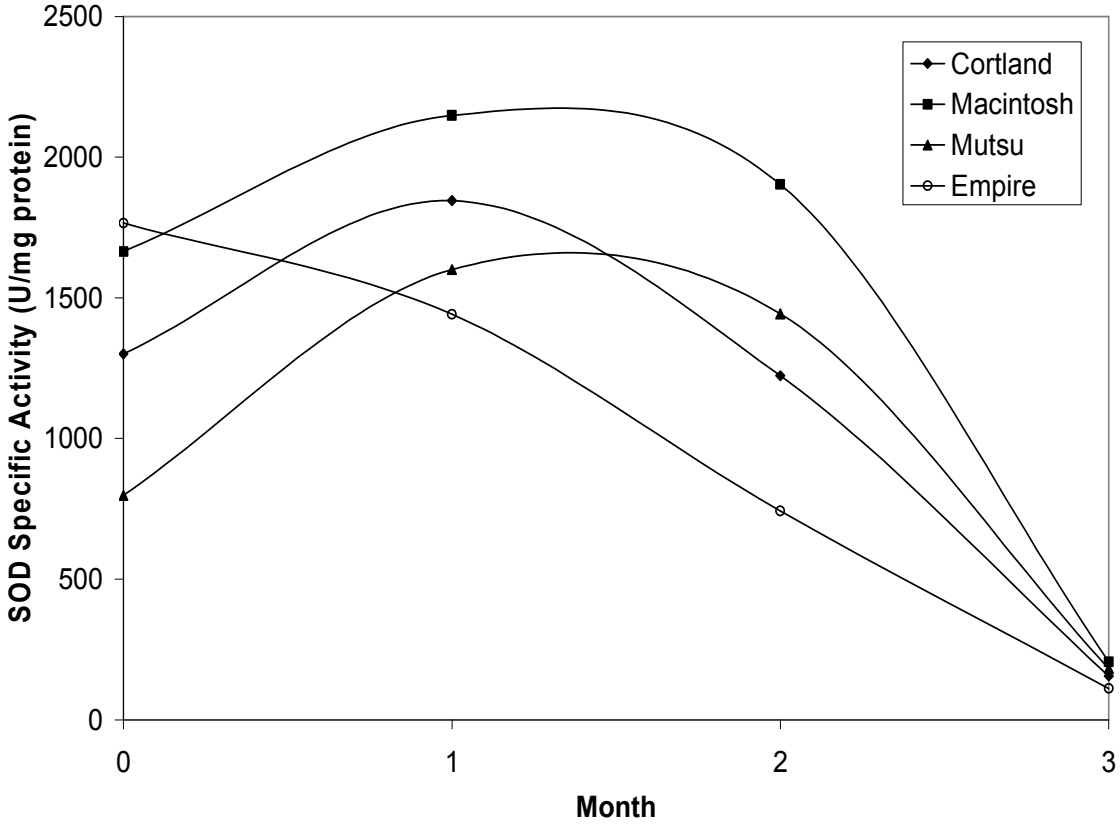


Figure 10. Superoxide dismutase specific activity (U/mg protein).

4.4.3. Proline Linked Pentose Phosphate Pathway (PLPPP) for Energy and Phenolic Synthesis.

During the initial month of storage different stress factors seem to induce a rise in proline biosynthesis (Fig. 11). This stimulation likely maintains lower steady state G6PDH activity in Cortland and Macintosh (Fig. 14). This indicates no new glucose is utilized for anabolic pathways. The increase in proline synthesis also induces PDH activity (Fig. 12). The increase in PDH activity would catalyze more proline catabolism allowing proline to act as a substitute reductant for NADH for mitochondrial oxidative phosphorylation for energy needs. This would allow for synthesis of ATP with less oxidative stress in tissues. However after 1 month of storage proline synthesis and PDH activity start to decline (Fig. 11 and 12) and SDH activity rises in almost all varieties during this period (Fig. 13) indicating a switch to the TCA cycle for ATP synthesis. This switch to the TCA cycle would likely generate more ROS therefore more oxidative stress. This higher ROS potential combined with falling SOD activity in the later stages (Fig. 10) likely leads to the rapid deterioration of apples seen towards the end of our 3 month storage period. During the 3rd month of storage, very low or no enzyme activity in all varieties for all enzymes was observed (Fig. 11-15).

In Empire and Mutsu varieties however a dramatic drop in G6PDH activity in the first month of storage was observed indicating that the PPP is less active and glucose mobilization is drastically reduced for anabolic pathways (Fig. 14). A less active PPP would result in less phenolic synthesis and this can be confirmed by the low phenolic content in both Mutsu and Empire varieties when compared to Macintosh and Cortland varieties where more stable low steady state G6PDH activity was exhibited for a longer

part of the storage period. Therefore, even though Empire and Mutsu have successfully switched to proline catabolism for energy needs they do not maintain an active PPP for anabolic support. This inactive PPP results in lower levels of reductant and phenolics synthesis or remobilization in the system causing poor antioxidant response (free radical and enzyme-linked) leaving these varieties open to oxidative damage via ROS. In addition in Empire variety deterioration begins earlier possibly because of the poor SOD activity which showed a steady fall in activity unlike all other varieties (Fig. 10).

The GPX activity over the storage period showed a general downward trend in almost all cases except for Mutsu variety which showed an initial increase in the first month followed by a decline like all other varieties (Fig. 15). GPX polymerizes synthesizes phenolic phytochemicals to form lignins (116). Lignins function as structural support and are usually synthesized during growth periods. It is possible that here lignin synthesis via GPX is reduced so as to have more free phenolics for antioxidant functions in the tissue to respond to oxidative stress linked post-harvest deterioration.

From the above results phenolic metabolite and enzyme factors affect the post-harvest preservation of apples. However further study is needed to discern the exact interconnection between each of the factors. Insights gleaned can be used to create various treatments that would boost innate systems (proline-linked PPP driven phenolics synthesis) responsible for post-harvest preservation. This would also help elucidate markers such high phenolic content that would allow for selection and breeding of varieties that would preserve better. The indication that phenolic help prolong post-harvest storage is very promising, as many studies have already shown phenolics to have a vast array of health benefits. Phenolics have been shown to reduce the risk of many

chronic oxidative stress linked diseases like Type II diabetes, cardiovascular disease and cancer (7-11). This dual benefit of phenolics would allow not only for selection of better preserving varieties of apples but also varieties with health benefits as well.

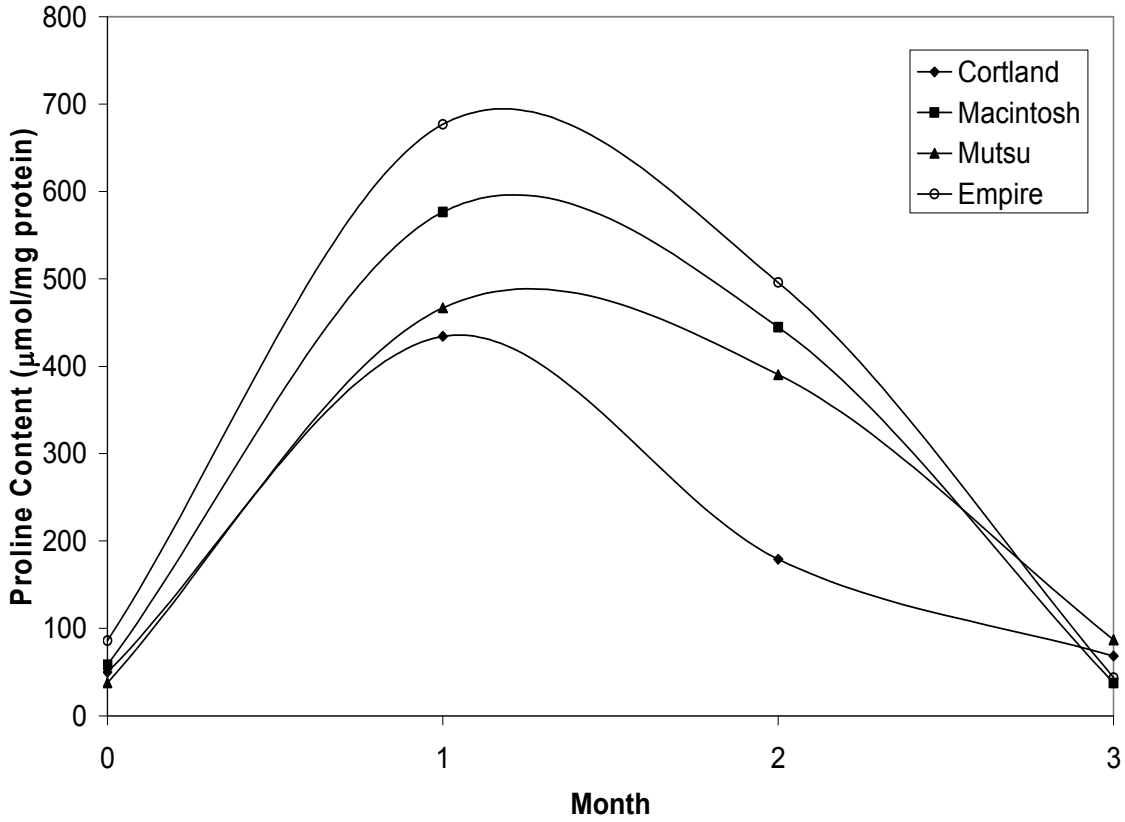


Figure 11. Proline content of apples over 3 months (µmol/mg protein).

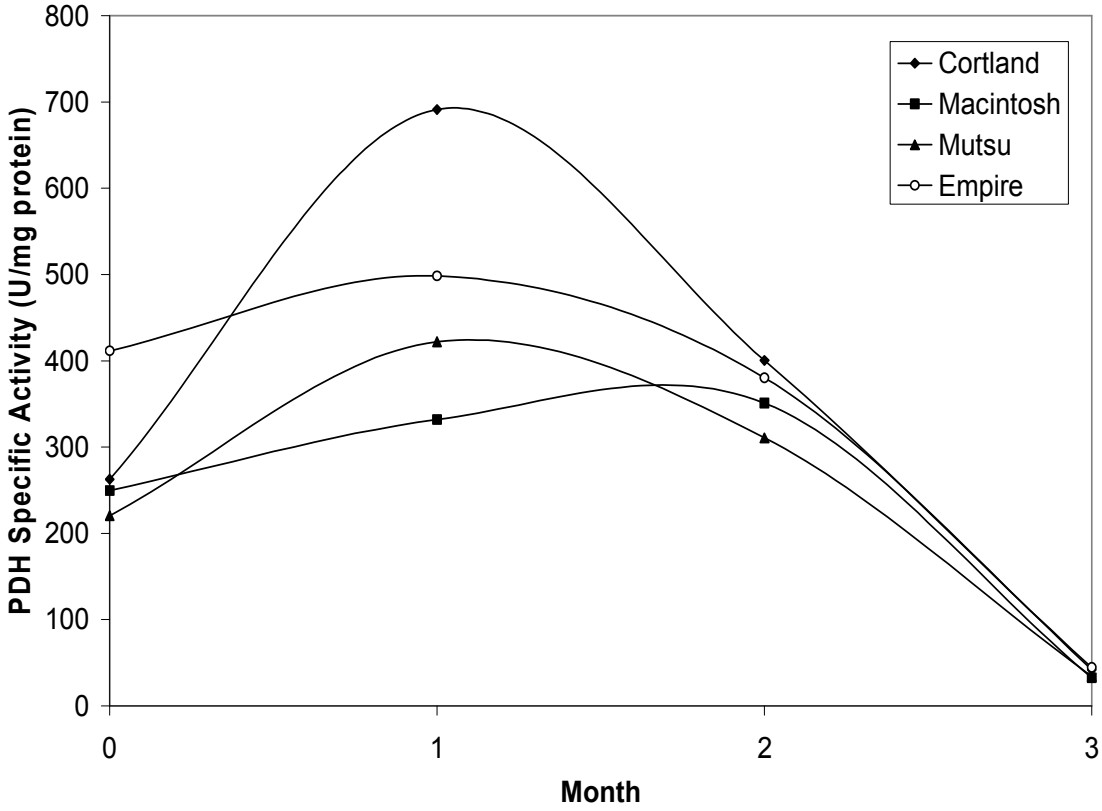


Figure 12. Proline dehydrogenase specific activity in apples over 3 months. (U/mg protein).

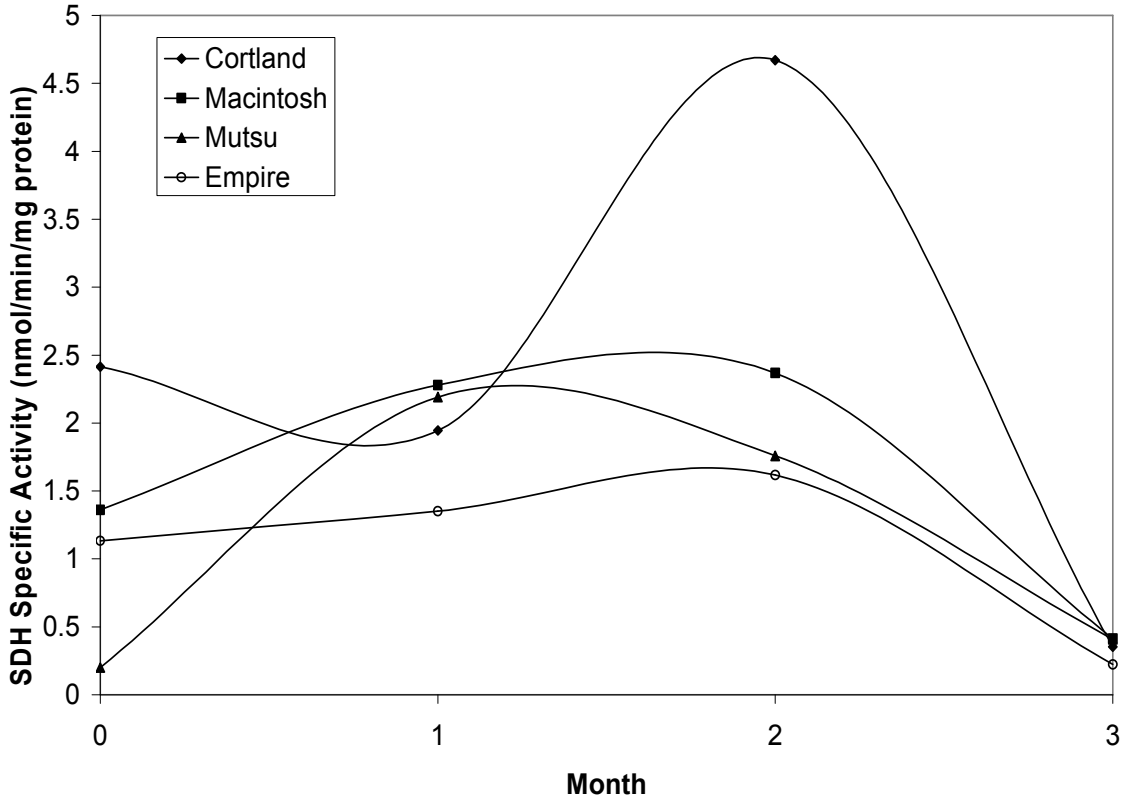


Figure 13. Succinate dehydrogenase activity in apples over 3 months (nmol/mg protein).

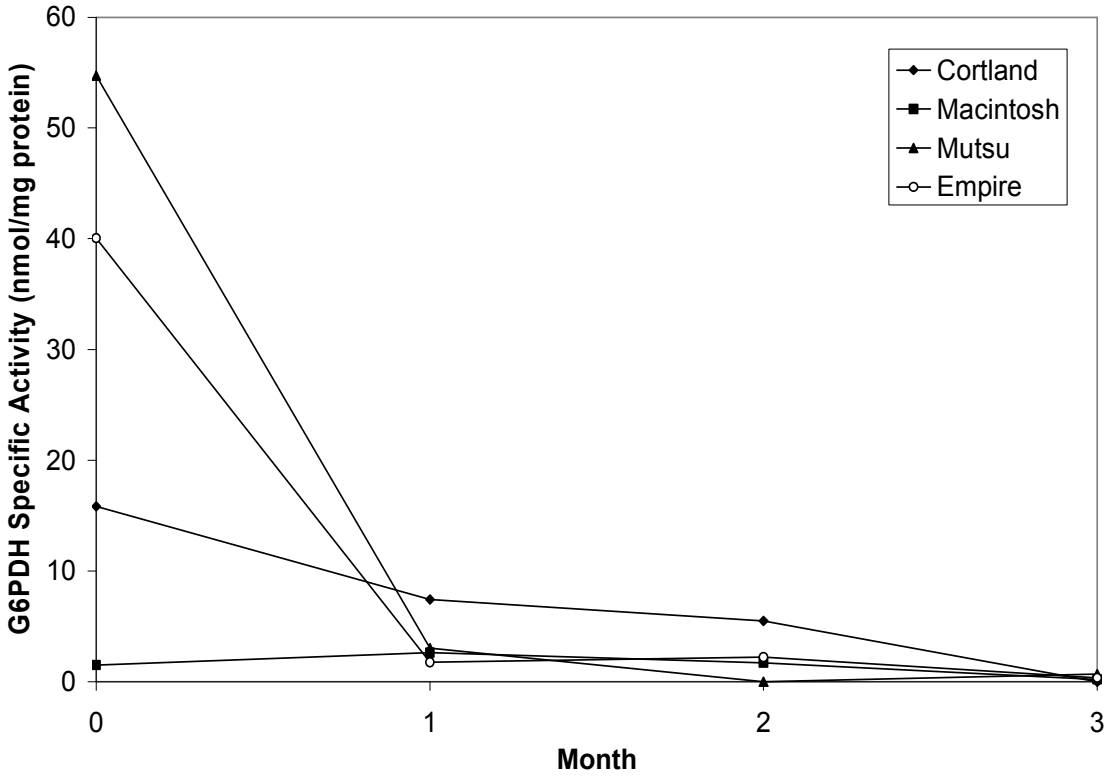


Figure 14. Glucose-6-phosphate dehydrogenase specific activity in apples over 3 months (nmol/mg protein).

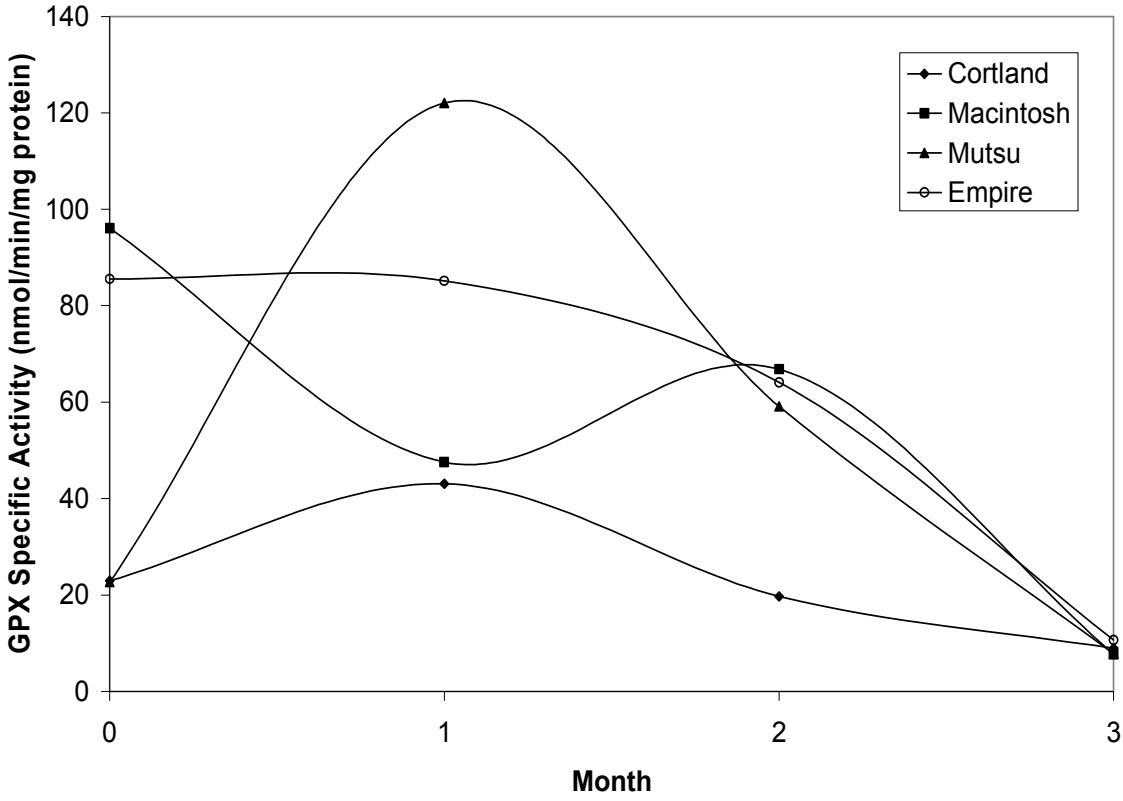


Figure 15. Guaiacol peroxidase specific activity in apples over 3 months (nmol/mg protein).

CHAPTER 5

HEALTH BENEFITS OF APPLE PHENOLICS FROM POST-HARVEST STAGES FOR POTENTIAL DIABETES MANAGEMENT USING *IN VITRO* MODELS

5.1. Abstract

Increasing number of studies have shown that regular intake of fruits and vegetables have clear links to reduced risk of chronic diseases like diabetes and cardiovascular disease. The beneficial effects in many cases have been attributed to the phenolic and antioxidant content of the fruits and vegetables. Apples are a major source of fiber and contain good dietary phenolics with antioxidant function. Previous epidemiological studies have indicated that intake of apples reduces the risk of developing Type II diabetes. Our studies indicate that this reduced risk is potentially due to modulation of postprandial glucose increase by phenolics present in apples via inhibition of α-glucosidase. Phenolic content was evaluated during 3 months of post-harvest storage of four varieties of apples and results indicated positive linkage to enhanced post-harvest preservation and α-glucosidase inhibition. These *in vitro* results along with existing epidemiological studies provide strong biochemical rationale for further animal or human clinical studies.

Key words:

Apple post-harvest preservation, phenolic, antioxidant activity, Type II diabetes, anti-diabetic, α-glucosidase.

5.2. Introduction

Growing evidence has suggested that the intake of fruits and vegetables is linked to reduced risk of chronic diseases like diabetes, cardiovascular disease and cancer (7-11). A recent study found apples to contain the second highest total phenolics content after cranberries, but the highest soluble phenolics (12). Apples also had the second highest free radical scavenging-linked antioxidant activity among the fruits investigated (12). Since the emerging oxidation-linked diseases can benefit from high intake of fruits and vegetables, high phenolic antioxidant-containing apples have the promise for enhancing human health through cellular protective functions. Apples are a popular fruit all over the world and have the potential to contribute as a significant source of phenolic antioxidants. In the United States, 22% of phenolic intake via fruit comes from apples (13). In Finland the main source of dietary phenolics are onions and apples and in the Netherlands tea, onions and apples are the biggest sources of phenolics (7, 9).

Abnormally high postprandial glucose levels or hyperglycemia has been linked to the onset of insulin independent diabetes mellitus (51). A good strategy for management of Type II diabetes is inhibition of enzymes that hydrolyze dietary polysaccharides in the gut which can significantly reduce the rise in blood sugar levels after a meal by reducing the absorption of monosaccharides by the entrocytes of the small intestine (52, 53). Enzymes that hydrolyze dietary polysaccharides and modulate gut absorption are pancreatic α-amylase and α-glucosidases (54, 55). However a common side effect of these enzyme inhibitory drugs like acarbose is the excessive inhibition of pancreatic α-amylase which can result in abdominal distention, flatulence and diarrhea (56, 57). These side effects are caused by abnormal fermentation of unhydrolyzed polysaccharides by gut

bacteria (56, 58). Therefore in order to reduce these side effects but still manage hyperglycemia a high α-glucosidase inhibition and low α-amylase inhibition is beneficial.

Acute complications in patients suffering from Type II diabetes are generally hyperglycemia-induced metabolic problems and infection. Long term effects of hyperglycemia are microvascular complications like nephropathy, diabetic neuropathy, sexual dysfunction and retinopathy and the macrovascular complication of hypertension (59). Recent studies have shown that hyperglycemia triggers generation of free radicals in mesangial cells in the renal glomerulus, neuron cells in peripheral nerves and capillary endothelial cells in the retina (60). The generation of free radicals in all these cells types causes oxidative stress which can be the cause of microvascular complications generally linked with hyperglycemia (60). Most cells are capable of reducing glucose transport inside the cell in hyperglycemic conditions so as to maintain a constant internal glucose level, however the cells usually damaged by hyperglycemia were found to be inefficient in keeping their internal glucose levels constant (61, 62). Therefore it is not only important to control postprandial hyperglycemia but also keep in check any cellular redox imbalances to prevent diabetic complications. Phenolic phytochemicals have been found to have potential in the management of oxidative stress linked chronic diseases and therefore have potential for managing complications of diabetes (5, 20, 46-48, 118).

A growing number of studies from our laboratory have found many common foods to have the correct profile of α-glucosidase inhibition and low α-amylase inhibition and free radical scavenging-linked antioxidant activity (15-17). This offers the potential for good postprandial blood glucose management without the common side-effects

associated with high α-amylase inhibition. In addition these same foods have free radical scavenging-linked antioxidant activity which can help maintain the redox balance in susceptible cells.

An epidemiological study by Knekt *et al* in 2001 (8) of more than 10,000 Finnish men and women showed intake of foods containing flavanoids was associated with lower risk of Type II diabetes. The study indicated the lower risk was associated with high intake of quercetin. The same study also states the strongest association between high flavonoid intake with lower risk for Type II diabetes was seen when the source of flavonoids was from apples and berries (8). Therefore a diet containing apples has the potential to reduce risk of Type II diabetes. Since apple is a common fruit with no known side effects, any α-glucosidase inhibiting effects, if found, are promising for Type II diabetes management.

Based on the above background this study explores the potential phenolic-linked anti-diabetic benefits of apples. Specifically phenolic-linked changes were investigated during post-harvest storage of four varieties of apple over three months (119). The objective of this study was to determine how the changes in phenolic content over the storage period which has relevance to post-harvest preservation (119) also has relevance for enhanced anti-diabetes-linked health benefits that could be attributed to the phenolic content. The health relevant parameters investigated were free radical-linked antioxidant activity and *in vitro* inhibition of enzymes α-glucosidase and α-amylase that are relevant to glycemic index control associated with Type II diabetes management.

5.3. Materials and Methods

The freshly picked apples were purchased from a local orchard (Clarkdale Orchards Deerfield, MA, US). Four of the popular varieties of apples were selected to be tested; Cortland, Macintosh, Empire and Mutsu. These were stored in a cold room (5°C) for 3 months.

5.3.1. Phenolic Extraction

Soluble phenolics were extracted from the peel and pulp of each variety separately. Phenolics were extracted using distilled water and 12% ethanol. The water extractions were done with 20 g of peel in 50 ml of water and 100 g of pulp in 50 ml of water. The ethanol extractions were done with 5 g of peel in 15 ml of 12% ethanol and 10 g of pulp in 20ml of 12% ethanol. Apples were first peeled then cut and weighed. Peel and pulp was then mixed with either distilled water or 12% ethanol. This was then homogenized for 2 min using a blender. Resulting mix was collected and centrifuged for 5 min. Supernatant was collected and stored at -20°C. This was done every month until the 3rd month of storage.

5.3.2. Total Phenolics Assay

The total phenolic content was determined by an assay modified from Shetty *et al*. (109). Briefly, one milliliter of extract was transferred into a test tube and mixed with 1 ml of 95% ethanol and 5 ml of distilled water. To each sample 0.5 ml of 50% (v/v) Folin-Ciocalteu reagent was added and mixed. After 5 min, 1 ml of 5% Na_2CO_3 was

added to the reaction mixture and allowed to stand for 60 min. The absorbance was read at 725 nm. The absorbance values were converted to total phenolics and were expressed in micrograms equivalents of gallic acid per grams fresh weight (FW) of the sample. Standard curves were established using various concentrations of gallic acid in 95% ethanol.

5.3.3. Antioxidant Activity by 1, 1-Diphenyl-2-Picrylhydrazyl Radical (DPPH) Inhibition Assay

To 3 ml of 60 μM DPPH in ethanol, 250 μl of each extract was added, the decrease in absorbance was monitored at 517 nm until a constant reading was obtained. The readings were compared with the controls, which contained 250 μl of 95% ethanol instead of the extract. The % inhibition was calculated by:

$$\%\ \text{inhibition} = \left(\left[\frac{A_{517}^{Control} - A_{517}^{Extract}}{\left[A_{517}^{Control} \right]} \right] \right) x100$$

5.3.4. α–Glucosidase Inhibition Assay

α-Glucosidase (EC 3.2.1.20) was purchased from Sigma Chemical Co. A volume of 50 μl of sample solution and 100 μl of 0.1 M phosphate buffer (pH 6.9) containing α–glucosidase solution (1.0 U/ml) were incubated in 96 well plates at 25^{o}C for 10 min. After pre-incubation, 50 μl of 5 mM p-nitrophenyl-α-D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) was added to each well at timed intervals. The reaction mixtures were incubated at 25^{o}C for 5 min. Before and after incubation, absorbance

readings were recorded at 405 nm by micro-array reader (Thermomax, Molecular device Co., Sunnyvale, CA) and compared to a control which had 50 µl of buffer solution in place of the extract. The α-glucosidase inhibitory activity was expressed as inhibition % and was calculated as follows:

$$\%\ \text{inhibition} = \left(\left[\frac{\Delta A_{405}^{Control} - \Delta A_{405}^{Extract}}{\left[\Delta A_{405}^{Control} \right]} \right] x100 \right)$$

5.3.5. α–Amylase inhibition Assay

Porcine pancreatic α-amylase (EC 3.2.1.1) was purchased from Sigma Chemical Co. A total of 500 µl of extract and 500 µl of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) containing α–amylase solution (0.5 mg/ml) were incubated at 25^{o}C for 10 min. After pre-incubation, 500 µl of a 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) was added to each tube at timed intervals. The reaction mixtures were then incubated at 25^{o}C for 10 min. The reaction was stopped with 1.0 ml of dinitrosalicylic acid color reagent. The test tubes were then incubated in a boiling water bath for 5 min, cooled to room temperature. The reaction mixture was then diluted after adding 10 ml distilled water and absorbance was measured at 540 nm.

$$\%\ \text{inhibition} = \left(\left[\frac{A_{540}^{Control} - A_{540}^{Extract}}{\left[A_{540}^{Control} \right]} \right] x100 \right)$$

5.3.6. Statistical Analysis

Analysis at every time point was carried out in triplicates. Means, standard errors and standard deviations were calculated using Microsoft Excel 2003.

5.4. Results and Discussion

5.4.1 Visual Observations

On visual examination of the post-harvest stored apples were observed for visual changes over a 3 month period (Fig. 16). Empire variety began to spoil much earlier than the other varieties. After the first month itself all the Empire apples had started to become soft and completely deteriorate. All other apple varieties investigated remained firm until two months, after which by the third month they began to deteriorate as well.

5.4.2. Phenolic Content of Apples

In all cases the phenolics content of peel extracts was much higher than that of pulp extracts (Fig. 17, 18) (119). Further 12% ethanol extracts had higher phenolic content than water extracts across all varieties and tissue types except in Macintosh in later stages of storage.

In peel extracts Macintosh had the highest water extractable phenolics (>1000 μg/g Fresh weight, FW) in the later part of the storage period (2^{nd} and 3^{rd} month). Cortland had intermediate content over the entire storage period (>600 μg/g FW). Empire and Mutsu in general had low phenolic content (400-600 μg/g FW) in the early stages (0-2^{nd} month) with an increase towards intermediate content (>600 μg/g FW) in the last month of storage. In the 12% ethanol extracts (peel), Macintosh in general had higher (>800 μg/g

FW) of phenolic content. Cortland was again intermediate (>600 μg/g FW) and Empire and Mutsu on the lower side (<600 μg/g FW).

In the pulp extracts the water extractable phenolics were generally less than 50μg/g FW across all varieties, except for Macintosh Cortland and Mutsu in the later stages of storage. In the 12% extracts (pulp) Macintosh, Mutsu and Cortland in general had over 100 μg/g FW in later stages, where as Empire was consistently lower at less than 100 μg/g FW through out the storage period.

5.4.3. Antioxidant Activity

The peel samples generally had higher free radical scavenging-linked antioxidant activity, except for Mutsu in the water extract (Fig. 19, 20). The peel in 12% ethanol extracts of Macintosh was superior with initial high inhibition of 45% that gradually reduced after the first month with a low final inhibition (<5%). Other varieties had an intermediate inhibition (25% to 30%) that was maintained through most of the storage period with the exception of empire which had a drop to 10% inhibition in the first month but regained close to original inhibition after and Cortland which had a drop in inhibition% on the third month to less than 5%.

In water extracts of peel, Macintosh had an initial low inhibition (10%) that increased to 30% by the second month of storage before dropping down to less than 5%. Cortland had a high initial inhibition (~25%) which dropped substantially in the first month of storage itself (5% to 10%), however inhibition increased over the rest of the storage period but never to initial levels. Empire and Mutsu both had low initial inhibition levels (<10%) which dropped slightly in the first month but then began to rise

for the rest of the storage period, Empire had a intermediate final inhibition (15% to 20%) but Mutsu still had a low inhibition level of 5%.

The pulp extracts, both water and 12% ethanol, generally had low DPPH inhibition that we was maintained steadily over the storage period.

5.4.4. α-Glucosidase and α-Amylase Inhibition

Peel extracts in general had higher α-glucosidase inhibitory activity than pulp (21, 22). In the 12% ethanol extracts of peel, Macintosh had the highest α-glucosidase inhibition (75%-90%) over the three month storage period. Cortland was the next highest (60%-80%) over storage time. Empire and Mutsu had an intermediate initial inhibition of around 60% followed by lower inhibition for rest of the storage period (<50%). The water extracts of peel generally had higher inhibition in the later stages of storage with Macintosh having the highest inhibition (>90%) at two months storage.

The pulp extracts in 12% ethanol had consistent low levels of inhibition (20% - 40%) over the storage period. The pulp water extracts had intermediate levels of inhibition (40% - 60%) but this was maintained over the storage period.

In all samples no detectable α-amylase inhibition was observed.

The phenolic content especially in late storage was highest in Macintosh and this was reflected in better post-harvest storage of this variety based on visual observations (119). Empire which had the lowest phenolic content deteriorated most rapidly with Cortland and Mutsu being intermediate.

It is significant that sample phenolic concentration (μg/ml) was proportional to the α-glucosidase inhibition trend this indicates a clear link between the two (Pearson Correlation Coefficient: 0.875, Fig. 23-25). In case where a dramatic rise or fall in phenolic concentration was observed a similar rise or fall the α-glucosidase inhibition followed. This trend is most evident in Macintosh where the phenolic content and α-glucosidase inhibition of the peel extracts show the most promising health relevant potential. Further evidence of this trend is the fact that, like phenolic content, inhibitory activity in the peel samples is much higher than that of pulp samples across all varieties. A similar relationship is seen between the antioxidant activity and phenolic content.

In this study Macintosh and Cortland appear to be superior varieties as they can preserve better which is linked to their phenolic content which in turn is closely linked to α-glucosidase inhibition. This means well stored apples with high phenolic content have potential for some glycemic index modulation while contributing antioxidants that can positively influence tissues susceptible to glucose-linked oxidative stress. The lack of α-amylase inhibition is also promising because this prevents common side effects that are associated with carbohydrates hydrolyzing enzyme inhibitors but still allows for glycemic modulation by allowing control over polysaccharide breakdown to monosaccharides.

Further advantages of this study is the indication towards mechanism of action where dietary phenolics with α-glucosidase inhibitory activity can counter negative effects of glucose-induced oxidative dysfunction a microvascular complication of Type II diabetes. This occurs via direct glycemic modulation by phenolics via inhibition of starch hydrolyzing enzymes and antioxidant activity which can help prevent oxidative damage in susceptible tissues. In this study these activities, α-glucosidase inhibition and

antioxidant activity; have shown strong ties to the phenolic content in the apples. Though these *in vitro* studies may not indicate clinical benefits but they provide strong biochemical rationale for animal and human clinical studies. These studies along with already existing epidemiological studies provide the biochemical basis for further clinical investigation.

The other interesting observation emerging out of this study is the correlation between high phenolic content apple varieties to extended post-harvest preservation. This also translates into health beneficial consequences in the context of higher antioxidant activity and Type II diabetes linked α-glucosidase inhibition. Therefore strategies to understand phenolic-linked post-harvest preservation and natural treatments to extend this preservation in selected varieties, such as Macintosh and Cortland in this study, can be basis for food ingredient design for health benefits. These strategies can then be extended to prolong post-harvest preservation and enhance phenolic linked health benefits of a wide variety of fruits and vegetables.

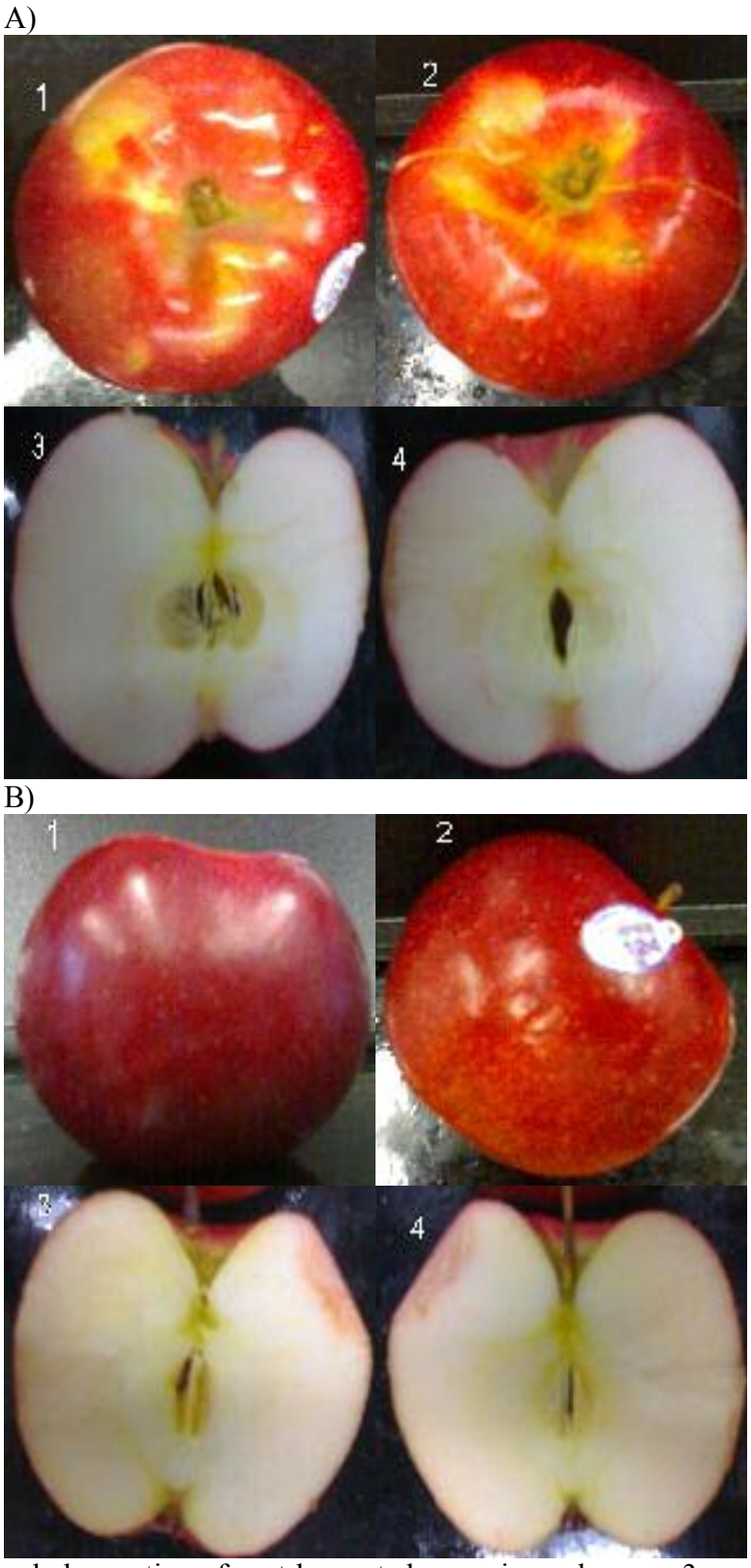
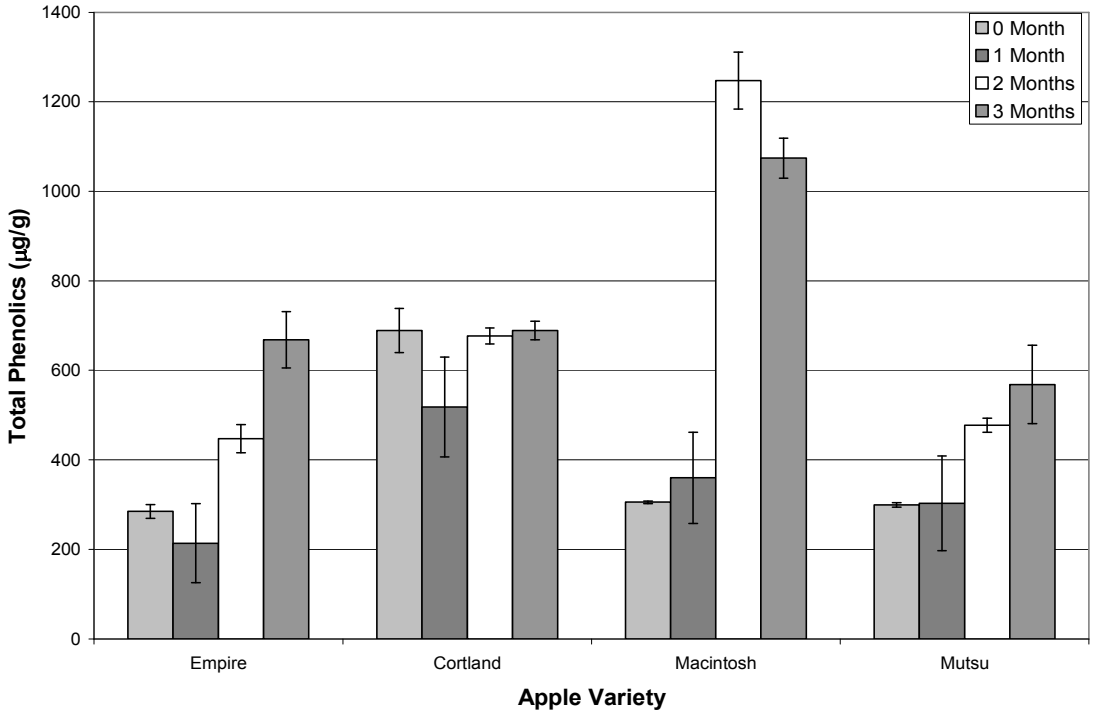


Figure 16. Visual observation of post-harvest changes in apples over 3 months. A) Macintosh B) Empire: 1) 0 Month 2-4) 3 Month.

A)



B)

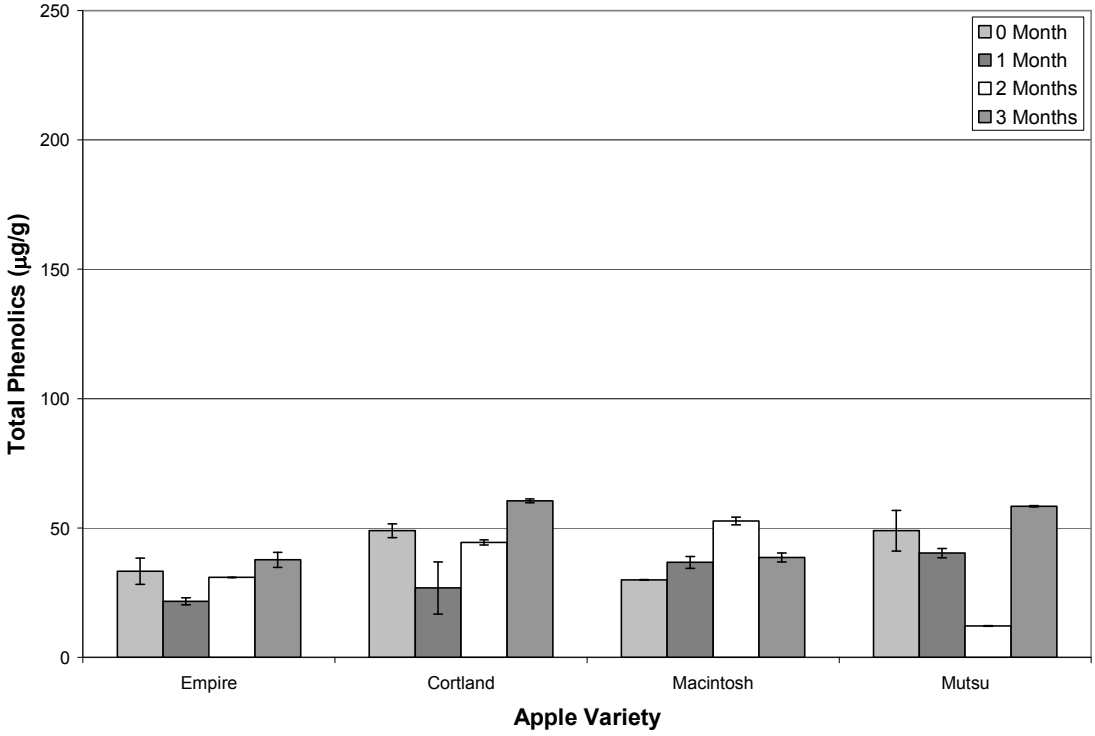
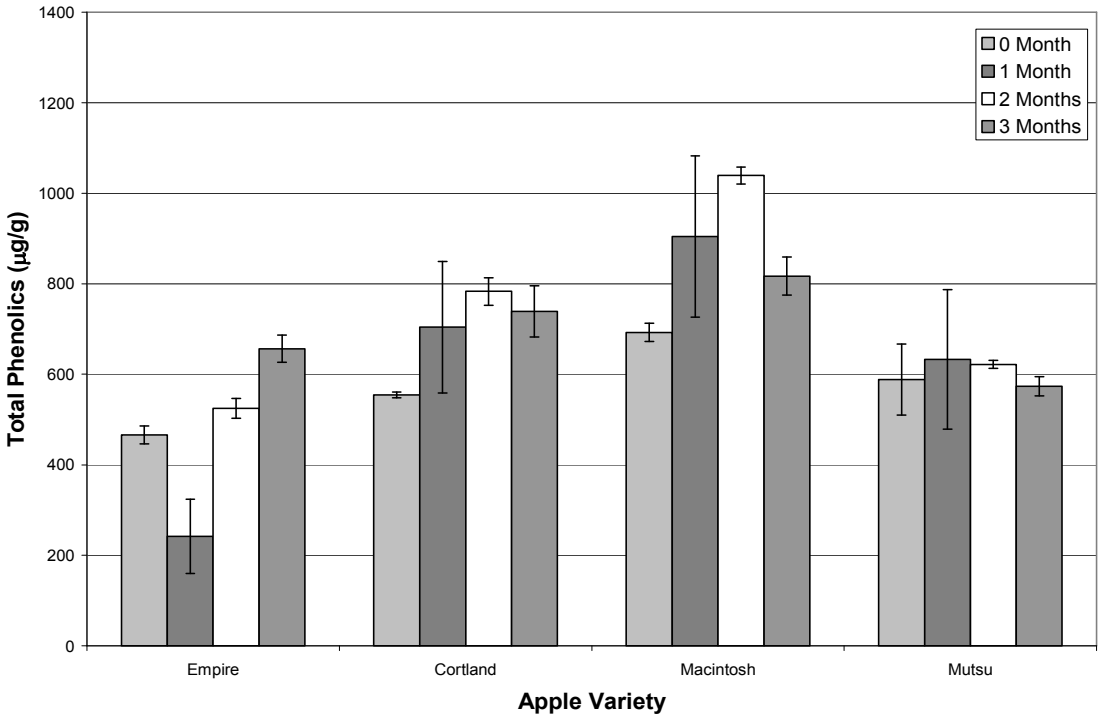


Figure 17. Total phenolic content of apples water extraction (µg/g FW).
A) Peel samples. B) Pulp samples.

A)



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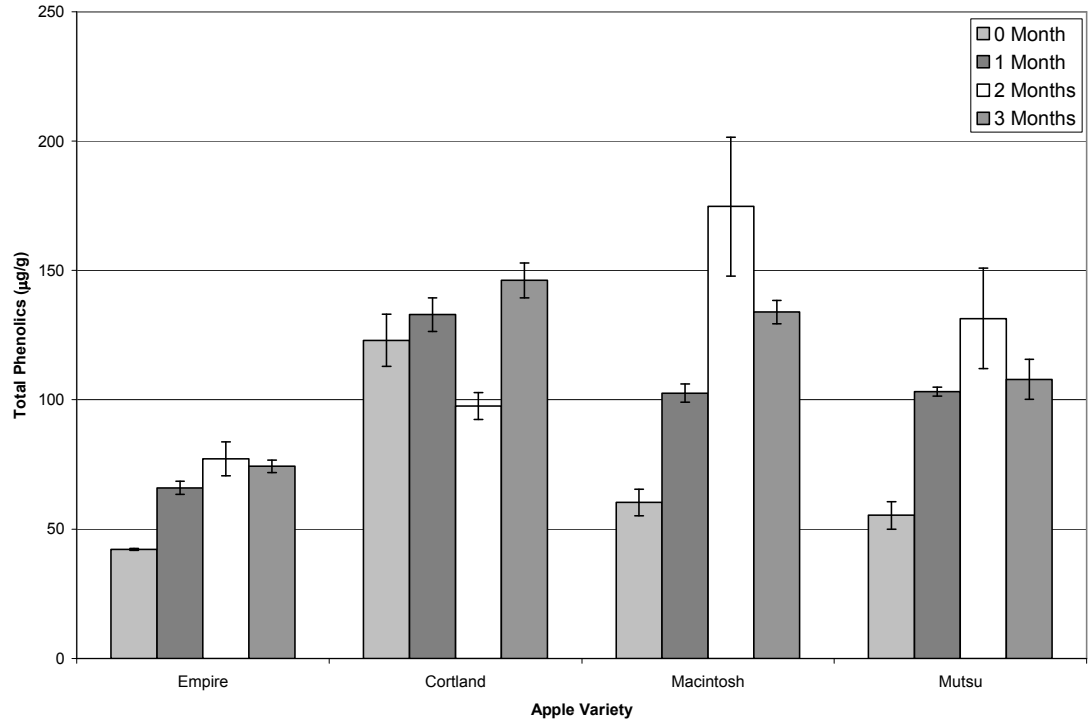
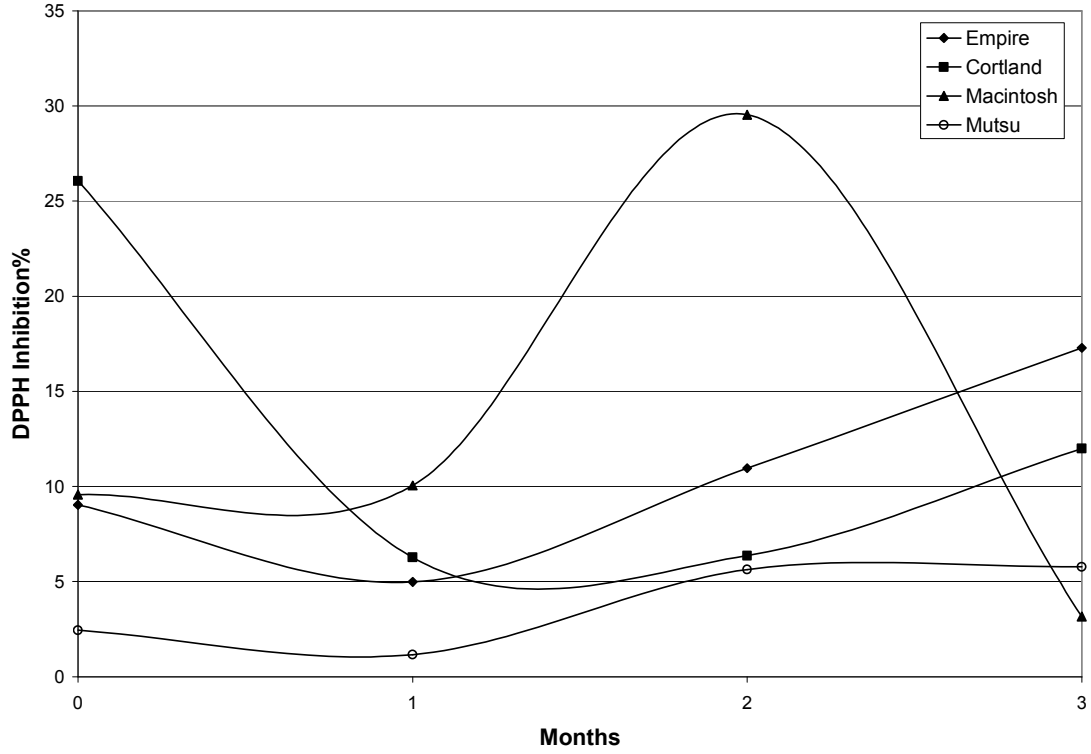


Figure 18. Total phenolic content of apples 12% ethanol extraction (µg/g FW). A) Peel samples. B) Pulp samples.

A)



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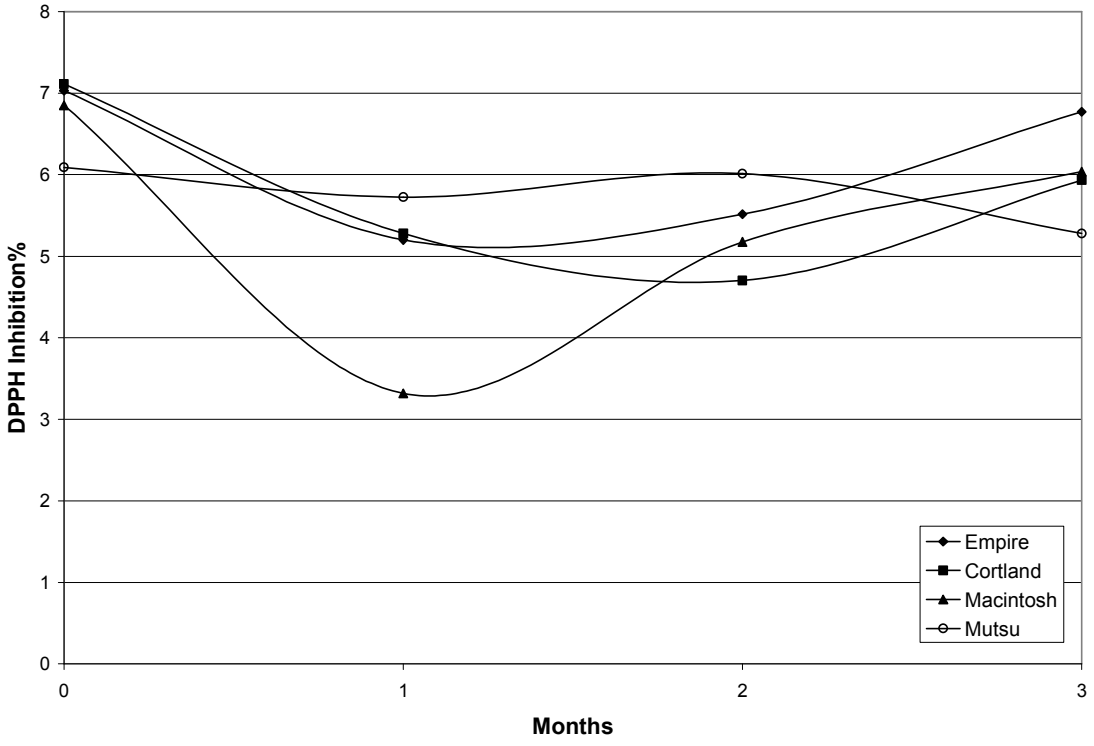
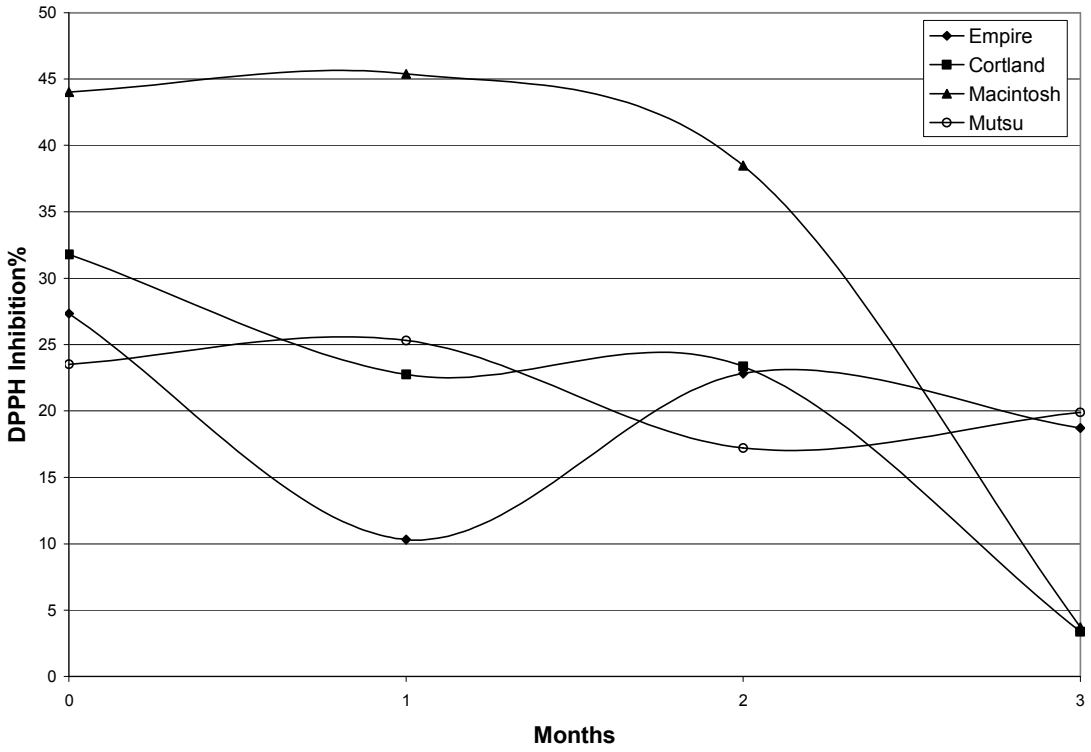


Figure 19. DPPH inhibition. Apple water extracts.
A) Peel samples. B) Pulp samples.

A)



B)

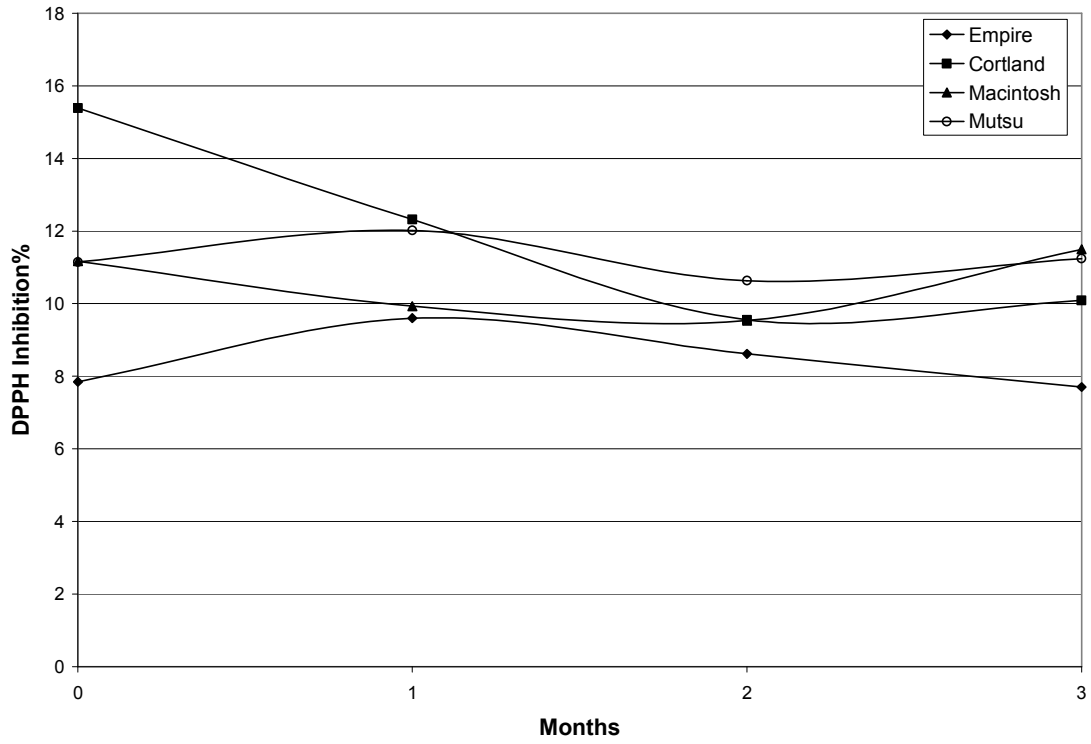
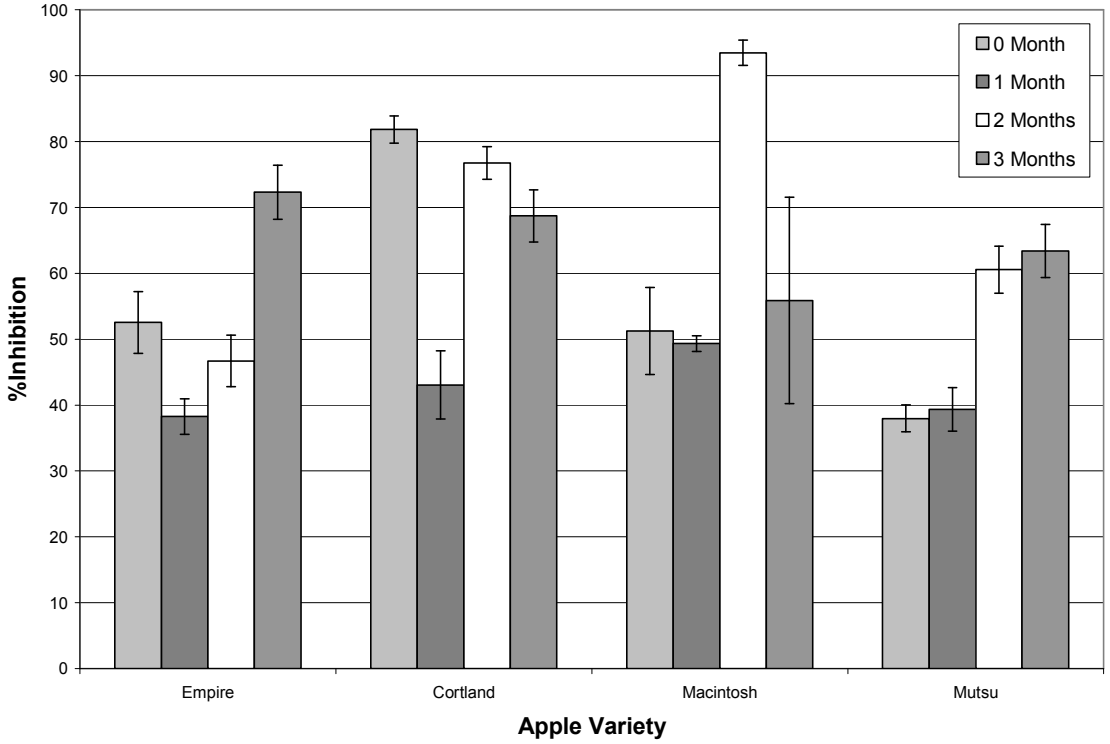


Figure 20. DPPH inhibition. Apple 12% ethanol extracts.
A) Peel samples. B) Pulp samples.

A)



B)

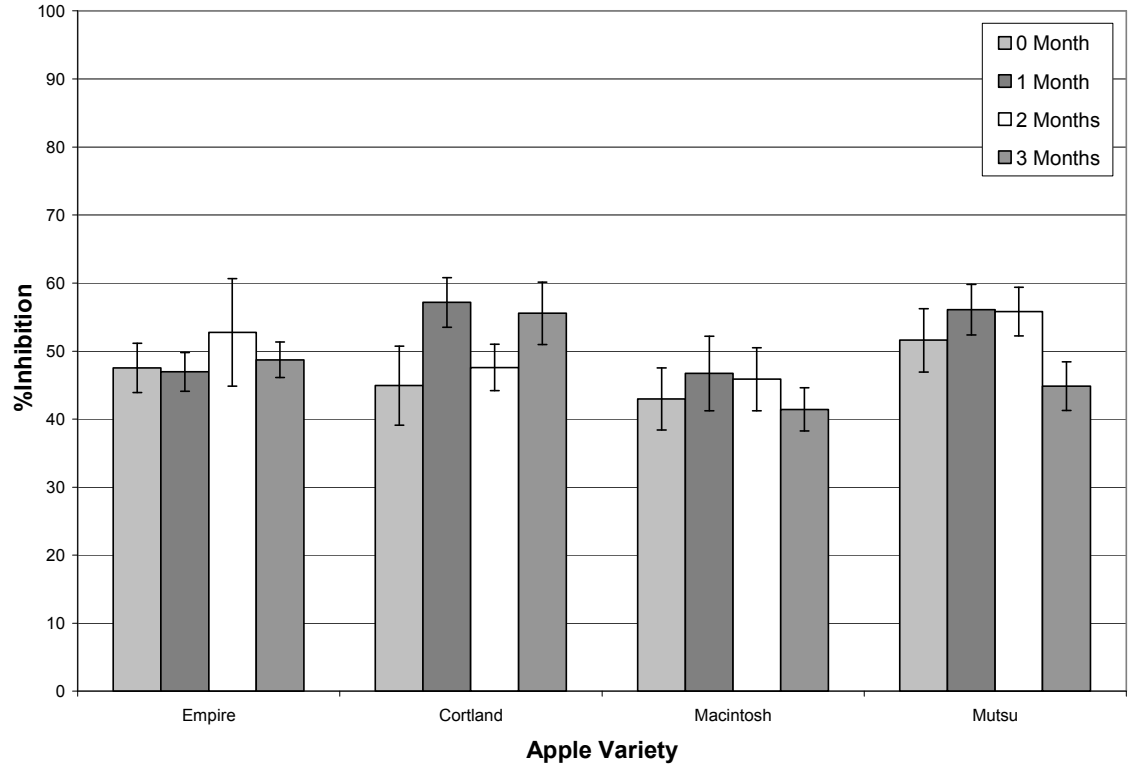
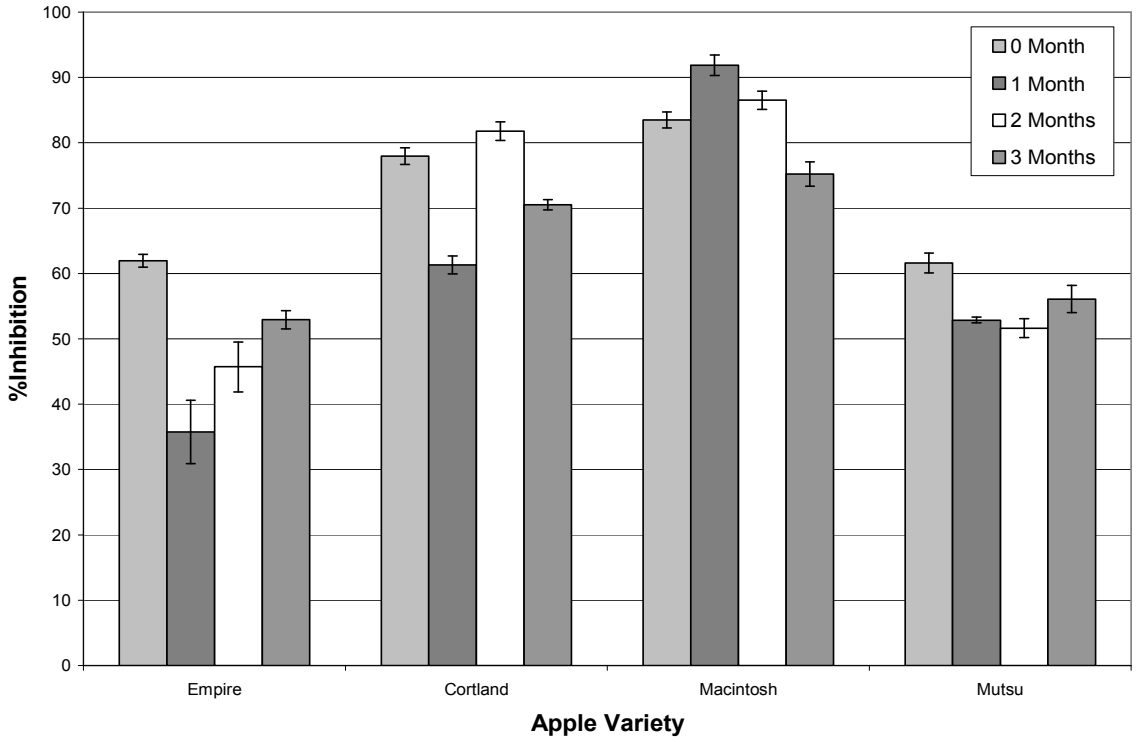


Figure 21. α-glucosidase inhibition. Apple water extracts.
A) Peel samples. B) Pulp samples.

A)



B)

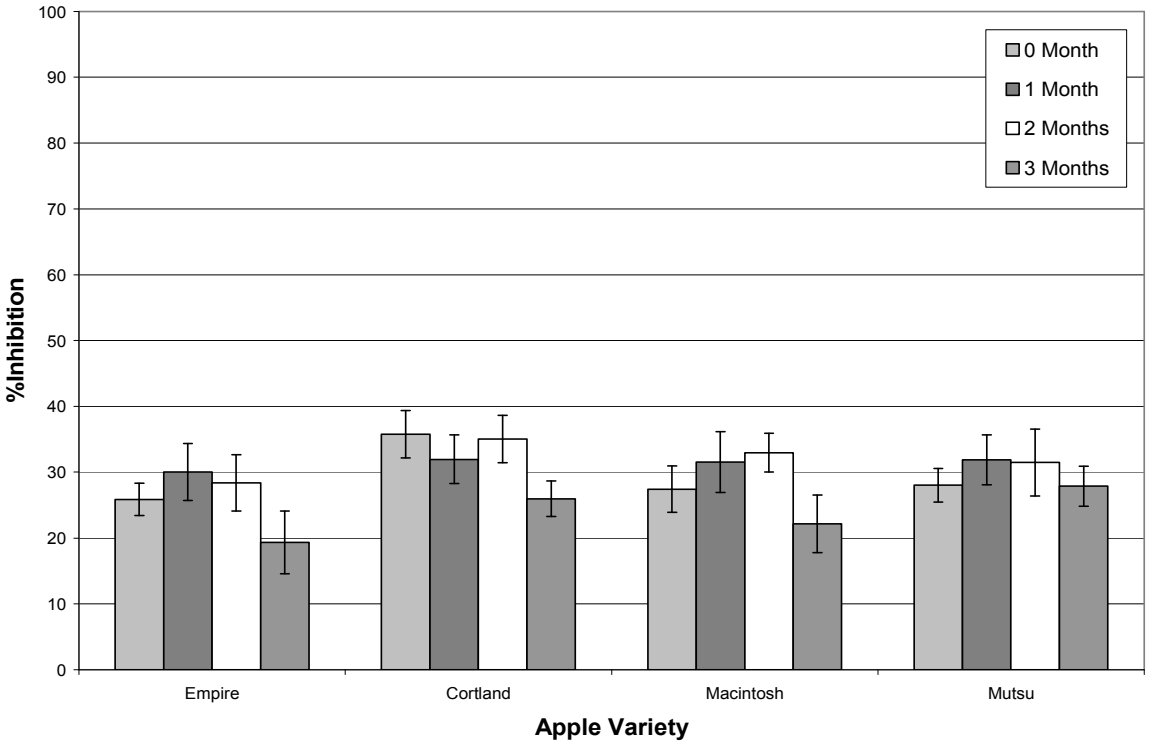


Figure 22. α-glucosidase inhibition. Apple 12% ethanol extracts.
A) Peel samples. B) Pulp samples.

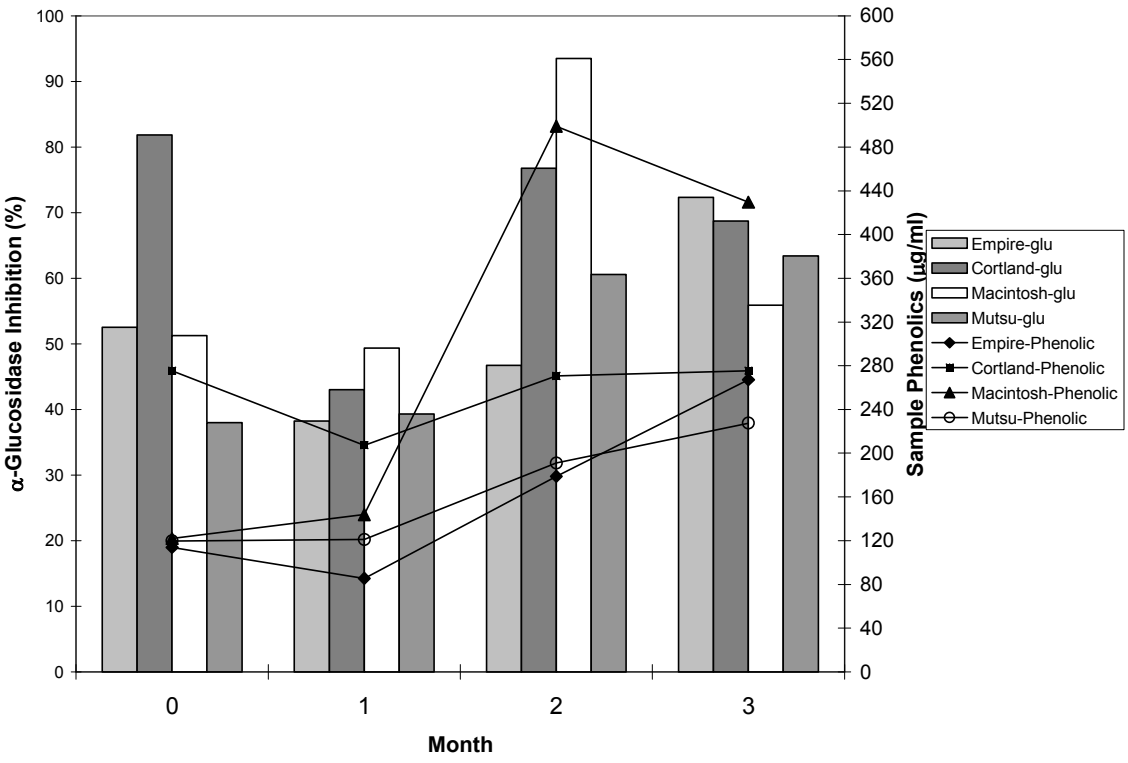


Figure 23. Sample phenolic concentration (µg/ml) and α-glucosidase inhibition. Apple peel water extracts.

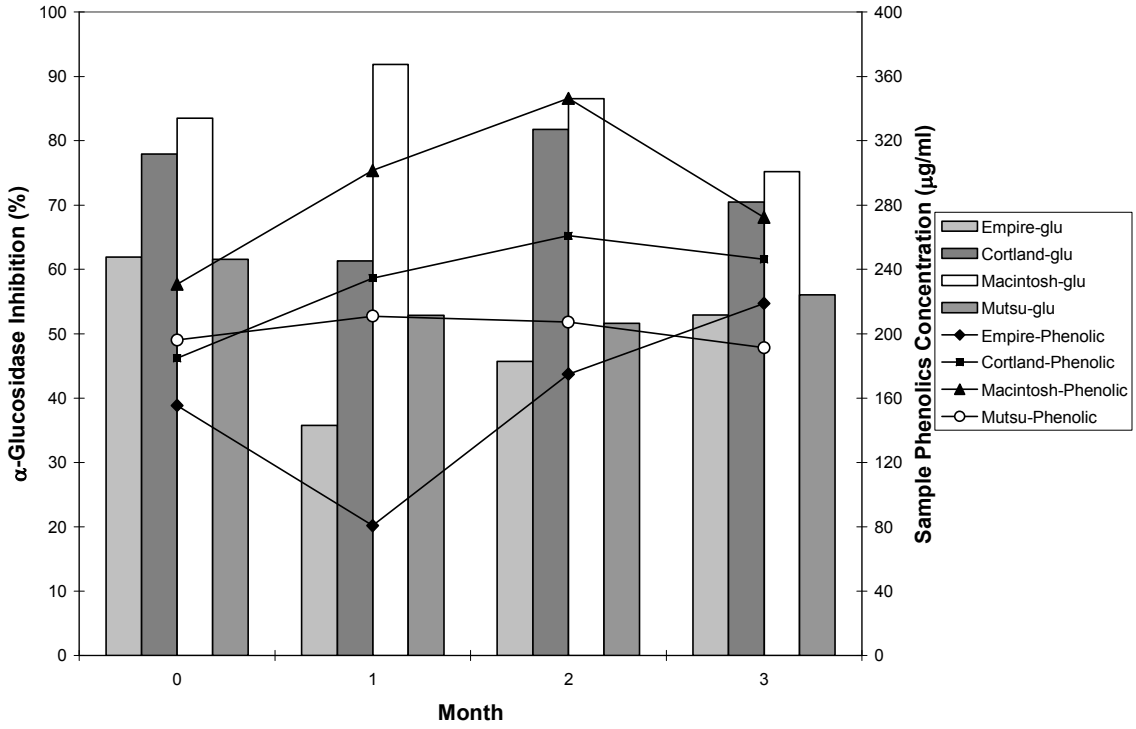


Figure 24. Sample phenolic concentration (μg/ml) and α-glucosidase inhibition. Apple peel 12% ethanol extracts.

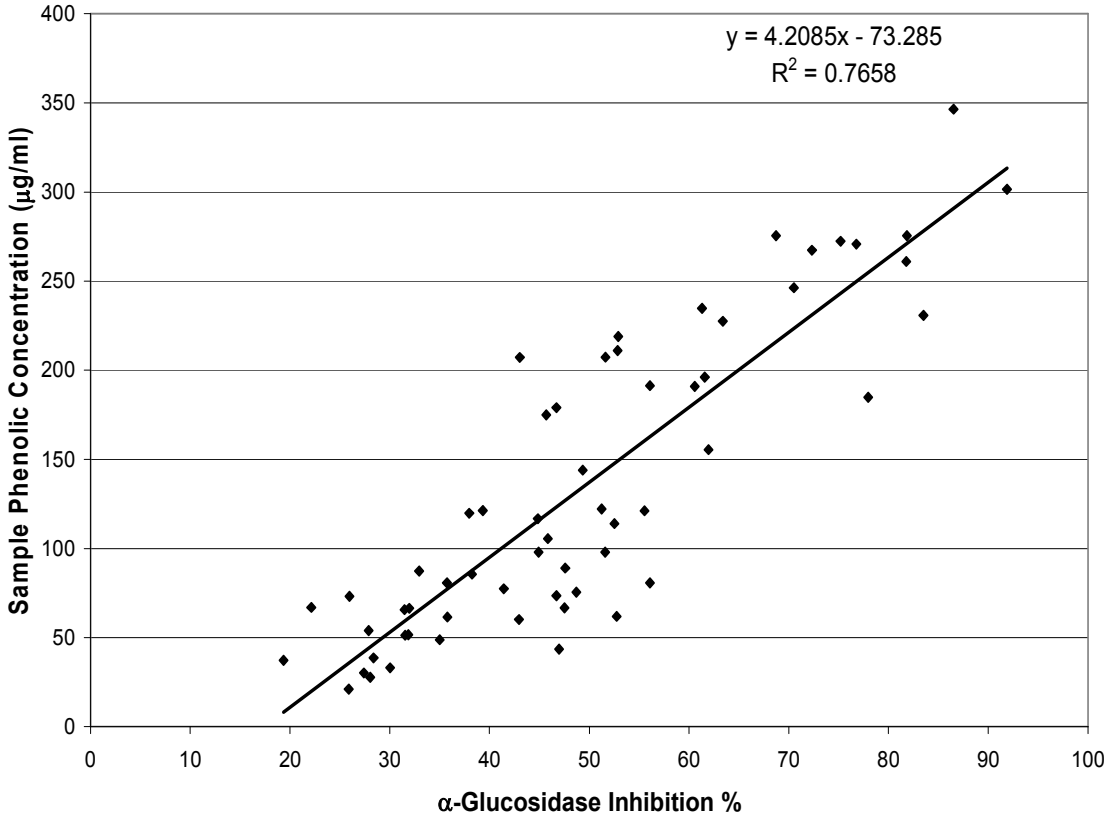


Figure 25. Correlation between sample phenolic concentration (µg/ml) and α-glucosidase inhibition.

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