INHIBITION OF ANAEROBIC RESPIRATION IN FRESH-CUT APPLE SLICES

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Joris Gerald Niilante Amissah

May 2010

INHIBITION OF ANAEROBIC RESPIRATION IN FRESH-CUT APPLE SLICES

Joris Gerald Niilante Amissah, Ph. D.

Cornell University 2010

Modified atmosphere packaging (MAP) extends the shelf life of fresh-cut fruits and vegetables. However, low oxygen (O_2) atmospheres associated with MAP can result in anaerobic fermentation and the accumulation of undesirable levels of acetaldehyde, ethanol and ethyl acetate. Alcohol dehydrogenase (ADH) catalyzes the conversion of ethanol to acetaldehyde. Inhibition of this reaction by nitric oxide (NO) has been demonstrated in rat and equine liver but inhibition of the reverse reaction has not been shown in yeast or plant tissue. This study investigated the effects of NO and sodium nitrite ($NaNO_2$) treatment on ADH activity in yeast and on ADH activity and shelf life of fresh-cut apples.

Yeast ADH activity was determined after pre-incubating the enzyme over time with or without 0.5 – 2 mM solutions of NO or $NaNO_2$. Fresh-cut apple slices were stored in 0.25 to 1% (v/v) NO (balance N_2) or 100% N_2 atmospheres in anaerobic vessels or high O_2 barrier pouches for 2 or 3 days at 6.1 °C. Slices were also treated with 1% NO or 2 mM $NaNO_2$, (with 100% N_2 or deionized water as control) packaged in 100% N_2 in pouches and stored for 6 weeks. Accumulation of ethanol, acetaldehyde and ethyl acetate were determined. Slice firmness and color were also measured.

Yeast ADH activity decreased in a dose and time-dependent manner with NO but was unaffected by $NaNO_2$. Ethanol accumulation in sliced apples was inhibited by 1% NO treatment whilst acetaldehyde increased, in both anaerobic vessels and pouches. Ethyl

acetate accumulation was inhibited only in anaerobic vessels. In the six-week study, slices treated with 1% NO or 2 mM $NaNO_2$ accumulated lower ethanol and higher acetaldehyde concentrations than the controls. Ethyl acetate accumulation was lower in NO-treated slices but unaffected by 2 mM $NaNO_2$. Treatment with NO or $NaNO_2$ resulted in darker slices (lower L* Values) than the controls, but did not affect slice firmness.

Our results suggest that NO and nitrite may be used to inhibit anaerobic respiration and extend the shelf life of fresh-cut apple slices and produce with low concentrations of phenolic compounds in which browning is not a major factor.

BIOGRAPHICAL SKETCH

The author was born in Accra, Ghana. He graduated from the University of Science and Technology (UST) in Kumasi, Ghana with a B.Sc. (Hons) Biochemistry degree in June 1998. He then worked in the Department of Biochemistry U.S.T. as an Analytical Biochemistry Teaching Assistant.

He was admitted to Cornell University and was awarded a Food Science Teaching Assistantship and an Institute for African Development Tuition Fellowship to pursue graduate studies in Food Science. He joined the Cornell Food Science Club and participated in the Institute of Food Technologists, Product Development and CollegeBowl competitions. His other extracurricular activities were volunteering during Graduate Student Orientation and mentoring incoming Food Science graduate students. In 2003 he earned a M.S. degree and was accepted into the Ph.D. program.

This dissertation is dedicated to my parents Jack and Lydia Amissah

and my sister Naalamle Amissah

ACKNOWLEDGMENTS

All praise, glory and thanks be to God for how far he has brought me. I am grateful to Professor J. H. Hotchkiss my Committee Chair for directing my graduate studies and for his creative ideas and hands on approach to resolving technical research challenges. Professor C.B. Watkins was most helpful with all aspects of postharvest physiology. Professor D.H. Streeter directed my training in entrepreneurship, the development of business plans and the commercial application of my research findings, for which I am most grateful. Analysis of apple slice firmness was made possible by Professor Rizvi, whilst Jacqueline Nock provided research support for the harvesting and controlled atmosphere storage of 'Red Delicious' apples. All technical assistance with building and modifying equipment for my research was provided by Tom Burke. My heartfelt gratitude goes to the Department of Food Science who provided financial support in the form of teaching and research assistantships. I also received a tuition fellowship from the Institute for African Development (IAD) for which I am most grateful.

My Aunt Awura-Abena and cousins Kobina and Kaku were a constant source of inspiration. Cyril and Efua Ofori ensured that their prayers, support and encouragement were never in short supply. My heartfelt gratitude goes to Uncle George whose time and resources were always available for my use. Aunt Biose and Bubu deserve special mention for their gifts, love and encouragement, whilst Sisi Yaa was consistent with her phone calls. I am also grateful for Jacqueline Vanderpuye-Orgle's tremendous support and wise counsel. I would also like to acknowledge the assistance provided by Brenda Werner and my labmates Joey, Julie and Zeki.

To my parents Jack and Lydia and my sister Naalamle, I say a very big thank you for your daily prayers, love, unwavering support, belief in me and constant encouragement. I am also indebted to Dolapo, who was consistent with her prayers, support and encouragement. My heartfelt gratitude and appreciation goes out to everyone who provided support and prayed for me. Thank you!

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AAT	Alcohol acetyl transferase
ACC	1-aminocyclopropane-1-carboxylic acid
ADH	Alcohol Dehydrogenase
ADH2	Alcohol Dehydrogenase 2
ATP	Adenosine triphosphate
cGMP	Cyclic guanosine monophosphate
GTP	Guanosine triphosphate
DETA/NO	2,2´ –(hydroxynitrosohydrazino)-bisethanamine
DTT	Dithiothrietol
EDTA	Ethylenediaminetetraacetic acid
ETC	Electron Transport Chain
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSNO	S-nitroso-L-glutathione
GMP	Guanosine monophosphate
GTP	Guanosine triphosphate
HNO_2	Nitrous acid
HNO_3	Nitric acid
LOX	Lipoxygenase
MAP	Modified atmosphere packaging
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide reduced form
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate reduced form
NH_3	Ammonia
NH_4^+	Ammonium

N_2	Nitrogen
N_2O	Nitrous oxide
N_2O_3	Dinitrogen trioxide
N_2O_4	Dinitrogen tetroxide
N_2O_5	Dinitrogen pentoxide
NO	Nitric oxide
NO_x	Oxides of nitrogen
NO_2	Nitrogen dioxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NOS	Nitric oxide synthase
$NaNO_2$	Sodium nitrite
O_2	Oxygen
PAR	4-(2-pyridylazo)-resorcinol
PBN	Phenyl N-tert-butylnitrone, N-*tert*-butyl-α-phenylnitrone
PDC	Pyruvate decarboxylase
PPO	Polyphenol oxidase
RNOS	Reactive nitric oxide species
ROS	Reactive oxygen species
Sin-1	3-morpholinosyl-nonimine
SNAP	S-nitroso-N-acetylpenicillamine
SNP	Sodium nitroprusside

CHAPTER ONE

LITERATURE REVIEW

1.1 Introduction

The minimal processing of whole produce into a convenient fresh-cut form has resulted in new produce marketing opportunities, an increase in the consumption of produce and rapid growth in the fresh-cut segment of the produce market. However the shelf life of fresh-cut produce is limited by senescence, microbial growth, unacceptable changes in color and texture, and the accumulation of acetaldehyde, ethanol and ethyl acetate as a result of anaerobic fermentation. Contamination and microbial growth in fresh-cut produce are minimized by the use of Good Manufacturing Practices during processing, whilst dipping solutions of ascorbic acid and calcium chloride may be used to delay the development of unacceptable changes in color and texture. Anaerobic fermentation however has remained a limiting factor, keeping the shelf life of most fresh-cut produce at a maximum of 3 weeks. As this is the same amount of time required for national distribution of fresh-cut produce from a central processing location to supermarket shelves and food service outlets, it is almost impossible to process and distribute fresh-cut produce nationally. The operations of the fresh-cut apple industry provide a classic example. Most of the U.S. apple harvest is obtained from Washington, New York and Michigan, thereby requiring the transportation of whole apples to many dispersed processing locations throughout the U.S. This is inefficient as only 50% of whole produce can be utilized as fresh-cut (Watada et al. 1996). Thus processing and distributing from a central location would reduce the cooling and transportation costs associated with the waste. Modified atmosphere packaging (MAP), with its low O_2 and elevated CO_2 levels is beneficial for extending the shelf life of fresh-cut produce (Watkins 2000). However, storing

fresh-cut produce in the low O_2 atmospheres associated with MAP may result in anaerobic fermentation and the production of acetaldehyde, ethanol and ethyl acetate which is undesirable. The accumulation of these fermentation compounds renders cut products organoleptically unacceptable. The constraints on fresh-cut shelf life have thus created the need for innovative processing and packaging methods that inhibit anaerobic respiration and extends the shelf life of fresh cut produce beyond the current 3 week maximum.

1.2 Fresh-cut produce

Fresh-cut produce also known as minimally processed fruits and vegetables are living respiring tissues that have undergone unit operations such as washing, peeling, slicing/cutting and shredding (Rolle and Chism 1987) and typically do not require further processing before consumption. Consumers are provided with the usable portion (about 50%) of raw produce in a convenient form (Watada et al. 1996). Convenience is an important factor driving the popularity and increased consumption of fresh-cut produce. For example in the 12 years prior to the 1989 introduction of fresh-cut (baby) carrots, the U.S. per capita consumption of carrots increased from 2.7 to 3.6 kg. However, in the 12 years after fresh-cut carrots became available, the per capita consumption of carrots increased 70%, from 3.6 to 6.4 kg (Perosio et al. 2001). Several factors account for the perishable nature of fresh-cut produce. During processing, microorganisms on the external surface of produce may be transferred onto cut (nutrient rich) surfaces resulting in a rapid increase in microbial populations. Also previously compartmentalized enzymes may come in contact with cut surfaces causing undesirable changes in color and texture. Modified atmosphere packaging has been used with some success to limit microbial growth, prevent undesirable changes and extend fresh-cut produce shelf life to the current 3 week maximum.

1.2.1 Modified atmosphere packaging of fresh-cut produce

In modified atmosphere packaging (MAP), the gas atmosphere around packaged produce is altered to obtain a final gas composition that differs from air (Al-Ati and Hotchkiss 2002). Modification of the package gas atmosphere can be attained by active or passive means. In active packaging, air is removed from the package, followed by the introduction of the desired gas mixture. However passive modification relies on the respiration of packaged produce to alter the package gas composition over a period of time. MAP has been used successfully to inhibit microbial growth (Gunes and Hotchkiss 2002) and extend the shelf life of whole and fresh-cut produce (Gunes et al. 2001; Kader et al. 1989). The optimum storage atmospheres for fresh-cut produce may differ from whole produce due to the removal of the skin and other barriers to gas diffusion (Kader et al. 1989; Watkins 2000). Thus elevated CO_2 and low O_2 levels that are injurious to whole produce may be used successfully to improve the quality and shelf life of fresh-cut produce (Gunes et al. 2001). Nonetheless, these high CO_2 and low O_2 atmospheres associated with MAP can result in anaerobic fermentation in whole and fresh-cut produce.

1.2.1.1 Respiration of fresh-cut produce

Respiration in fresh-cut produce increases due to the wounding sustained during their preparation (Brecht 1995). Consequently, fresh-cut produce have respiration rates that are generally 1.05 – 3.45 times higher than in intact produce and show an increase with temperature, which is dependent on the type of produce (Watada et al. 1996). Kiwifruit for example, show an increase in respiration in response to cutting which is not the case in banana (Watada et al. 1990). Watada et al. (1996), noted that storage atmosphere affected the rate of respiration of fresh-cut produce. Respiration rate is

reduced when fresh-cut produce is stored in low O_2 and elevated CO_2 atmospheres. Though beneficial in reducing respiration, the low O_2 and elevated CO_2 atmospheres associated with MAP of fresh-cut produce result in anaerobic fermentation.

1.2.1.2 Anaerobic respiration of fresh-cut produce

Anaerobic respiration occurs when fresh-cut produce is exposed to storage atmospheres that contain low O_2 (< 1%) and/or elevated CO_2 concentrations. During anaerobic respiration, pyruvate which is the end product of glycolysis, is converted to acetaldehyde in a reaction catalyzed by pyruvate decarboxylase. Alcohol dehydrogenase (ADH) then catalyzes the reduction of acetaldehyde to ethanol, followed by the conversion of ethanol to ethyl acetate catalyzed by alcohol acetyltransferase (AAT) (Ke et al. 1994). Low concentrations of acetaldehyde, ethanol and ethyl acetate are desirable in the aroma of fruits and vegetables, although the accumulation of these compounds is undesirable (Kader et al. 1989). Anaerobic respiration of fresh-cut produce also increases with temperature. Fresh-cut carrots stored in 0.5 or 2% O_2 (balance N_2) atmospheres had higher levels of PDC and ADH activity and accumulated higher levels of acetaldehyde and ethanol at 15 °C compared with fresh-cut carrots stored at 5 °C (Kato-Noguchi and Watada 1997). The O_2 threshold of produce and the cultivar used are important in determining which fresh-cut fruit and vegetables have a greater tendency to undergo anaerobic fermentation (Ke et al. 1991; Lakakul et al. 1999).

1.3 Alcohol Dehydrogenase (ADH)

Alcohol dehydrogenase (ADH), (E.C. 1.1.1.1) is a zinc metalloenzyme that catalyzes the reversible oxidation of alcohols to aldehydes using the cofactors NAD and NADH respectively (Scandalios 1977). ADH activity is present in microorganisms and a

wide range of animal and plant tissues (Sund and Theorell 1963). ADH catalyzes the conversion of alcohol to aldehyde in animal tissue and the reverse reaction in plant tissue.

1.3.1 Biochemical properties of ADH

The structure and chemical composition has been determined for ADH from yeast and horse liver. Yeast ADH is a tetramer with a molecular weight of 150,000 (Hayes and Velick 1954) and is known to have much greater activity than horse liver ADH which is a dimer, about half the size of the yeast enzyme, with a molecular weight of 80,000 (Eklund et al. 1974). Both forms of ADH contain zinc, however there were initial disagreements in the literature as to the number of zinc atoms and their possible role (catalytic and/or structural) in ADH. Initial reports suggested that yeast ADH had four zinc atoms, with one zinc atom per subunit (Vallee and Hoch 1955; Veillon and Sytkowski 1975). The zinc atoms were thought to have a catalytic role (Hoch and Vallee 1956) or a structural role in binding the four monomers and providing stability for the quaternary structure of the enzyme (Kagi and Vallee 1960). Subsequent research indicated that horse liver ADH had four zinc atoms, with two zinc atoms per subunit (Akeson 1964). Two of the zinc atoms were involved in catalytic activity while the other two stabilized the quaternary structure of the enzyme (Drum et al. 1967). It was later found that yeast ADH had eight zinc atoms, with two zinc atoms per ADH subunit. (Klinman and Welsh 1976). Based on the similarities Jornvall et al. (1975) observed in the structures of horse liver and yeast ADH, Klinman and Welsh (1976) concluded that the zinc atoms of yeast ADH also had catalytic and structural roles. Removing the structural zinc atom by pre-incubating yeast ADH with 0.02 to 100 mM dithiothrietol (DTT) had no effect on enzyme activity even though ADH became more sensitive to heat denaturation. At these concentrations, DTT had no

effect on the zinc atom bound to the active site however; treating yeast ADH with 0.1 to 100 mM ethylenediaminetetraaetic acid (EDTA) removed the zinc atom bound to the active site in a dose dependent manner resulting in a loss of activity.

The optimum pH of ADH depends upon the reaction being catalyzed and the cofactors used in the reaction. Bartley and Hindley (1980) reported a pH optimum of 5.5 for the reduction of acetaldehyde to ethanol by apple ADH using NADPH as cofactor and 5.5 - 6.0 using NADH as cofactor. There was no ADH activity when NADP was used as a cofactor in the reverse reaction (ethanol to acetaldehyde) whilst activity increased as pH was increased from 7 to 10 using NAD as cofactor (Bartley and Hindley 1980). Melon ADH has similar pH optima, 5.7 for acetaldehyde reduction (with either NADH or NADPH as cofactors) and 9.6 for the oxidation of ethanol (NAD cofactor) (Rhodes 1973). Yeast ADH is denatured at acidic pH and precipitates out of solution at pH less than or equal to pH 5 (van Eys et al. 1957).

1.3.2 Role of ADH in animal tissue metabolism

ADH is synthesized in mammalian liver and catalyzes the conversion of alcohols (which are toxic to animal tissue) to aldehydes (Sund and Theorell 1963). ADH is also important in vision and catalyzes the oxidation of vitamin A (retinol) (Mezey and Holt 1971).

1.3.3 Role of ADH in plant tissue metabolism

ADH in plant tissue is important in anaerobic respiration and catalyzes the conversion of acetaldehyde to ethanol. Plants are able to obtain energy in the form of adenosine triphosphate (ATP) from glycolysis during anaerobic respiration. This is an important source of energy for plant cells during anaerobic conditions that may exist in MAP

packages, the metabolism and germination of seeds (Scandalios 1977) and under environmental stress conditions such as flooding of roots (Chan and Burton 1992).

1.3.4 Benefits of ADH

The presence of ADH has numerous benefits to plant tissue. ADH is involved in the reaction pathways that produce aroma compounds in fruits such as apple (Bartley and Hindley 1980). ADH enables glycolysis to proceed in environments with low or no O_2 such as MAP of fresh-cut fruits and vegetables. Anaerobic respiration allows for the generation of reduced amounts of ATP from glycolysis compared with aerobic respiration thus making it possible for plant tissue to tolerate hypoxia and anaerobic conditions. In addition, ADH catalyzes the conversion of acetaldehyde (which is toxic to plant cells) to ethanol.

1.3.5 Problems associated with ADH during storage of fresh-cut produce

Anaerobic fermentation results when fresh-cut produce is kept in low O_2 or completely anaerobic environments (Ke et al. 1994). The accumulation of fermentation volatile compounds acetaldehyde, ethanol and ethyl acetate limit fresh-cut shelf life by causing undesirable off flavors and odors in the packaged product (Kader et al. 1989). The problems associated with anaerobic respiration have been addressed in the past by including low levels of O_2 in the package atmosphere to delay the onset of anaerobic respiration in packages of fresh-cut produce. Also low temperatures are maintained during the transport and storage of MAP fresh-cut produce as ADH activity is known to increase with temperature (Kato-Noguchi and Watada 1997). To try and address this problem, models have been developed to match package film permeability with the respiration rate of packaged fresh-cut produce, to

ensure that the packages do not become anaerobic during storage (Lakakul et al. 1999).

1.4 Oxidation states of nitrogen

There are nine oxidation states of nitrogen ranging from -3 to +5 (Hotchkiss 1989). The oxides of nitrogen (NO_x) which exist in five (+1 to +5) of these oxidation states (Figure 1) are important in mammalian and plant physiology and can be converted from one oxidation state to another by chemical or enzymatic reactions.

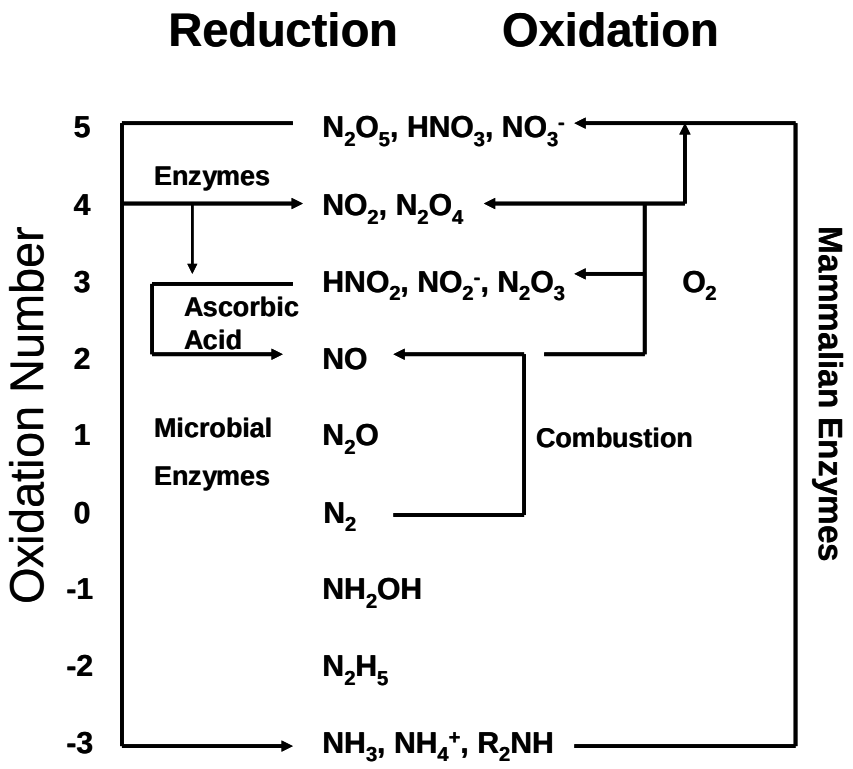

Figure 1: Oxidation states of nitrogen (Hotchkiss 1989).

N_2 is oxidized to NO during combustion reactions, but in the presence of O_2, NO is oxidized, forming oxides of nitrogen (NO_x) including NO_2, N_2O_3 and N_2O_4 which are

nitrosating agents with +3 and +4 oxidation states (Hotchkiss 1989). NO_x with the same oxidation number exist in a state of equilibrium with each other and can be converted to a higher or lower oxidation state by redox reactions. NO_3^- which is the most oxidized form of nitrogen can be reduced to NO_2 by microbial enzymes (Hotchkiss 1989) or plant nitrate reductase (Dean and Harper 1986; Harper 1981; Yamasaki et al. 1999). NO_2 can then be converted back to NO by ascorbic acid (Hotchkiss 1989) a reaction that takes place in both plants and animals. In addition, plants can convert NO_2 to NO by nitrate reductase (Yamasaki et al. 1999) or by carotenoids in the presence of light (Cooney et al. 1994). NO (+2) may also form S-nitrosothiols (+3) or nitrates (+5) of tyrosine *in vivo* (Gow et al. 2004).

1.4.1 Nitric oxide

NO is a small, free radical gas that diffuses readily across cell membranes (Feldman et al. 1993). Initially thought to be an atmospheric pollutant derived from combustion reactions, NO was later found to be synthesized by both animal and plant cells (Furchgott 1988; Ignarro et al. 1988; Leshem and Haramaty 1996; Palmer et al. 1987), where it functions as an effective signaling molecule, that is important in the bioregulation of many physiological processes.

1.4.2 Chemistry of the oxides of nitrogen

NO modifies proteins by a) binding to metal centers b) oxidizing thiols and tyrosine c) the nitration of tyrosine, tryptophan and amines and d) the S-nitrosylation of thiol groups, these modifications may reversibly activate or inactivate enzymes (Gow et al. 2004). NO binds reversibly to iron in the ferrous and ferric states (Cooper 1999) and this is important in many of the physiological reactions in animals and plants. For example NO activates soluble guanylate cyclase by binding to ferrous heme. The

activated enzyme catalyzes the conversion of guanosine triphosphate (GTP) to cyclic GMP thus regulating neurotransmission, the relaxation of smooth muscle and the inhibition of blood platelet aggregation and adhesion (Ignarro 1990; Moncada et al. 1991). On the other hand, NO inhibits cytochrome oxidase and catalase by binding to ferrous and ferric heme respectively (Cooper 1999). NO also reacts with iron sulfur centers, inhibiting iron-sulfur enzymes such as aconitase, complex I and II of the electron transport chain (Stadler et al. 1991). NO reacts with thiol groups to form S-nitrosothiols which have been shown to inhibit enzyme activity. NO inhibits aldehyde dehydrogenase (Moon et al. 2005) and ribulose-1,5-bisphosphate carboxylase/oxygenase activity (Abat et al. (2008) by S-nitrosylation.

1.4.3 Role of the oxides of nitrogen in animal metabolism

The de-novo synthesis of NO by mammals was discovered by several researchers (Furchgott 1988; Ignarro et al. 1988; Palmer et al. 1987). NO is synthesized by the conversion of L-arginine to L-citrulline catalyzed by NO synthase (NOS). Three isoforms of NOS which can be designated to two groups, have been identified in mammals i) constitutive NOS which is synthesized by brain (neuronal NOS, nNOS) and endothelial cells (eNOS) and ii) inducible NOS which is synthesized by macrophages (iNOS) (Wink and Mitchell 1998). Constitutive forms of NOS which require Ca^{2+} and calmodulin, synthesize low levels of NO (< 1 μM), while iNOS is induced by cytokines to produce high concentrations of NO (> 1μM) (Wink and Mitchell 1998). The *in vivo* reactions of NO may be grouped into two i) direct effects where low levels of NO are directly involved in biochemical reactions and ii) indirect effects where reactive NO species (RNOS) formed from the reaction of high levels of NO with O_2 or superoxide ion (O_2^-) participate in oxidation, nitrosation and nitration reactions (Wink and Mitchell 1998). An important direct effect is the role of NO as a

signaling molecule in animals (Wink and Mitchell 1998). NO is involved in vasodilation, blood pressure regulation, the relaxation of smooth muscle, penile erection and neurotransmission. NO revolutionized medicine with the use of nitroglycerin and viagra to treat hypertension and erectile function respectively (Leshem 2000).

1.4.4 Role of the oxides of nitrogen in plant metabolism

A decade after the endogenous synthesis of NO was discovered in mammals, plants were also found to synthesize NO (Leshem and Haramaty 1996). Plants possess NOS activity (Barroso et al. 1999; Ribeiro et al. 1999) and produce NO by catalyzing the conversion of L-arginine to L-citrulline. Another enzymatic source of NO in plants is the reduction of NO_3^- to NO_2^- by NAD(P)H-dependent nitrate reductase, which further reduces NO_2^- to NO (Dean and Harper 1988; Harper 1981; Yamasaki et al. 1999). NO is also produced in plants by non enzymatic chemical reactions such as the reduction of NO_2^- by ascorbic acid. Carotenoids in the presence of light have also been demonstrated to reduce NO_2^- to NO (Cooney et al. 1994). Figure 2 below, provides a summary of NO production in plants by enzymatic and non-enzymatic reactions.

Similar to animals, NO functions as an important signaling molecule in plants, activating guanylate cyclase and catalyzing the formation of cGMP from guanosine triphosphate (GTP). NO signaling is involved many diverse physiological processes in plants which include the germination of seeds (Giba et al. 1998), the stimulation of stomatal closure (Garcia Mata et al. 2003; Garcia Mata and Lamattina 2001) and the inhibition of hypocotyl and internode growth (Beligni and Lamattina 2000). NO is also an endogenous regulator of plant growth; Leshem et al. (1998) showed that growth in NO treated pea leaf discs, increased with NO concentration up to an

optimum level of 10^{-6} M, after which growth promotion decreased and was inhibited by NO concentrations greater than 10^{-5} M.

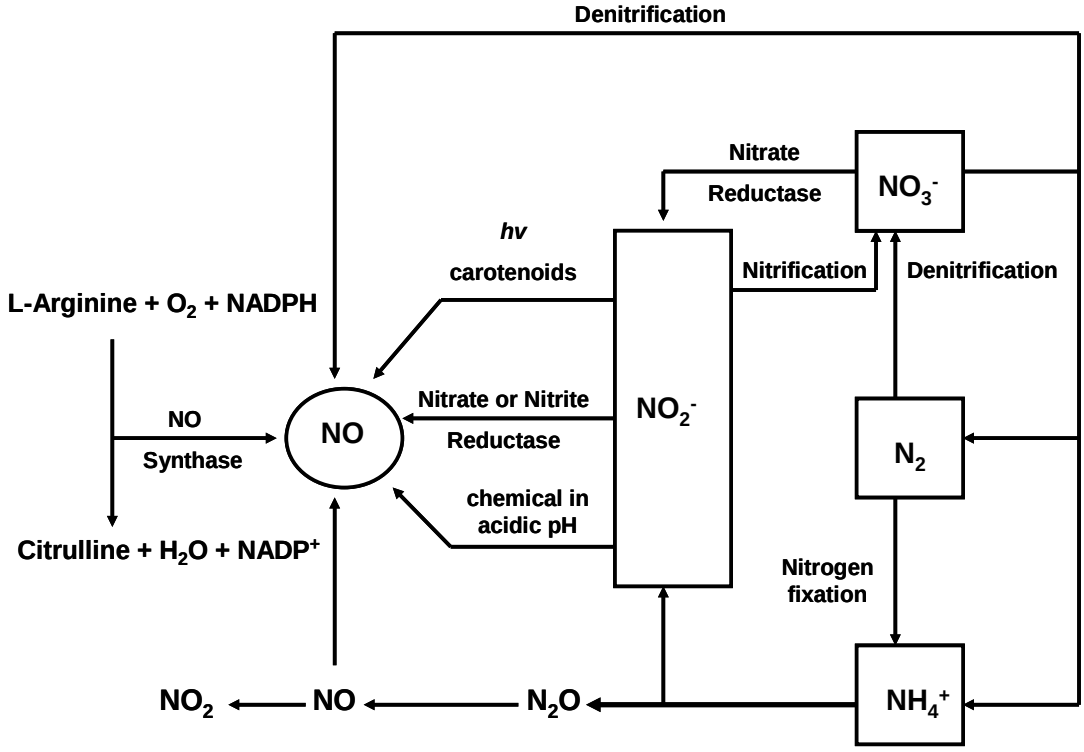

Figure 2: NO biosynthesis in plants (Wojtaszek 2000).

In addition, Leshem et al. (1998) suggested a stress-coping role for NO due to the existence of a stoichiometric relationship between NO and ethylene emission from heat stressed alfalfa plantlets. NO emission increased with the duration of heat stress whilst ethylene emission decreased. Table 1 below, provides a summary of the effects of NO on physiological processes of plants.

Table 1: Nitric Oxide Effects on Physiological Processes of Plants (Beligni and Lamattina 2001)

Tissue or Organ	Effects on Physiological Processes	Species	Optimum NO Concentration (Moles)
Seeds	Stimulates germination	*Paulownia tomentosa*	10^{-6}
	Inhibits respiration after imbibition	Soybean	
	Inhibits aleuronic cell death	Barley	
Roots	Growth	Maize	10-10
	Induces the formation of lateral and adventitious roots	Cucumber	10^{-9} to 10^{-5}
		Lavandula spp	10^{-6}
Tuber	Tuberization	Potato	10^{-6}
Hypocotyls	Inhibits growth under low light conditions	Lettuce *Arabidopsis thaliana*	10^{-6}
Stems	Inhibits internode growth under low light conditions	Potato	10^{-6}
Leaves	Stimulates de-etiolation	Wheat and barley	10^{-6}
	Delays senescence	Pea	5×10^{-6}
	Stomatal closure	Wheat, *Vicia faba*	10^{-6}
	Leaf expansion	Pea	5×10^{-6}
	Stimulates defense responses	*Arabidopsis*, tobacco	2×10^{-6}
	Inhibits cell death	Potato	10^{-6}

Physiological and biological effects of NO on plants (Beligni and Lamattina 2001)

1.4.5 Nitric oxide and plant respiration

The cytochrome oxidase pathway in plant mitochondria is inhibited by NO in a reversible and dose dependent manner however, the alternative oxidase pathway is unaffected by NO (Millar and Day 1996; Yamasaki et al. 2001). In earlier studies with rat mitochondria and synaptosomes, Brown and Cooper (1994) and Cleeter et al. (1994) demonstrated the inhibition of cytochrome oxidase by NO. This inhibition was dependent on O_2 concentration, suggesting that NO competed with O_2 for binding to cytochrome oxidase (Brown and Cooper 1994). The cytochrome oxidase pathway is coupled to the synthesis of adenosine triphosphate (ATP). Thus a decline in ATP synthesis would be expected when cytochrome oxidase is inhibited by NO. Evidence for NO inhibition of ATP synthesis in plant mitochondria was provided by Yamasaki et al. (2001). NO has also been shown to decrease the membrane potential of plant mitochondria (Yamasaki et al. 2001; Zottini et al. 2002). A reduction in mitochondrial membrane potential is associated with cell death in animals (Zamzami et al. 1995). Thus after incubating carrot cells for 24 hours with (the NO donor) 1mM sodium nitroprusside (SNP), Zottini et al. (2002) observed a 20% decrease in the number of viable cells. They attributed this relatively low level of cell death (compared with that expected from animal cells) to the presence of the alternative oxidase pathway in plant mitochondria. When the cytochrome pathway is inhibited by NO, The alternative oxidase minimizes oxidative stress on mitochondria by reducing the formation of reactive oxygen species (ROS) (Maxwell et al. 1999). Thus the alternative oxidase pathway may enable plants tolerate high NO levels that are toxic to animal cells.

Even though cytochrome oxidase is reversibly inhibited by low (nanomolar to micromolar) concentrations of NO, complex I and II of the cytochrome pathway are inhibited by much higher NO concentrations (Brown 2001). In addition the glycolytic

enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been identified as a target for NO inhibition by S-nitrosylation in the medicinal plant *Kalanchoe pinnata* (Abat et al. 2008). However most of the literature with regards to NO inhibition of enzymes in glycolysis and the Krebs cycle are derived from earlier studies with mammalian systems. NO inhibition of GADPH is well known in mammalian systems (Dimmeler and Brune 1993; Dimmeler et al. 1992; Molina y Vedia et al. 1992; Zhang and Snyder 1992). Further, NO has been shown to increase maximum GADPH activity and accelerate glucose uptake and metabolism via glycolysis, Krebs cycle and the pentose phosphate pathway but high concentrations of NO (> 0.5 mM) inhibit GAPDH activity (Mateo et al. 1995). By speeding up glycolysis, it is possible that low concentrations of NO may increase the accumulation of fermentation volatile compounds during anaerobic respiration in plant tissue. While higher NO levels may counter the effects of the increased rate of glycolysis, by inhibiting GAPDH activity. It is possible high NO levels may also inhibit ADH activity as previous research has shown structural similarities between the coenzyme binding domains of horse liver ADH and GAPDH l(Eklund et al. 1974).

1.4.6 Nitrite and plant respiration

Plant respiration is not inhibited by $NaNO_2$ (Millar and Day 1996; Yamasaki et al. 2001). However inhibition of plant respiration can be achieved by first converting $NaNO_2$ to NO by non enzymatic or enzymatic reactions. It is known that sodium nitrite is reduced by ascorbate to form NO (Stohr and Ullrich 2002) and plant nitrate reductase catalyzes the conversion of $NaNO_2$ to NO in the presence of NADH (Yamasaki and Sakihama 2000).

NO [produced from reacting 10 mM ascorbate with $NaNO_2$ (2.5 – 20 mM)] has been demonstrated to inhibit the cytochrome pathway in plant mitochondria (Millar and

Day 1996; Yamasaki et al. 2001). NO generated from $NaNO_2$ by nitrate reductase has also been shown to inhibit the cytochrome pathway in plant mitochondria (Yamasaki et al. 2001). The alternative pathway in plants is not inhibited by either $NaNO_2$ alone or when combined with ascorbate (Millar and Day 1996; Yamasaki et al. 2001). In addition, NO generated from $NaNO_2$ by nitrate reductase, does not inhibit the alternative pathway in plant mitochondria (Yamasaki et al. 2001).

1.4.7 Horticultural use of nitric oxide

The horticultural use of NO as a postharvest application to extend the vase/shelf life of flowers, whole and fresh-cut produce resulted from the discovery by Leshem et al. (1998) that unripe fruit and fresh flowers have a higher endogenous NO content than ripe fruit and senescing flowers respectively. Unripe avocado and banana were shown to have 10 and 4 times greater endogenous NO than their respective ripe fruit, whilst fresh waratah and geraldton wax flowers emitted about 2.5 times more NO than their corresponding senescing flowers (Leshem et al. 1998). The maturation and senescence of produce and flowers was associated with a decrease in endogenous NO, leading Leshem et al. (1998) to hypothesize that senescence or maturation could be delayed by the exogenous application of NO. In subsequent experiments to test this hypothesis, Leshem et al. (1998) reported an extension of postharvest life (average 117%) among six fruit and vegetables fumigated with low concentrations (0.05 to 0.25 μmol/L) of NO for short periods of time (2 to 16 hours) prior to storage in air at 20 °C. Postharvest life extension was also observed in carnations supplied with NO donors [10^{-3} M N-tert-butyl-α-phenylnitrone (PBN) or 3 morpholinosyl-nonimine (Sin-1)] (Leshem et al. 1998). Bowyer et al. (2003) questioned whether the effects of NO was due to contact between the flowers and gaseous NO released from solution or the uptake of the NO donor and subsequent NO release *in vivo*, as this could not be

determined from the experimental design of Leshem et al. (1998). Both methods extended the postharvest vase life of carnations, however *in vivo* delivery was found to be more effective than fumigation (Bowyer et al. 2003). Postharvest life extension was also observed in eight other flower species (Badiyan et al. 2004), demonstrating that the effects of NO were not limited to carnations.

1.4.7.1 Postharvest use of nitric oxide

Considerable research interest in the use of NO to extend the postharvest life of flowers, whole and fresh-cut fruit and vegetables developed in the decade following the groundbreaking publication by Leshem et al. (1998). Fumigation with NO resulted in significant extensions to the postharvest life of strawberries (Soegiarto and Wills 2006; Wills et al. 2000), broccoli, green bean and bok choy (Soegiarto and Wills 2004), apple slices (Pristijono et al. 2006; Wills et al. 2007), lettuce (Wills et al. 2007; 2008) and peaches (Zhu et al. 2006). Fumigation with NO concentrations greater or less than the optimum (which varied amongst species and ranged from 5 to 500 μL/L) resulted in a decline in the maximum postharvest life obtained. Fumigation with higher than optimum NO concentrations caused browning in strawberries (Wills et al. 2000). Dipping whole and cut produce in aqueous solutions of NO or NO donors such as 2,2′-(hydroxynitrosohydrazino)-bisethanamine (DETA/NO) and SNP extended the postharvest life of strawberry (Zhu and Zhou 2007), longan fruit (Duan et al. 2007), kiwifruit (Zhu et al. 2008), peach slices (Zhu et al. 2009), lettuce (Wills et al. 2007; 2008), apple slices (Pristijono et al. 2008), and sliced banana (Cheng et al. 2009). Similar to the results obtained in flowers, aqueous solutions of DETA/NO were more effective (than fumigation) in extending the shelf life of fresh-cut apples and lettuce (Wills et al. 2007). NO donor compounds may be better suited than gaseous NO for

commercial use in the horticulture and food processing industries due to their greater effectiveness, ease of use and safety considerations.

The postharvest application of low concentrations (1 – 500 μL/L) of NO has been shown to maintain or enhance the edible quality of fruits and vegetables by inhibiting enzyme systems that accelerate ripening and/or senescence. The effects of NO on browning are concentration dependent, with low concentrations of NO inhibiting browning on the cut surfaces of apple (Pristijono et al. 2006), lettuce (Wills et al. 2008) and peach (Zhu et al. 2009). Measurements of polyphenol oxidase (PPO) activity in NO-treated fruit confirmed the inhibition of PPO activity by NO (Duan et al. 2007; Zhu et al. 2009). However NO accelerates browning when applied to produce in high concentrations (Wills et al. 2000). The contrasting effects of NO in inhibiting and accelerating browning may be explained by the inhibition of PPO activity by low concentrations of NO (Duan et al. 2007; Zhu et al. 2009) whilst high concentrations of NO react directly with phenolic compounds to form quinones (Urios et al. 2003) which may then polymerize to form brown pigments (melanins). Thus treatment with high concentrations of NO may not be suitable for produce that contain levels of phenolic compounds. NO (5 and 10 μL/L) inhibited 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase activity in peach fruit (Zhu et al. 2006). ACC is converted to ethylene in a reaction catalyzed by ACC oxidase. With the inhibition of ACC oxidase, ethylene levels in NO treated peach fruit would be expected to decrease. This was the case, with Zhu et al. (2006) reporting decreased ethylene levels and decreased lipoxygenase (LOX) activity in NO treated peach, though the mechanism of inhibition was not determined.

Surface area is an important factor in the absorption of NO by produce. Soegiarto et al. (2003) reported a 130-fold difference in the rate of absorption of NO per 100 g of produce, from 70 – 90 mmol/min in leafy vegetables to < 1 mmol/min in lime which has a thick skin. On the other hand a 15-fold difference was observed (13 to <1 mmol/min) when absorption per 100 cm^2 of produce was taken into consideration (Soegiarto et al. 2003). These results suggest that NO absorption would be enhanced in dense produce items such as apple by increasing the surface area to volume ration for a given weight of produce. Benefits that may be derived from increasing produce surface area to volume ratio include: reduced fumigation times as well as the use of lower concentrations of NO to obtain the desired postharvest life extension. The physiological effects of the postharvest application of NO to produce are summarized in Table 2 below. The effects of NO listed in Table 2, were obtained by treating small amounts of produce (10 – 250 g) with large volumes of NO (4 L). Low μL/L concentrations of NO were utilized in these experiments. However, the large NO headspace in relation to the treated produce and the total number of moles of NO present in the treatment vessel suggest that produce can be successfully treated with much higher NO concentrations for short periods of time, less than the 2 hours fumigation time used by most researchers in Table 2.

Table 2: Postharvest Treatment With Nitric Oxide

Plant Material	Weight (g)	[NO] µL/L	Volume (L)	Moles of NO*	Treatment Time (Hr)	Effects	Reference
Produce and flowers	10 – 200	1 - 500	4	1.64×10^{-7} - 8.18×10^{-5}	2	Reduced water loss	(Ku et al. 2000)
Strawberry	250	5 - 10	4	8.18×10^{-7} - 1.64×10^{-6}	2	Delayed fruit softening and the onset of rotting	(Wills et al. 2000)
Carnations	20	1 - 5	4	1.64×10^{-7} - 8.18×10^{-7}	2	Reduced mold growth, browning and petal color loss	(Bowyer et al. 2003)
Broccoli	100	4000	4	6.54×10^{-4}	2	Delayed yellowing	(Soegiarto and Wills 2004)
Green Bean	100	100	4	1.64×10^{-5}	2	Delayed yellowing	
Bok Choy	100	50	4	8.18×10^{-6}	2	Delayed yellowing	
Strawberry	200	10	4	1.64×10^{-6}	2	Reduced mold growth and loss of red color	(Soegiarto and Wills 2006)
Lettuce	100	10	4	1.64×10^{-6}	24	Inhibited surface browning	
Apple	150	10	4	1.64×10^{-6}	1	Inhibited surface browning	(Pristijono et al. 2006)
Lettuce	40 – 45	500	4	8.18×10^{-5}	1	Inhibited surface browning	(Wills et al. 2008)

* Moles of NO was calculated using 25 °C and a 4L headspace and does not account for the volume occupied by produce or flowers

1.4.8 Nitric oxide inhibition of ADH activity

The activity of ADH from mammalian tissue is reversibly inhibited by NO (0.1 – 1 mM) (Gergel and Cederbaum 1996). NO inhibition of rat ADH activity had been observed earlier by Dimmeler and Brune (1993). Inhibition of ADH was dose and time dependent, requiring the pre-incubating of ADH with NO for at least 20 minutes.

1.4.8.1 Inhibition of ADH activity in animal tissue

ADH activity was inhibited by 50% when the enzyme was pre-incubated at 37 °C for 30 minutes, with the NO donor (0.25 mM) Sin-1 (Dimmeler and Brune 1993). As horse liver ADH and GAPDH have similar coenzyme binding domains (Eklund et al. 1974) and NO was known to inhibit GAPDH activity by auto-ADP-ribosylation (Brune and Lapetina 1989; Dimmeler et al. 1992). Dimmeler and Brune (1993) investigated whether NO inhibition of ADH was also due to auto-ADP-ribosylation. Their results showed that for the same concentration of Sin-1 (0.25 mM), auto-ADP-ribosylation of ADH was 2.5 times less than in GAPDH, even though the inhibition of enzyme activity was 50 and 60% for ADH and GAPDH respectively. Auto-ADP-ribosylation alone could not be used to explain the decrease in ADH activity observed by Dimmeler and Brune (1993), suggesting the existence of another mechanism in addition to auto-ADP-ribosylation, for NO inhibition of ADH.

Gergel and Cederbaum (1996) reported the inhibition of ADH activity in intact rat hepatocytes or cytosol incubated with the NO donor S-nitroso-N-acetylpenicillamine (SNAP). They also found that ADH activity in rat hepatocytes was inhibited by endogenously synthesized NO. In further experiments with ADH isolated from equine liver, NO inhibition which was observed to be both concentration and time dependent, resulted in S-nitrosylation and the release of zinc from the active site of ADH. In

addition, Gergel and Cederbaum (1996) demonstrated that the effects of NO inhibition could be reversed by the reduction of nitrosylated-ADH with dithiothreitol (DTT), followed by incubation with $ZnCl_2$ to replace zinc at the active site. After DTT and $ZnCl_2$ treatment, ADH activity was still inhibited 30%, which may be accounted for by auto-ADP-ribosylation of the enzyme. (Mohr et al. 1996) confirmed that S-nitrosylation is responsible for the reversible inhibition of GAPDH while auto-ADP-ribosylation is not reversible and occurs after S-nitrosylation. Compared with GAPDH, ADH is less susceptible to auto-ADP-ribosylation (Dimmeler and Brune 1993), thus the main mode of interaction between ADH and NO is by the reversible S-nitrosylation of ADH.

NO inhibits ADH conversion of ethanol to acetaldehyde in animal tissue (Gergel and Cederbaum 1996). NO has also been shown to inhibit the (reverse reaction) conversion of acetaldehyde to ethanol by ADH2 in *Entamoeba histolytica* (a protozoan parasite) (Siman-Tov and Ankri 2003). Inhibition of ADH2 was also reversed by incubation with DTT (Siman-Tov and Ankri 2003). These results may suggest that NO could inhibit ADH conversion of acetaldehyde to ethanol in plant tissue, extend shelf life and enhance the edible quality of fresh-cut produce.

1.4.8.2 Inhibition of ADH activity in plant tissue

While some work has been conducted on the general effects of NO on the shelf life of commercially viable plant products, little effort has been made to understand the biochemical basis for these effects or the effect of NO on anaerobic respiration in plant materials. Previous research on NO was carried out in low (< 1%) O_2 atmospheres for periods of time up to 2 hours due to the rapid oxidation of NO to NO_2 and other oxides of nitrogen and a concern for the undesirable effects of anaerobic

respiration in the absence of O_2. The half life ($t_{½}$) of NO in air was reported as 5 seconds (Snyder 1992), whilst subsequent research reported a $t_{½}$ of 18 hours in 0.3% O_2 and 3.5 hours in air (Soegiarto et al. 2003). Previous researchers did not investigate the effects of NO under anaerobic conditions and thus missed the opportunity to discover the possible role of NO in inhibiting anaerobic respiration in plant tissue.

CHAPTER TWO

OBJECTIVES

2.1 Overall objective

The overall objective of this research is to determine the effect of nitric oxide and nitrite on yeast and apple alcohol dehydrogenase (ADH) activity and to determine whether these compounds can be used to extend the shelf life of fresh-cut apples.

2.1.1 Role of nitric oxide and nitrite in yeast ADH activity

Determine whether nitric oxide and/or nitrite inhibit yeast alcohol dehydrogenase activity.

2.1.2 Role of nitric oxide and nitrite in apple ADH activity

Determine if nitric oxide and nitrite inhibit anaerobic respiration in fresh-cut apples by inhibiting alcohol dehydrogenase and subsequent ethanol accumulation.

2.1.3 Shelf life extension of fresh-cut apples

Determine if treatment with nitric oxide and/or nitrite will extend the shelf life of fresh-cut apples.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Overview general methods

3.1.1 Preparation of nitric oxide stock solutions

Nitric oxide stock solutions were prepared by measuring 19 mL of degassed 0.1 M phosphate buffer pH 7.1, into a 20 mL vial which was sealed with a septum stoppered screw cap. The vial headspace was flushed with N_2 (15 mL/min) for 3 min to obtain less than 0.1% O_2 (checked with gas chromatography). The vial was placed on ice and cooled to 1 °C. Nitric oxide (100%, 2 mL) was then injected into the phosphate buffer. The stock solution was kept on ice until diluted to the desired NO concentration with degassed 0.1 M phosphate buffer (pH 7.05, 1 °C). The concentration of nitric oxide was determined using the Greiss reagent assay (Green et al. 1982).

3.1.2 Greiss reagent assay

Greiss Reagent (1:1 v/v solution of N-(1-Naphthyl)ethylenediamine dihydrochloride [NED 0.1% in distilled water] and sulfanilamide [1% sulfanilamide in 5% concentrated H_3PO_4]) (Green et al. 1982) was used to assay NO in solution. NO solution 9 μL was added to 1.8 mL Greiss reagent and absorbance measured at 546 nm (after incubation at room temperature, 25 °C for 15 minutes) using a Jenway 6300 Spectrophotometer (Jenway Ltd. Essex, UK). A standard curve was prepared from the absorbance of known concentrations of $NaNO_2$ (9 μL) added to Greiss reagent.

Greiss reagent was also used to determine the concentration of NO in prepared gas mixtures. 1.8 mL of Greiss reagent was pipetted into a 2 mL glass vial. The vial was sealed with a screw cap top containing a rubber septum. NO gas mixtures (0.1 mL) were injected into the vial and dissolved in solution to form nitrite which turned the

Greiss reagent purple. Absorbance (546 nm) was measured after incubating vials at room temperature 25 °C for 15 minutes. A standard curve was prepared from the absorbance of known concentrations of $NaNO_2$ added to Greiss reagent.

3.2 Effect of nitric oxide and nitrite on yeast ADH

3.2.1 Nitric oxide and yeast ADH activity

Yeast ADH (0.001 mg/mL, 0.45 units/mL) 25 µL was incubated with 875 µL (0.5, 1 and 2 mM, pH 7.05) NO solution for 0, 5, 15, 30, 45 and 60 minutes at 30 °C. Phosphate buffer 875 µL (0.1 M, pH 7.05) served as control. ADH activity was then determined by adding 50 µL of 4 mM β-NADH to the pre-incubated enzyme solution followed by 50 µL (45 mM) acetaldehyde to start the reaction. The conversion of acetaldehyde to ethanol by yeast ADH was monitored (in triplicate) by measuring absorbance (340 nm) at 2 second intervals for 2 minutes. The decrease in absorbance was linear with time during this period and the molar extinction coefficient of 6220 M^{-1} cm^{-1} for β-NADH was used to calculate the activity of ADH.

3.2.2 Sodium nitrite and yeast ADH activity

Yeast ADH (0.001 mg/mL, 0.45 units/mL) 25 µL was incubated with 875 µL (0.5, 1 and 2 mM, pH 7.05) $NaNO_2$ solution for 0, 5, 15, 30, 45 and 60 minutes at 30 °C. ADH activity was determined as outlined in method 3.2.1.

3.3 Michaelis Menten kinetics of yeast ADH

3.3.1 Michaelis Menten kinetics of yeast ADH in the presence of nitric oxide

Yeast ADH (0.001 mg/mL, 0.45 units/mL) 25 µL was pre-incubated for 20 minutes in 875 µL phosphate buffer 0.1 M with or without (0.5, 1 and 2 mM NO). NADH (4

mM, 50 μL) was added to the pre-incubated enzyme followed by 50 μL acetaldehyde (4.5, 9, 18, 36 and 72 mM) to start the reaction. The rate of formation of ethanol from acetaldehyde by yeast ADH was determined in triplicate. A LineWeaver Burke plot was used to determine the Vmax, Km and the type of inhibition exhibited by NO.

3.4 Apple preparation

'Delicious' apples harvested from Cornell Orchards were stored in controlled atmospheres (CA) of 2 – 2.2% O_2, and 1.4 – 1.5% CO_2, (balance N_2) at 1° C for 1 to 5 months. Apples were removed from CA, stored in air at 2 ºC and processed within 2 weeks. Prior to processing, apples were removed from cold storage and kept overnight at room temperature (25 ºC). The apples were washed in deionized water (25 ºC), peeled and placed in deionized water (25 ºC) until they were cored (1cm diameter) and sliced into 8 or 16 wedges each, using a hand operated corer/slicer and a sharp knife.

3.4.1 Color Measurement

Average color values (CIE L*, a* and b*) of the apple slices were measured with a color meter (Minolta Chroma Meter, Model CR-100, Minolta Camera Co. Ltd. Ramsey, NJ) which was calibrated with a standard white tile (Y = 87.8, x = 0.310, y = 0.317). Color was measured using the method of Amissah et al. (2006).

3.4.2 Firmness Measurement

Firmness was measured using a back extrusion cell in the procedure described by Bourne and Moyer (1968). An Instron Universal Testing Machine (Model 1122, Instron Corporation, Canton, MA) fitted with a 5.8 cm I.D. and 9.7 cm high stainless steel cup and a 4.9 cm wide plunger was used. Each slice was cut into two pieces and

70g placed in the cup, then extruded at 50 $mm.min^{-1}$ with the plunger. Firmness was the maximum force expressed as Newtons (N).

3.4.3 Analysis of acetaldehyde, ethanol and ethyl acetate in apple tissue

Slices were treated with liquid nitrogen (LN_2) and pulverized in a Hamilton Beach blender (Scovill Inc. Washington, NC). Triplicate 5g portions of pulverized apple were placed in septum stoppered glass vials and 0.25 mL of aqueous 4.16 mM 1-propanol added as an internal standard. Vials were stored at -20 ºC until analysis, then heated at 64 ºC for 20 min. The concentration of acetaldehyde, ethanol and ethyl acetate in the headspace was determined by gas chromatography (Varian 6000 (Walnut Creek, CA); DB™ Wax fused silica capillary column 15 m x 0.53 mm with a 1.0 μm film (J&W Scientific, Folstom, CA). The column temperature was maintained at 65 °C. Peak areas were compared to the 1-propanol standard by integration (Varian 4290, Walnut Creek, CA).

3.4.4 Minimum NO concentration required to inhibit ADH in 400 g apple tissue

'Delicious' apples were prepared as above and each apple was sliced into 16 wedges. Apple slices (400 g) were packed into 3.3 L stainless steel anaerobic vessels (Torbal model AJ-2, Torsion Balance Co. Clifton, NJ) fitted with a vacuum/pressure gauge and Swagelok regulating stem valves at the inlet and outlet ports). The vessels were flushed with 100% N_2 for 10 minutes at a flow rate > 700 mL/min to obtain an atmosphere with less than 0.1% O_2. Vacuum (25 torr) was pulled on all vessels and released with 0.25, 0.5, 0.75 and 1% (v/v) NO, (balance N_2) or 100% N_2 (control). NO headspace concentrations were determined using the greiss reagent assay (above). Table 3 (below) shows the total number of moles of NO in the headspace. The

anaerobic vessels were stored at 6.1 ºC for 2 days. Apple slices were analyzed for the accumulation of acetaldehyde, ethanol and ethyl acetate and color as described earlier.

Table 3: NO headspace concentrations (Moles/L) in anaerobic pouches (0.4 L) and vessels (3.3 L), containing 100, 200 or 400 g of apple slices.

NO headspace Concentration		Apple		Pouch or Vessel Headspace	
% (v/v)	Moles/L of NO*	Weight (g)	Volume (L)	Volume (L)	Total NO (Moles)
1	4.1×10^{-4}	100	0.13	0.27	1.1×10^{-4}
1	4.1×10^{4}	200	0.27	0.13	5.3×10^{-5}
1	4.1×10^{-4}	400	0.53	2.77	11.4×10^{-4}
0.75	3.1×10^{-4}	400	0.53	2.77	8.6×10^{-4}
0.5	2.0×10^{-4}	400	0.53	2.77	5.5×10^{-4}
0.25	1.0×10^{-4}	400	0.53	2.77	2.8×10^{-4}
0.125	5.1×10^{-5}	400	0.53	2.77	1.4×10^{-4}

* Concentration of NO (Moles/L) was calculated using a temperature of 25 °C.

3.4.5 In situ inhibition of apple ADH by 1% NO

Apples were removed from cold storage and kept overnight at (25 °C), and then washed, peeled and dipped in deionized water (25 ºC), sliced into 16 wedges and again dipped in deionized water. Slices were blotted dry with cheese cloth and 100 or 200 g packaged in triplicate in 13 x 22 cm (0.4L) pouches, made from a high O_2 barrier plastic film (CVP Systems, Cold Stream, IL). The film was a laminate of nylon (0.8 mil), ethyl vinyl acetate (1.2 mil) and surlyn (2 mil) with gas transmission rates ($cc/m^2/24hr$) of 28 to 38 for O_2, 4 to 7 for N_2 and 108 to 128 for CO_2.

The pouches were flushed with 1% NO (balance N_2) for 2 minutes and heat sealed using a Food Saver vacuum/heat sealer (Tilia, Inc., San Francisco, CA). The O_2 content of the pouches was less than 0.1% (checked by GC). Table 3 (above) shows the total number of moles of NO in the pouch headspace. Control pouches were flushed with 100% N_2 for 2 minutes. The pouches were stored at 6.1 ºC for 3 days.

Apple slices were analyzed for the accumulation of acetaldehyde, ethanol and ethyl acetate as described above.

3.4.6 Shelf life of fresh-cut apple treated with NO or sodium nitrite

Eight wedges/apple were prepared as above. Apple slices (650 g) were placed in 3.3 L anaerobic vessels and flushed with 100% N_2 for 10 minutes at a flow rate > 700 mL/min to obtain an atmosphere with < 0.1% O_2. The composition of the atmosphere in the anaerobic vessel was determined using a gas chromatograph Varian Aerograph moduline 2700 (Varian, Walnut Creek CA) with a molecular sieve N_2/O_2 5A packed column. Vacuum (25 torr) was pulled on all vessels and released with 100% N_2 (control) or 1% (v/v) NO (balance N_2). Anaerobic vessels were stored at 25 ºC for 20 minutes, then flushed with 100% N_2 for 10 minutes at a flow rate > 700 mL/min. Slices (200 g) were packed (in triplicate) in 20 x 22 cm pouches made from a high O_2 barrier plastic film as above. The pouches were flushed with 100% N_2 for 2 minutes and heat sealed as above. The O_2 content of the pouches was less than 0.1% as determined by GC. The pouches were stored in the dark at 6.1 ºC. For sodium nitrite treatment, apple slices were dipped in deionized water (control) or 2mM $NaNO_2$ for 20 minutes, then blotted dry with cheese cloth. Apple slices (200 g) were packed (in triplicate) in high O_2 barrier (20 x 22 cm) pouches, flushed with 100% N_2 for 2 minutes and heat sealed. The pouches were stored at 6.1 ºC.

The average color values (CIE L*, a* and b* values) of six slices from each pouch, three readings per slice were measured weekly in triplicate (using method 3.4.1). Firmness was measured weekly in triplicate (using method 3.4.2) with one firmness reading per pouch. Slices were also analyzed weekly in triplicate for the accumulation of acetaldehyde, ethanol and ethyl acetate, using method 3.4.3 with modifications. The

column temperature was maintained at 55 °C and standard curves were prepared for each compound.

3.4.7 ADH activity of fresh-cut apple treated with NO or sodium nitrite

Apples were prepared using (method 3.4) and sliced into wedges (8 or 16 per apple) or horizontally 1.5 or 3 mm thick. The horizontal slices (200 g) were placed on stainless steel rods with 0.5 mm stainless steel separators between slices. Apples (200 g) were placed in 3.3 L anaerobic vessels and treated with 1% NO (balance N_2) or 100% N_2 (control) for 20 minutes. The experiment was repeated by dipping apple slices (200 g) in solutions of 2 mM $NaNO_2$ or deionized water (control) for 20 minutes.

ADH activity of sliced apple was determined using the method of Ke et al. (1994) with modifications. ADH activity was determined in triplicate and each replicate analyzed 3g of tissue from 3 wedges or 3 horizontal slices. Apple tissue (3g) was homogenized in 10 mL of 100 mM 2-(N-morpholino)ethane-sulfonic acid (MES) buffer (pH 6.0) with added 2 mM dithiothrietol (DTT) and 1% (w/v) polyvinylpyrolidone (PVP), filtered through four layers of cheese cloth, then centrifuged at 27,000 g for 10 minutes at 4 °C. The supernatant was decanted and placed on ice as enzyme extract. ADH activity of the enzyme extract was then analyzed in triplicate at 30 °C by adding 0.05 mL of 80 mM acetaldehyde to a mixture of 0.8 mL of 100 mM MES buffer (pH 6.0), 0.05 mL of 1.6 mM NADH and 0.1 mL of enzyme extract. The decrease in absorbance (340 nm) due to the oxidation of NADH was measured using a Jenway spectrophotometer (Jenway Ltd. Felsted, UK).

3.5 Statistical analysis

The data was analyzed using Minitab Release 15 (Minitab Inc., State College, PA). The General Linear Model was used to determine the treatment and interaction effects. Tukey's test was used to compare the different factor levels. Data for ethanol accumulation were analyzed by regression.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Results

4.1.1 Effect of nitric oxide and nitrite on yeast ADH activity

4.1.1.1 Nitric oxide and yeast ADH activity

The activity of yeast ADH (100% = 22.62 μM/min) pre-incubated with buffer only (control) decreased with time and became significant ($p \leq 0.05$) after 45 minutes. NO treatment (0.5 – 2 mM) inhibited yeast ADH activity in a dose and time dependent manner ($p \leq 0.05$; Figure 3). Compared with the control, NO (1 and 2 mM) inhibition was significant ($p \leq 0.05$) at the start of the experiment but 0.5 mM NO required at least 5 minutes pre-incubation time.

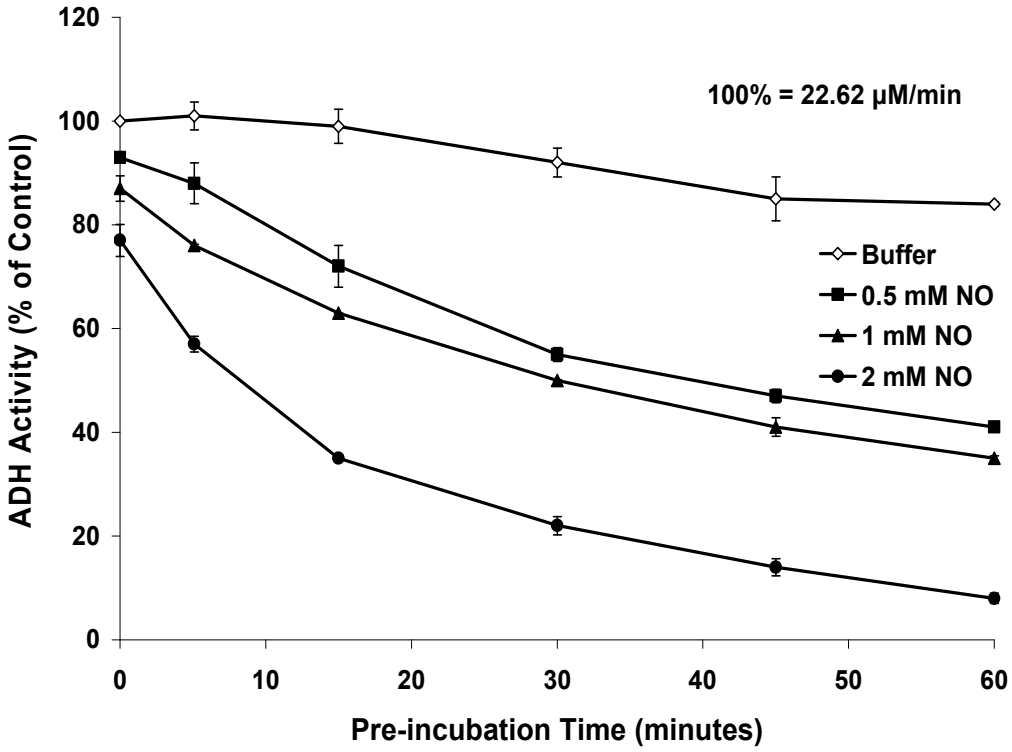

Figure 3: Activity of yeast ADH pre-incubated with or without 0.5 – 2 mM NO for 0 – 60 minutes. 100% activity = 22.62 μM/min.

4.1.1.2 Sodium nitrite and yeast ADH activity

Yeast ADH activity however, was not inhibited by pre-incubation with $NaNO_2$ (at pH 7.05) for 0 - 60 minutes ($p \leq 0.05$; Figure 4).

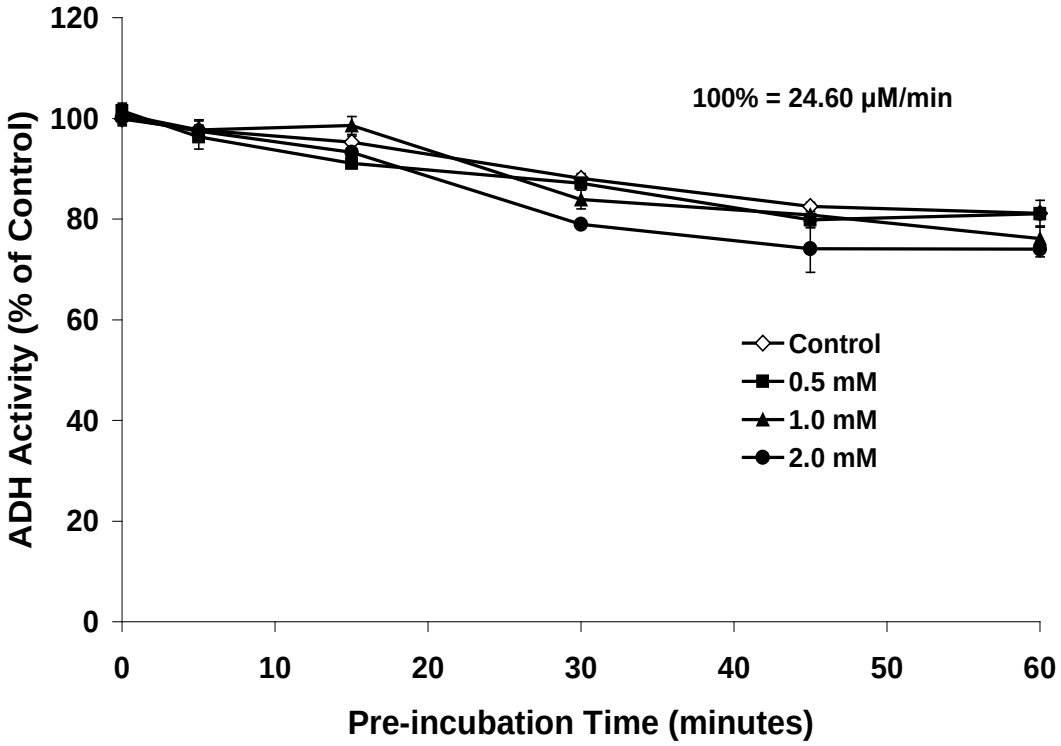

Figure 4: Activity of yeast ADH pre-incubated with or without 0.5 – 2 mM $NaNO_2$ for 0 – 60 minutes. 100% activity = 24.60 µM/min.

4.1.2 Michaelis Menten kinetics of yeast ADH

4.1.2.1 Michaelis Menten kinetics of yeast ADH in the presence of nitric oxide

Michaelis Menten constants Vmax and Km were determined for yeast ADH after pre-incubation for 20 minutes with or without NO (0.5 – 2 mM). NO was observed to inhibit yeast ADH activity in a dose dependent manner (Figure 5). The LineWeaver Burke plot (Figure 5) indicates that NO inhibits yeast ADH in a noncompetitive manner. These results suggest that the inhibition is reversible and NO does not bind to

the active site of yeast ADH. The value of Vmax decreased with increasing NO concentration ($p \leq 0.05$), but Km was not affected, confirming the noncompetitive nature of NO inhibition (Table 4).

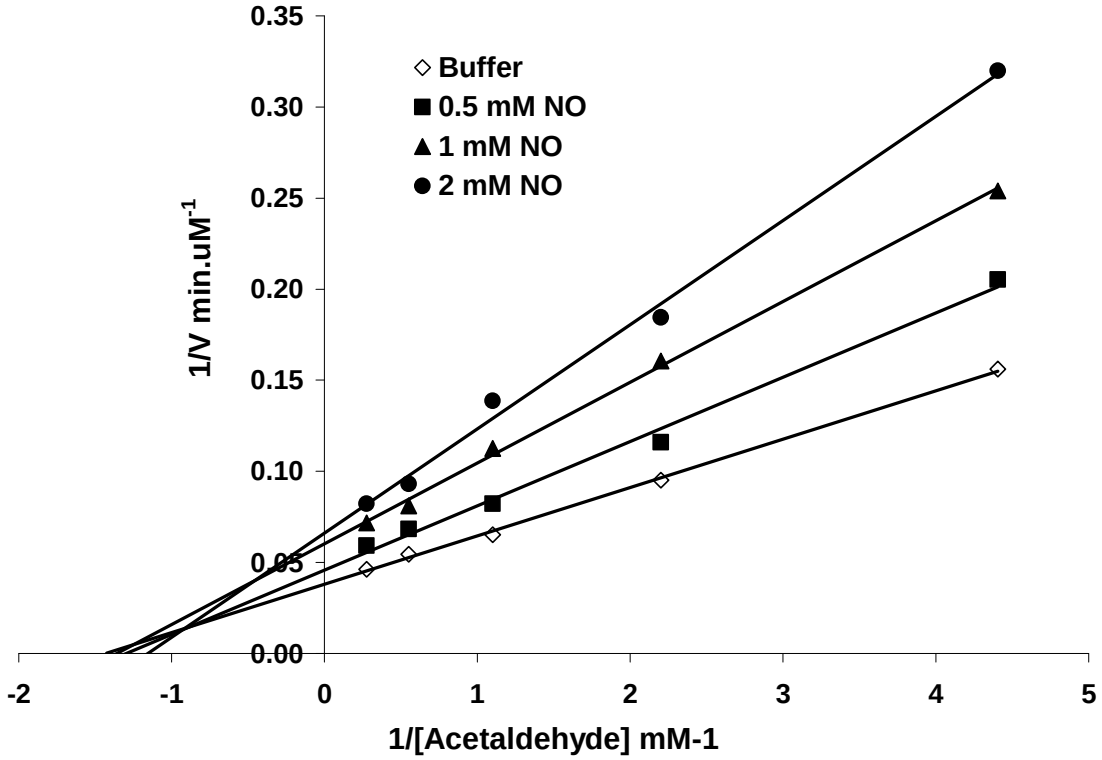

Figure 5: LineWeaver Burke plot of yeast ADH pre-incubated with or without 0.5 – 2 mM NO for 20 minutes.

Table 4: Km and Vmax values for Yeast ADH pre-incubated for 20 minutes with or without 0.5 – 2 mM NO

	Km (mM)	Vmax µM.min^{-1}
Buffer	0.70	26.32 a
0.5 mM NO	0.78	21.87 b
1 mM NO	0.73	16.61 c
2 mM NO	0.87	15.14 c

Means with different letters in the same column are significantly different ($p \leq 0.05$)

4.1.3 Minimum NO concentration required to inhibit ADH activity in 400 g apple tissue

Ethanol accumulation when 400 g of apple slices were packed in 3.3 L anaerobic vessels and treated with 1% NO (v/v, headspace) was inhibited 52% compared to slices treated in N_2 only ($p \leq 0.05$; Figure 6). Treatment with NO concentrations less than 1% (v/v, headspace, balance N_2) had no significant effect on ethanol accumulation under the conditions of the experiment although the general trend was that ethanol content decreased as NO concentration increased (Figure 6). Slices treated with NO concentrations of 0.25 to 1% (v/v headspace) accumulated 42% more acetaldehyde compared to slices treated with N_2 only ($p \leq 0.05$; Figure 6). Ethyl acetate accumulation was inhibited 35% in slices treated with 1% NO (v/v headspace) compared with slices treated with N_2 only ($p \leq 0.05$; Figure 6). Treatment with NO concentrations less than 1% had no significant effect on ethyl acetate accumulation (Figure 6).

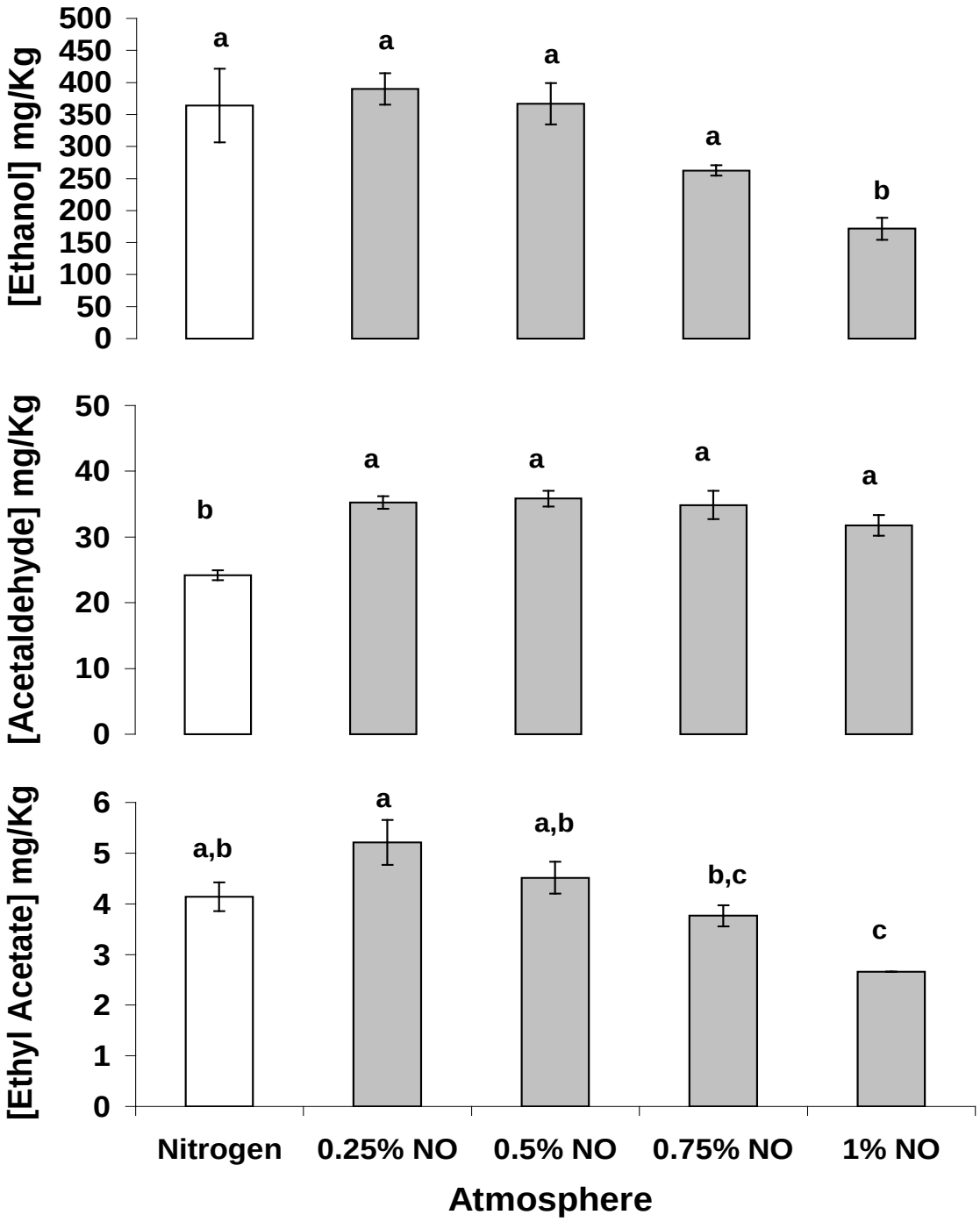

Figure 6: Ethanol, acetaldehyde and ethyl acetate concentration (mg/Kg) of sliced 'Delicious' apples (400 g) stored in 3.3 L anaerobic vessels in 100% N_2 (□) or 0.25 – 1% (v/v) NO (■), (balance N_2) atmospheres at 6.1 °C for 2 days. Bars represent the standard error (± S.E.). Means for the same compound with different letters are significantly different ($p < 0.05$).

4.1.4 In situ inhibition of apple ADH by 1% NO

Ethanol accumulation when 100 or 200 g of apple slices were packaged in 0.4 L high-barrier pouches and treated with 1% NO (v/v; headspace) was inhibited 28% compared to slices treated with N_2 only ($p \leq 0.05$; Figure 7). Slices treated with 1% NO also accumulated twice as much acetaldehyde ($p \leq 0.05$) as those treated with N_2 only (Figure 7). There was no significant effect of NO treatment on ethyl acetate accumulation although ethyl acetate levels tended to be lower in NO-treated slices (Figure 7). Acetaldehyde, ethanol, and ethyl acetate accumulation (in slices stored in 0.4 L pouches) were unaffected by the weight of apple (100 or 200 g) treated with 1% NO. Initial acetaldehyde, ethanol and ethyl acetate concentrations were all below the limit of detection.

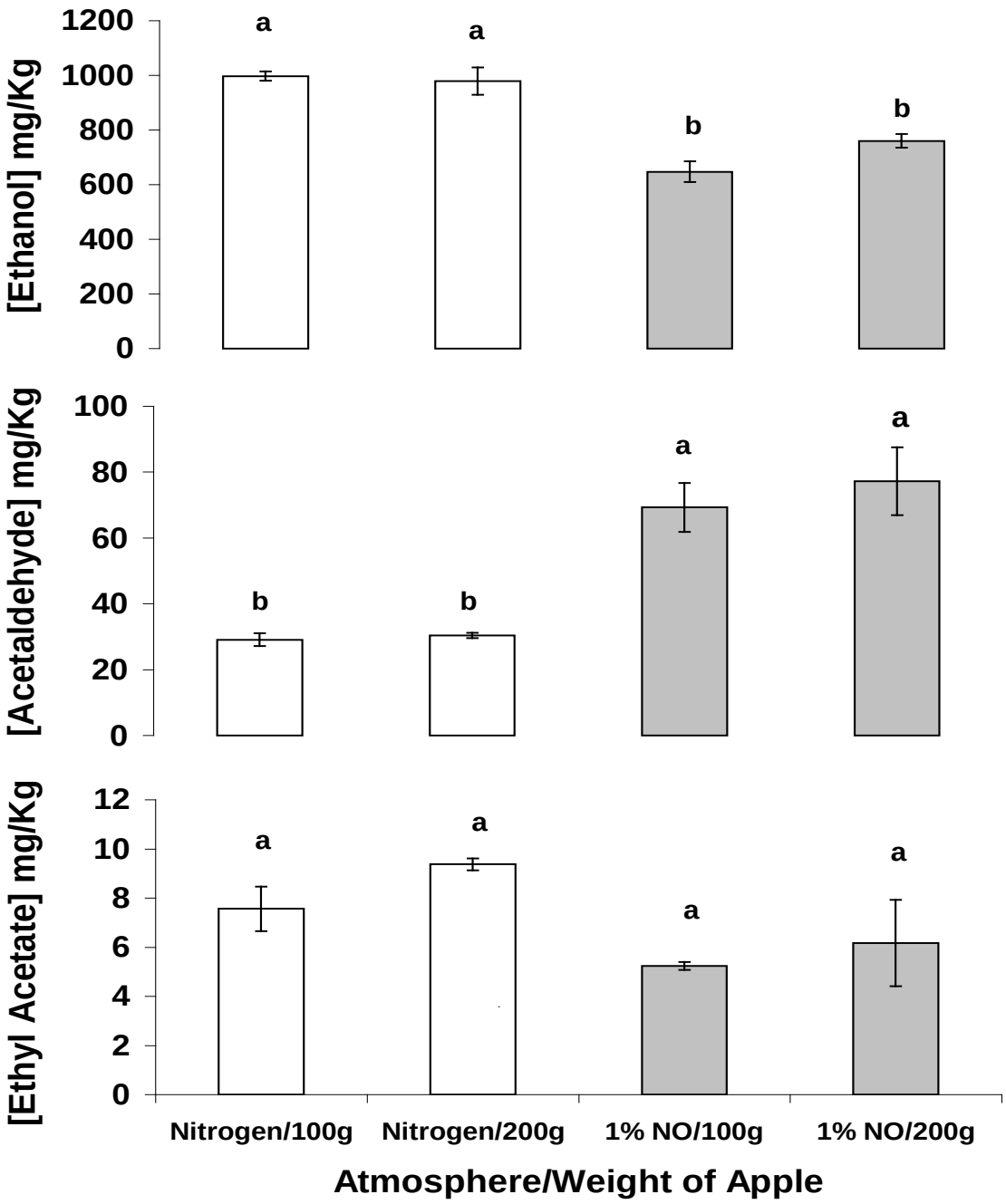

Figure 7: Ethanol, acetaldehyde and ethyl acetate concentration (mg/Kg) sliced 'Delicious' apples (100 and 200 g) stored in high O_2 barrier pouches in 100% N_2 (□) or 1% (v/v) NO (■), (balance N_2) atmospheres at 6.1 °C for 3 days. Bars represent the standard error (± S.E.). Means for the same compound with different letters are significantly different ($p < 0.05$).

4.1.5 Shelf life of fresh-cut apple treated with NO or sodium nitrite

Nitric oxide treated slices accumulated less ethanol than slices treated with N_2 only when packaged in 20 x 22 cm (high O_2 barrier) pouches and stored at 6.1 °C (Figure 8). In slices treated with N_2 only or 1% v/v NO (balance N_2) there was a linear increase in ethanol accumulation (Figure 8) with storage time. The accumulation of ethanol is described by the linear regression model: $Y = 957 + 331(a) – 606(b)$ with $R^2 = 80\%$, where Y = Ethanol accumulation; a = Time and b = Treatment. On average, NO treatment decreased ethanol accumulation by 606 mg/kg over the 6 week storage period.

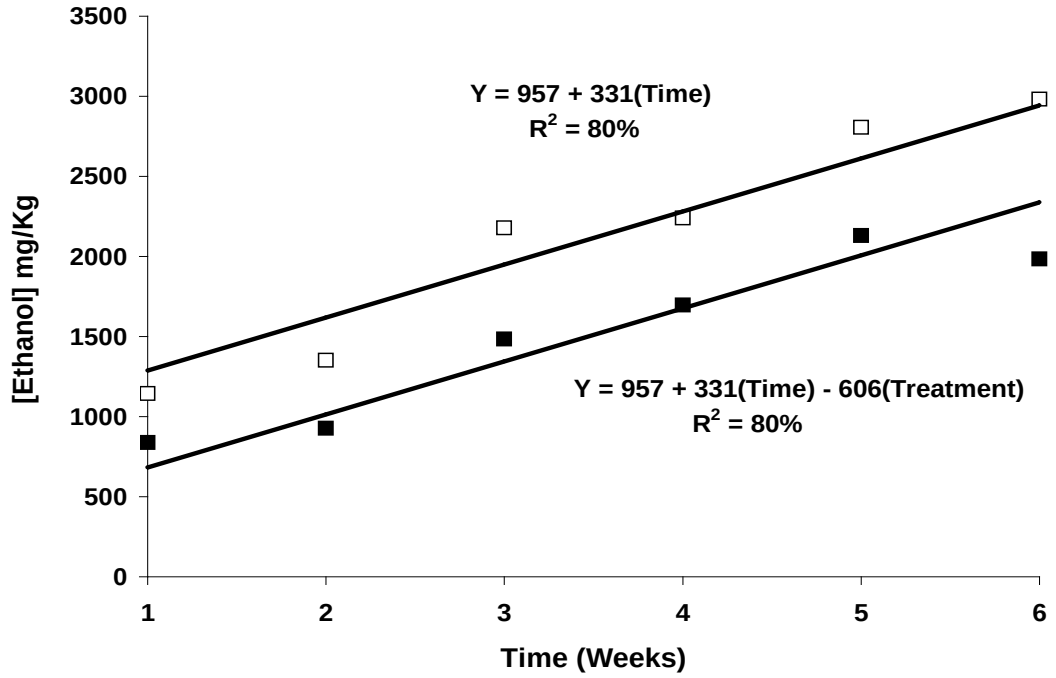

Figure 8: Ethanol concentration (mg/Kg) of sliced 'Delicious' apples treated with N_2 (□) only or 1% NO (■) (balance, N_2) atmosphere for 20 minutes and stored for 1 to 6 weeks in 100% N_2 atmosphere.

Similar results were observed for slices treated with deionized water or 2 mM $NaNO_2$. Slices treated with 2 mM $NaNO_2$ accumulated less (Figure 9) ethanol than slices treated with deionized water. There was a linear increase in ethanol accumulation with storage time in slices treated with either deionized water or sodium nitrite. The accumulation of ethanol is described by the linear regression model: $Y = 718 + 598(a) - 441(b)$ with $R^2 = 91\%$, where Y = Ethanol accumulation; a = Time and b = Treatment. On average treatment with 2 mM $NaNO_2$ decreased ethanol accumulation by 441 mg/kg over the 6 week storage period.

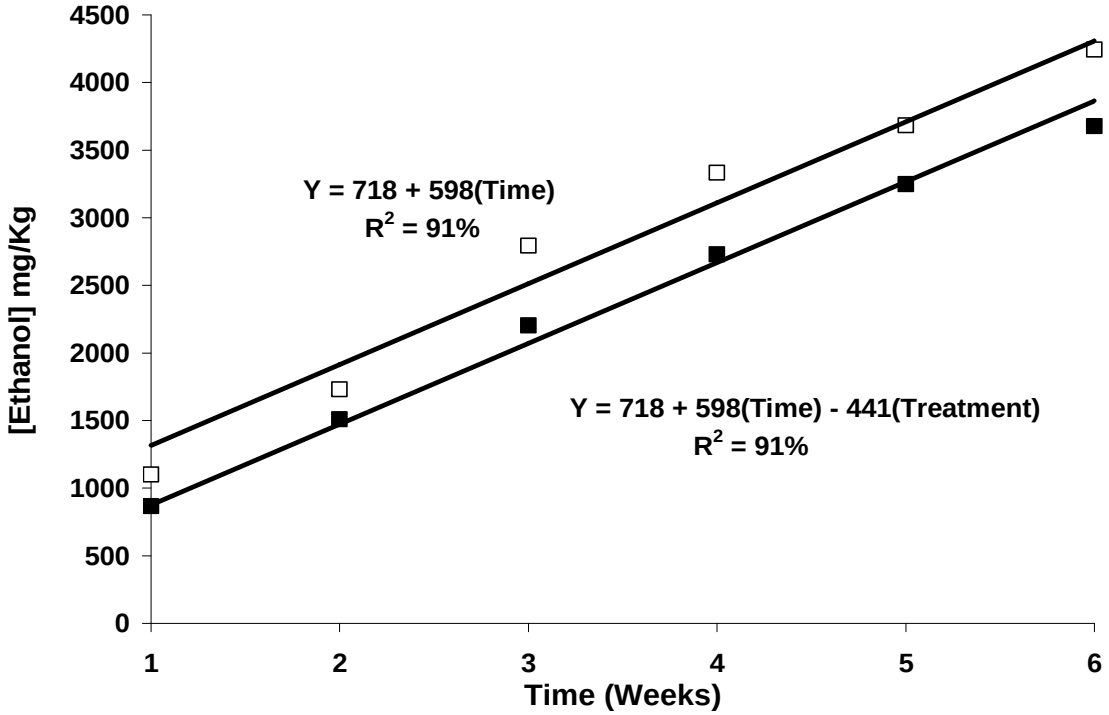

Figure 9: Ethanol concentration (mg/Kg) of sliced 'Delicious' apples treated with deionized water (□) or 2 mM $NaNO_2$ (■) for 20 minutes and stored for 1 to 6 weeks in 100% N_2 atmosphere.

Acetaldehyde accumulation in nitric oxide treated slices was higher ($p \leq 0.05$, Figure 10) than in N_2 treated slices. Acetaldehyde accumulation in slices treated with N_2 or 1% nitric oxide (balance N_2) was not affected by storage time. Slices treated with 2

mM $NaNO_2$ accumulated higher ($p \leq 0.05$, Figure 11) concentrations of acetaldehyde than slices treated with deionized water. Acetaldehyde accumulation was unaffected by storage time in slices treated with 2 mM $NaNO_2$, but decreased in the sixth week in slices treated with deionized water ($p \leq 0.05$, Figure 11).

Slices treated with 1% NO (balance N_2) accumulated less ethyl acetate than slices treated with N_2 only ($p < 0.05$, Figure 12). Sodium nitrite treatment however, had no effect on ethyl acetate accumulation when compared to deionized water treatment (Figure 13). Ethyl acetate accumulation was unaffected by storage time in slices treated with 1% NO (balance N_2) or N_2 only (Figure12), but increased with storage time in slices treated with deionized water or 2 mM $NaNO_2$, reaching a maximum in the third week and decreasing thereafter ($p \leq 0.05$, Figure 13).

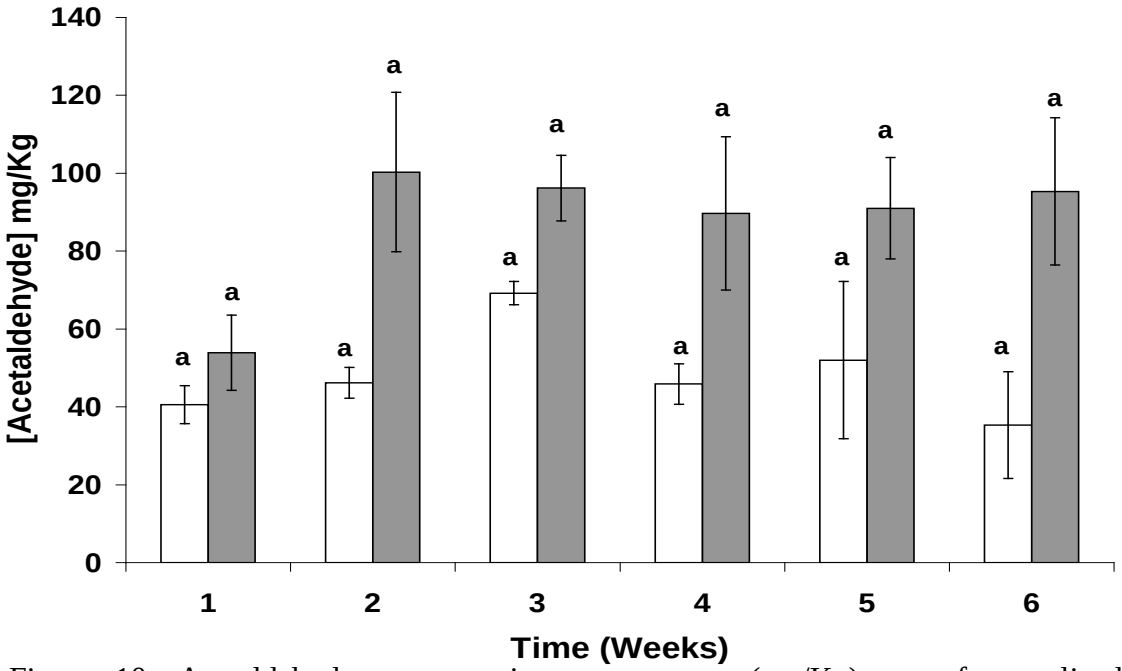

Figure 10: Acetaldehyde concentration (mg/Kg) of sliced 'Delicious' apples treated with N_2 (□) only or 1% NO (■) (balance, N_2) atmosphere for 20 minutes and stored for 1 to 6 weeks in 100% N_2 atmosphere. Bars represent the standard error (± S.E.). Means for the same treatment with the same letters are not significantly different ($p < 0.05$).

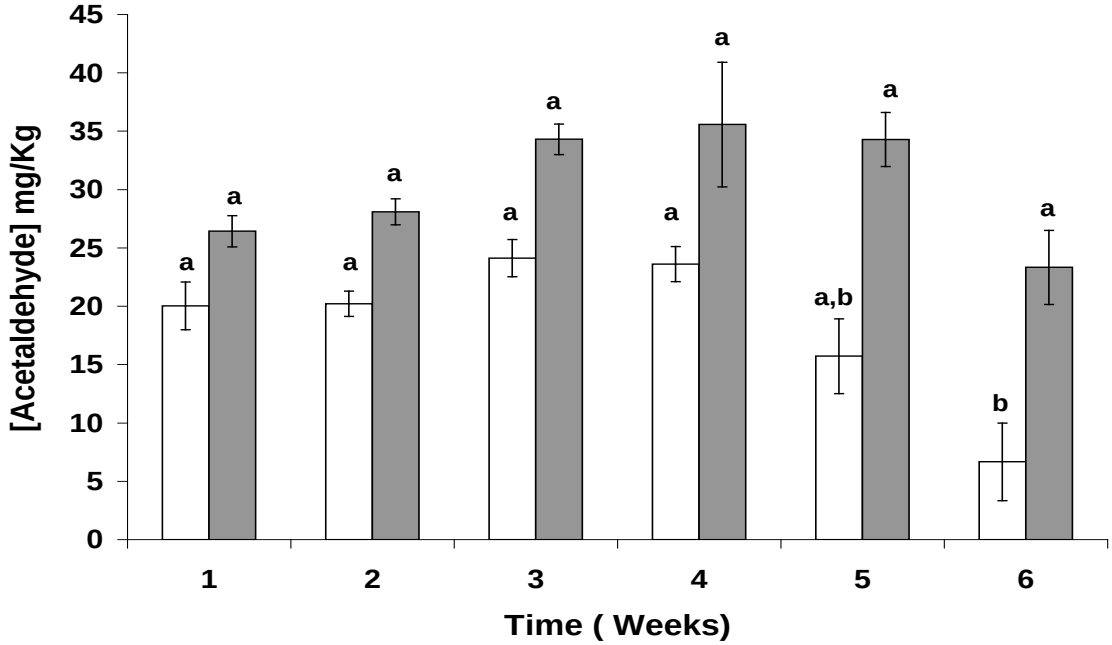

Figure 11: Acetaldehyde concentration (mg/Kg) of sliced 'Delicious' apples treated with deionized water (□) or 2 mM $NaNO_2$ (■) for 20 minutes and stored for 1 to 6 weeks in 100% N_2 atmosphere. Bars represent the standard error (± S.E.). Means for the same treatment with different letters are significantly different ($p < 0.05$).

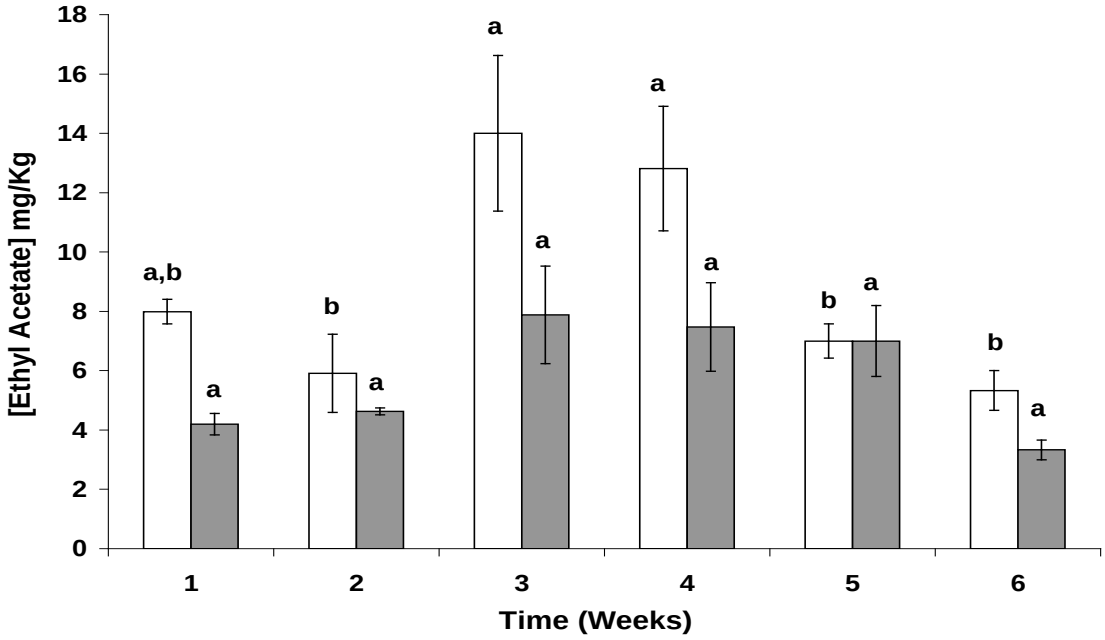

Figure 12: Ethyl acetate concentration (mg/Kg) of sliced 'Delicious' apples treated with N_2 (□) or 1% NO (■) (balance, N_2) atmosphere for 20 minutes and stored for 1 to 6 weeks in 100% N_2 atmosphere. Bars represent the standard error (± S.E.). Means for the same treatment with different letters are significantly different ($p < 0.05$).

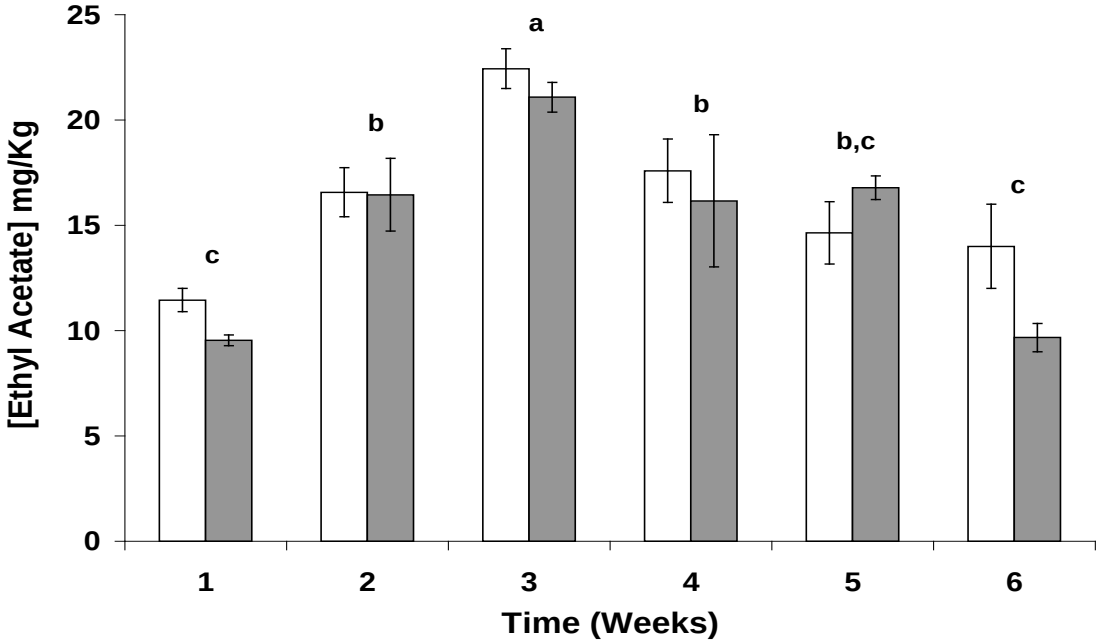

Figure 13: Ethyl acetate concentration (mg/Kg) of sliced 'Delicious' apples treated with deionized water (□) or 2 mM $NaNO_2$ (■) for 20 minutes and stored for 1 to 6 weeks in 100% N_2 atmosphere. Bars represent the standard error (± S.E.). Means for Time (weeks) with different letters are significantly different ($p < 0.05$).

Firmness of slices treated with N_2 or 1% NO (balance N_2) decreased ($p \leq 0.05$, Figure 14) with storage time. Compared to N_2 treatment, there was no effect of nitric oxide treatment on slice firmness (Figure 14). Similar results were obtained for slices treated with deionized water or 2 mM $NaNO_2$. Firmness of slices treated with deionized water or 2 mM $NaNO_2$ decreased ($p \leq 0.05$, Figure 15) with storage time. Compared with deionized water treatment, there was no effect of 2 mM $NaNO_2$ treatment on slice firmness ($p \leq 0.05$, Figure 15).

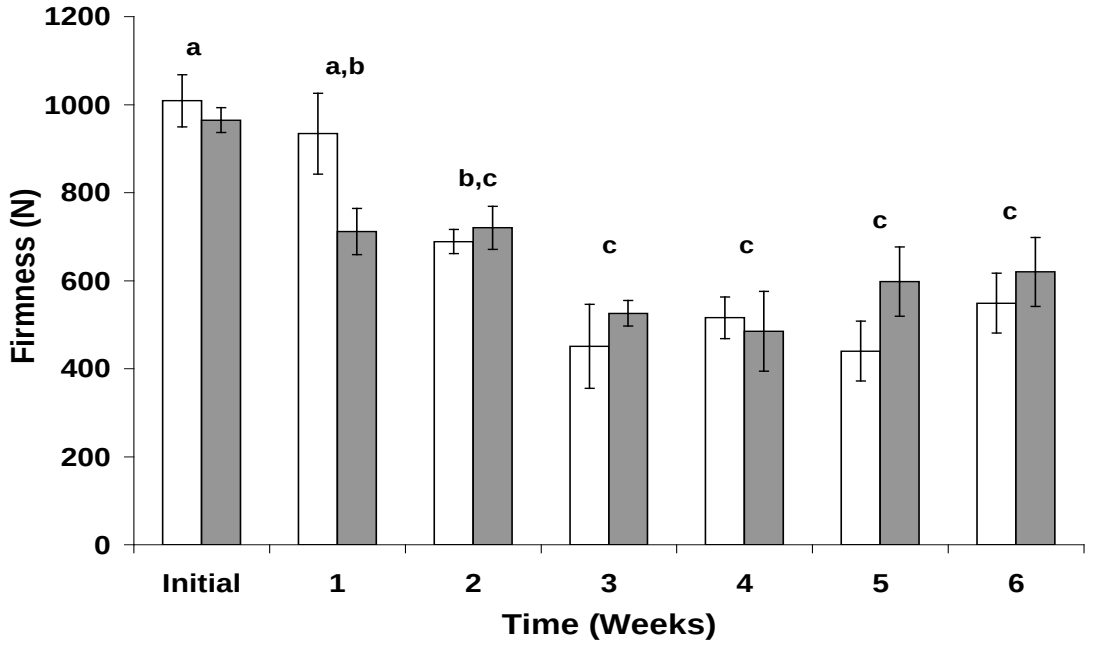

Figure 14: Firmness (N) of sliced 'Delicious' apples treated with N_2 (□) only or 1% NO (■) (balance N_2) atmospheres for 20 minutes and stored in 100% N_2 atmosphere for 1 to 6 weeks. Initial firmness was determined at the start of the experiment. Bars represent the standard error (± S.E.). Means for the storage time (weeks) with different letters are significantly different ($p < 0.05$).

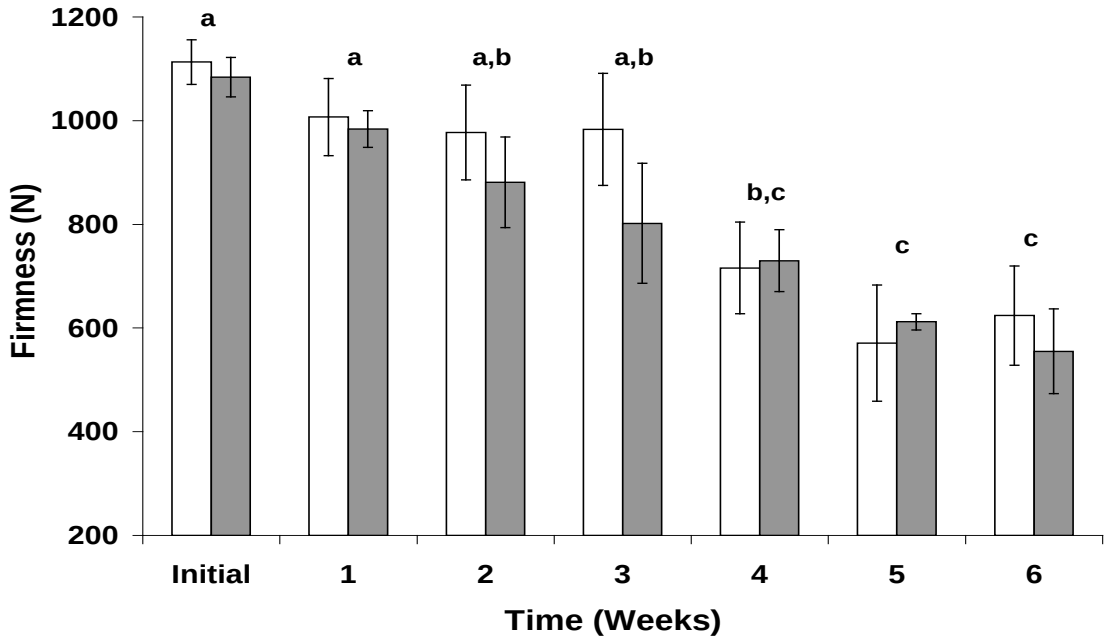

Figure 15: Firmness (N) of sliced 'Delicious' apples treated with deionized water (□) or 2 mM $NaNO_2$ (■) for 20 minutes and stored in 100% N_2 atmosphere for 1 to 6 weeks. Initial firmness was determined at the start of the experiment. Bars represent the standard error (± S.E.). Means for the storage time (weeks) with different letters are significantly different ($p < 0.05$).

4.1.5.1 L* Value of fresh-cut apple treated with NO or sodium nitrite

The highest L* Value (lightest flesh color) was observed in the initial apple slices immediately after preparation and before storage in anaerobic vessels or high barrier polymer pouches, in 100% N_2 or 0.25 to 1% NO atmosphere ($p \leq 0.05$; Figures 16, 17 and 18). Flesh darkening occurred in all slices treated with NO and N_2 only and stored in anaerobic vessels ($p \leq 0.05$; Figure 16). Slices treated with NO were darker than slices treated with N_2 only ($p \leq 0.05$; Figure 16), with lower L* Value (darker flesh color) as NO concentration was increased. The difference in color between N_2 and NO treated slices was visible to the naked eye.

Flesh darkening occurred in slices treated with N_2 or 1% NO for 20 minutes and stored in 100% N_2 in pouches ($p \leq 0.05$, Figure 17), with a decrease in L* Value as storage time increased. Slices treated with nitric oxide were darker than slices treated with N_2 ($p \leq 0.05$, Figure 17). Similar results were obtained for slices treated with deionized water or 2 mM $NaNO_2$. Slices treated with 2 mM $NaNO_2$ were lighter than slices treated with deionized water immediately after preparation, however they were darker ($p \leq 0.05$, Figure 18) than slices treated with deionized water throughout the 6 week storage period. L* Value decreased ($p \leq 0.05$) with storage time in slices treated with deionized water or 2 mM $NaNO_2$.

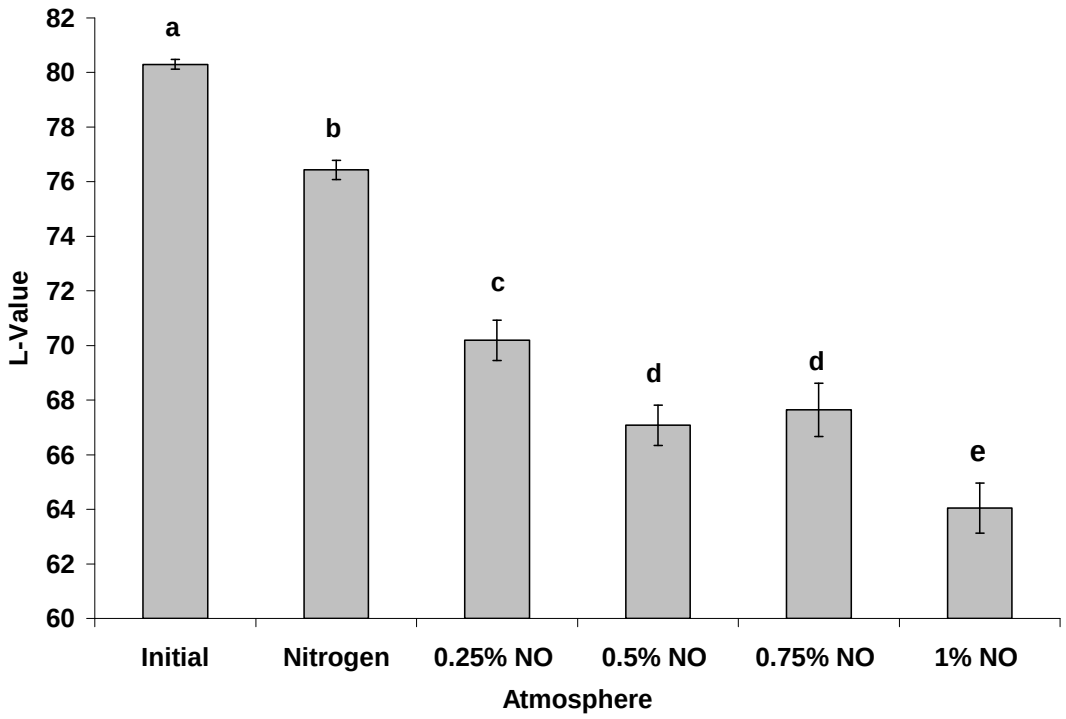

Figure 16: L* Value of sliced 'Delicious' apples 400 g (1/16th Wedges) stored in modified atmosphere for 2 days at 6.1 °C. Bars represent the standard error (± S.E.). Means with different letters are significantly different ($p < 0.05$).

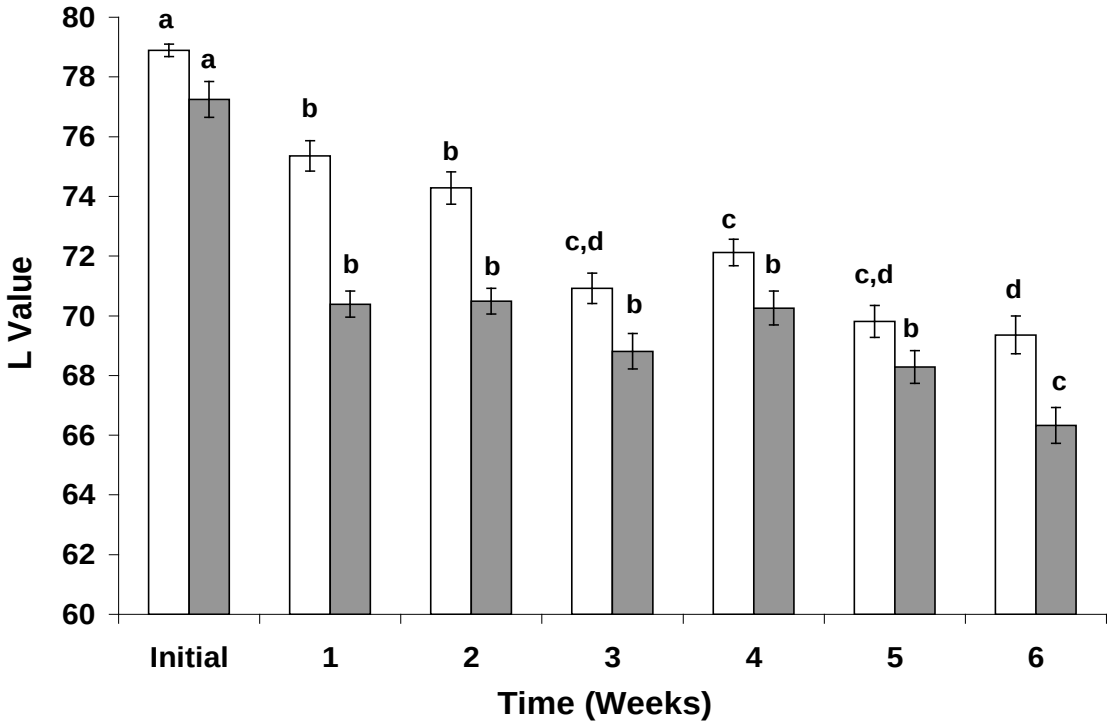

Figure 17: Lightness (L* Value) of sliced 'Delicious' apples treated with N_2 (□) only or 1% NO (■) (balance N_2) atmospheres for 20 minutes and stored in 100% N_2 atmosphere for 1 to 6 weeks. Initial L* Value was determined at the start of the experiment. Bars represent the standard error (± S.E.). Means for the same treatment with different letters are significantly different ($p < 0.05$).

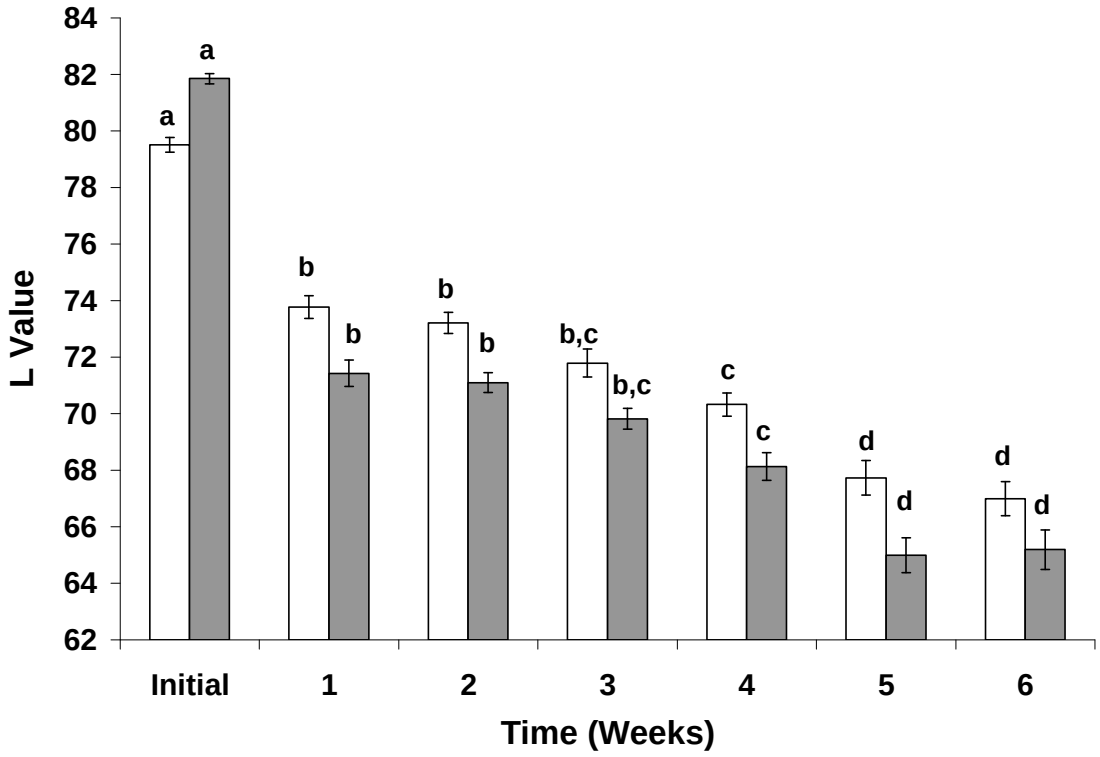

Figure 18: Lightness (L* Value) of sliced 'Delicious' apples treated with deionized water (□) or 2 mM $NaNO_2$ (■) for 20 minutes and stored in 100% N_2 atmosphere for 1 to 6 weeks. Initial L* Value was determined at the start of the experiment. Bars represent the standard error (± S.E.). Means for the same treatment with different letters are significantly different ($p < 0.05$).

4.1.6 ADH activity of fresh-cut apple treated with NO or sodium nitrite

ADH activity of the apple slices was not affected by N_2 treatment. However, compared with the N_2 controls, NO treatment inhibited ADH activity in Delicious apples ($p \leq 0.05$; Figure 19). Increasing the surface area to volume ratio of the apple slices by decreasing slice thickness (from $1/8^{th}$ to $1/16^{th}$ wedge or 3 mm to 1.5 mm) resulted in a decrease in ADH activity even though (for each slice thickness) the same weight of apple was treated ($p \leq 0.05$; Figure 19). The lowest ADH activity was

obtained in the 1.5 mm thick slices which were the thinnest and had the largest surface area to volume ratio.

Similar results were obtained in the slices treated with deionized water or 2 mM sodium nitrite treated. ADH activity was not affected by dipping in deionized water for 20 minutes. However treatment with 2 mM $NaNO_2$ inhibited ADH activity in all slices ($p \leq 0.05$; Figure 20). Increasing the surface area to volume ratio of the apple slices by cutting thinner slices ($1/8^{th}$ to $1/16^{th}$ wedge or 3 mm to 1.5 mm) resulted in a greater inhibition of ADH activity ($p \leq 0.05$; Figure 20). The 1.5 mm thick slices had the lowest ADH activity (Figure, 20).

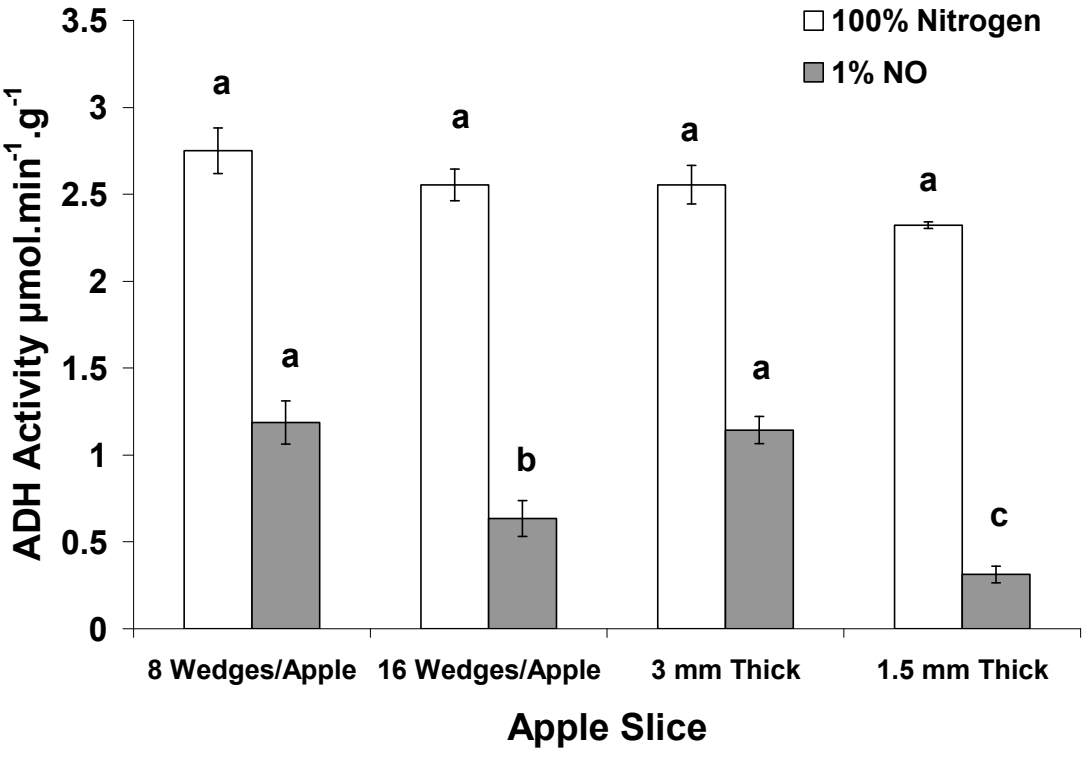

Figure 19: ADH activity $\mu mol.min^{-1}.g^{-1}$ of 200 g of 'Delicious' apple slices treated with 100% N_2 (□) only or 1% NO (■) (balance N_2) atmospheres for 20 minutes in 3.3 L anaerobic vessels. Bars represent the standard error (± S.E.). Means for the same treatment with different letters are significantly different ($p < 0.05$).

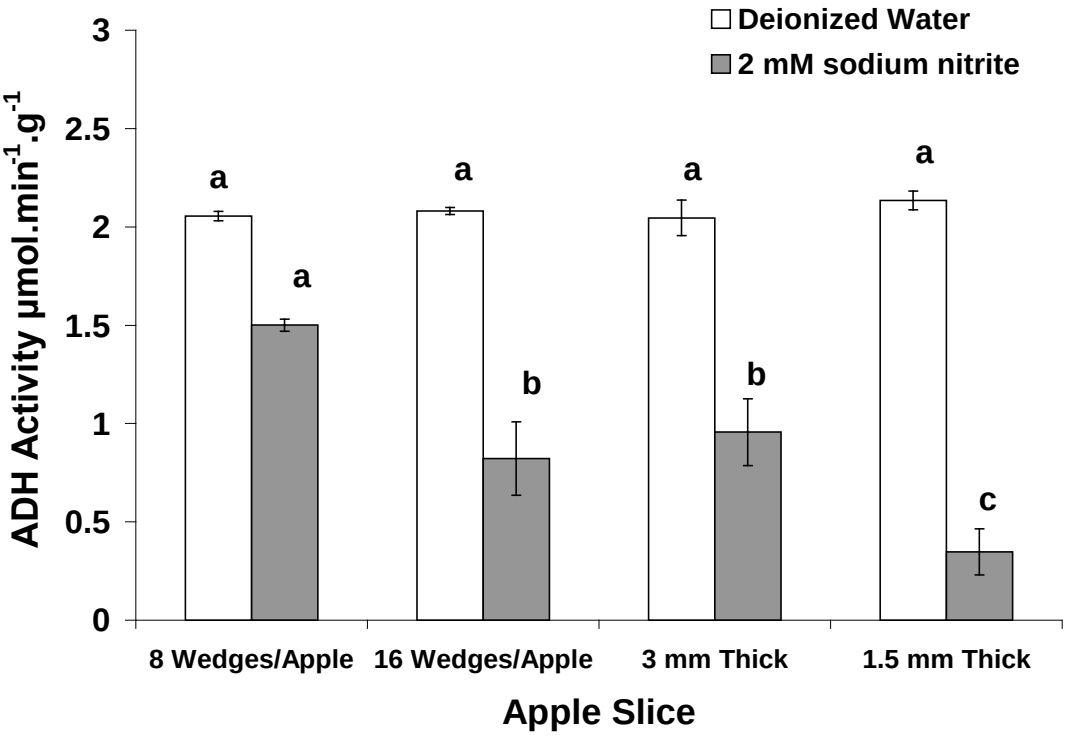

Figure 20: ADH activity µmol.min^{-1}.g^{-1} of 200 g of 'Delicious' apple slices treated with deionized water (□) or 2 mM $NaNO_2$ (■) for 20 minutes. Bars represent the standard error (± S.E.). Means for the same treatment with different letters are significantly different ($p < 0.05$).

4.2 Discussion

Yeast ADH activity was inhibited by NO (0.5 – 2 mM) at pH 7.05 in a dose and time dependent manner but was not affected by pre-incubation with $NaNO_2$ (0.5 – 2 mM). $NaNO_2$ was not converted to NO under the conditions of the experiment. It is known that $NaNO_2$ is converted to NO under acidic conditions (Hotchkiss 1989), but not at neutral pH 7.05 that yeast ADH activity was determined. $NaNO_2$ may inhibit ADH activity under acidic conditions. Prior research by Gergel and Cederbaum (1996) demonstrated the dose and time dependent NO inhibition of rat and horse liver ADH activity. Results from the LineWeaver Burke plot and Michaelis Menten constants (Vmax and Km), indicate that NO inhibits yeast ADH in a noncompetitive manner and suggests that the inhibition is reversible. Though the Michaelis Menten kinetics of

yeast ADH with NO have not been determined previously, Gergel and Cederbaum (1996) reported on the reversible nature of NO inhibition of ADH. Their results also suggested that NO did not bind directly to the active site but inhibited ADH by S-nitrosylation of the enzyme and the removal of zinc. NO inhibition of ADH in plant tissue has not been demonstrated previously, however, Siman-Tov and Ankri (2003) showed that ADH2 conversion of acetaldehyde to ethanol was inhibited by NO in *Entamoeba histolytica*. The same reaction (acetaldehyde to ethanol) occurs in plants and is catalyzed by ADH suggesting that NO may inhibit ADH and enhance the edible quality of plant tissue. Thus fresh-cut apples were treated with NO to determine whether ADH activity and the accumulation of ethanol would be inhibited.

Nitric oxide (1%) inhibited ADH in apple tissue when packaged in 0.4 L pouches or 3.3 L anaerobic vessels. As expected, ADH inhibition resulted in a decrease in ethanol accumulation and an increase in acetaldehyde. Ethyl acetate accumulation was inhibited 35% in apple slices treated with 1% NO in the 3.3 L anaerobic vessels, but was not affected in apple slices treated with 1% NO in 0.4 L pouches. This may be related to the greater inhibition of ethanol in the stainless steel anaerobic vessels compared to in the pouches. The pouches had a smaller headspace than the anaerobic vessels and thus fewer, total number of moles of NO than were present in the pouches than in the anaerobic vessels (Table 3). At 1% NO, the number of moles of NO per 100 g of apple tissue was higher in the anaerobic vessels compared to the pouches. The pouches also allow some degree of gas permeation compared to the anaerobic vessels. Thus, some NO may have permeated out of the pouches.

Apple slices treated with 1% NO or 2 mM $NaNO_2$, that were packaged in pouches and stored at 6.1 °C, accumulated higher concentrations of acetaldehyde over the six week

storage period than N_2 and deionized water treated slices respectively. This is consistent with the inhibition of ADH, which catalyzes the conversion of acetaldehyde to ethanol. Nitric oxide inhibition of the reverse reaction (ethanol to acetaldehyde) which is also catalyzed by ADH has been demonstrated *in vitro* by Gergel and Cederbaum (1996) using rat and equine ADH. As would be expected from an inhibition of ADH conversion of acetaldehyde to ethanol, the concentration of ethanol in 1% NO and 2mM $NaNO_2$ treated slices was consistently lower than in slices treated with N_2 and deionized water respectively, throughout the 6 week storage period. Ethyl acetate accumulation was less in 1% NO treated slices compared to N_2 treated slices, but, unexpectedly, was not affected by treatment with 2 mM $NaNO_2$. In the presence of acid, nitrite is converted to nitric oxide and nitrate (Stohr and Ullrich 2002). $NaNO_2$ in contact with the acidic apple surface is converted to NO which, in turn, inhibits ADH, resulting in an increase in acetaldehyde and a decrease in ethanol concentrations. It is possible that sodium nitrite concentrations higher than 2 mM (in contact with apple slices) may be required to generate enough NO to reduce ethyl acetate accumulation.

Apple slices treated with NO were significantly darker than slices treated with N_2 (Figure 16). Increasing NO concentration resulted in further darkening (Figure 16). Similar results were obtained in the shelf life study. Slices treated with 1% NO or 2 mM $NaNO_2$ were darker than slices treated with N_2 or deionized water when stored for 1 to 6 weeks in 100% N_2 atmosphere (Figures 17 and 18). It was later determined in subsequent research that browning (due to NO or $NaNO_2$) was not inhibited by dipping the apple slices in (1 – 10 mg/L) ascorbic acid prior to treatment with 0.25 - 1% NO or 2mM $NaNO_2$. Pristijono et al. (2006) reported that fumigation with 10 to 100 µL/L nitric oxide for 1 to 6 hours inhibited browning, whilst browning was

observed when slices were fumigated with 500 µL/L nitric oxide. It is possible that the low concentrations of NO (10 to 100 µL/L) inhibit polyphenol oxidase on the cut apple surface, whilst higher NO concentrations cause browning due to the reaction of NO with phenolic compounds to form quinones as reported by Urios et al. (2003). The low NO concentrations used by Pristijono et al. (2006) did not inhibit ADH in our work.

There was no effect of any treatment compared to controls on slice firmness. Firmness decreased with storage time of all slices. Zhu et al. (2006) also observed a decrease in firmness with storage time in peaches fumigated with N_2 or NO (5 to 15 µl/l) for 3 hours. They found NO (5 to 10 µl/l) treated fruits were firmer than fruits treated with N_2 after storage for 6 and 30 days at 25 and 5 °C respectively. Since NO delays maturation and senescence when applied exogenously to whole fruits and vegetables (Leshem et al. 1998), delayed maturation and senescence may account for the NO treated peaches being firmer than those treated with N_2.

Previous researchers reported the benefits of treating produce postharvest with low 10 – 500 uL/L NO for about 2 hours. High levels of NO were reported to limit shelf life. It is possible that in addition to the concentration of NO, the total number of moles of NO in contact with produce may play an important role in the observed postharvest effects of NO. Even though most of the previous researchers on the postharvest use of NO report using low levels of NO (10 – 500 uL/L under anaerobic conditions for short periods of time, they also exposed small amounts of produce to NO in a large container. For example, Pristijono et al. (2006) treated six apple slices (about 150 g) in a 4 L container with 10 – 500 uL/L. The large headspace suggests that even though the concentration of NO used was low, the 4 L container volume and the time spent in the

vessel (1 - 2 hours) enables the apples to absorb a large number of moles of NO. By taking the total number of moles into consideration, it is possible that produce may be successfully treated with much higher concentrations of NO for short periods of time (up to 20 minutes). From the results of Gergel and Cederbaum (1996), Siman-Tov and Ankri (2003) and this study, inhibition of ADH occurs with 0.15 – 2 mM NO.

ADH activity was inhibited by treatment with 1% NO atmospheres or 2 mM $NaNO_2$ (Figure 19 and 20). When the same weight of apple was treated (200 g), greater levels of ADH inhibition were obtained when the apples were sliced thinner, increasing the surface area to volume ratio and the effectiveness of the treatment with 1% NO or 2 mM $NaNO_2$. Soegiarto et al. (2003) reported the importance of surface area in the absorption of NO by produce. As the inhibition of ADH activity increased with an increase in apple surface area to volume ration, this suggests that compared with thick slices or whole produce, lower fumigation times and NO concentrations may be used to inhibit ADH activity in thin slices of produce. In addition to greater ADH inhibition, the reduced NO concentrations and fumigation times associated with thin slices of produce may have the added benefit of reduced browning. Similar to pasteurization where high temperature for short periods of time may be used instead of low temperature for long periods of time, inhibition of ADH activity requires high levels of NO (up to 1%) for short periods of time ($\leq$ 20 minutes). The effectiveness of NO or $NaNO_2$ treatment in inhibiting ADH activity is enhanced by treating thin slices of produce compared with whole or thick slices of produce.

4.3 Conclusion

Yeast ADH activity is inhibited by NO (0.5 – 2mM) in a time and dose dependent manner, but is not affected by the same concentration of $NaNO_2$. NO inhibition of

yeast ADH is noncompetitive, suggesting that the inhibition may be reversible. NO inhibits ADH activity and the accumulation of ethanol in plant tissue. NO (1%) and 2mM $NaNO_2$ inhibit alcohol dehydrogenase in apple tissue resulting in the accumulation of acetaldehyde and a decrease in ethanol and ethyl acetate accumulation. However, at the NO concentrations required for inhibition of ADH, there was an acceleration of undesirable browning. NO and nitrite may be used successfully to extend shelf life of produce with low concentrations of phenolic compounds. NO may be effective at inhibiting ADH in produce items in which browning is not a major factor.

CHAPTER FIVE

COST/BENEFIT ANALYSIS OF THE INHIBITION OF ANAEROBIC RESPIRATION IN FRESH-CUT PRODUCE

5.1 Introduction

Fresh-cut fruits and vegetables available on the retail market have been minimally processed (washed, peeled, sliced and cut) providing consumers with a convenient ready to eat product (Rolle and Chism 1987). Unfortunately, these processed produce have a maximum shelf life of just three weeks, which makes it difficult to produce and distribute nationwide from a central processing location, and still maintain freshness. To get around this problem, majority of fresh-cut produce in the U.S. is processed and distributed within a 100 mile radius (FIND/SVP 1995) to minimize the time spent in distribution.

5.1.1 Fresh-cut produce industry overview

Target consumers are middle to affluent health conscious dual income households with busy lifestyles who appreciate the convenience associated with fresh-cut produce (FIND/SVP 1997). Retailers and food service operators (restaurants, schools and hospitals) receive their fresh-cut produce from growers and distributors, with consumers serving as the final link in the distribution chain. Most of the fresh-cut produce sales (70%) is handled by food service operators (FIND/SVP 1995). The National Association of Fresh Produce Processors data indicates that local processors (100 mile distribution radius) and regional processors (500 mile distribution radius) are responsible for 60 and 25% of the fresh-cut produce market share respectively, with on-site processing accounting for the remaining 15% (FIND/SVP 1995).

5.1.1.1 Market Size

The U.S. retail fresh-cut produce sales estimated by (FIND/SVP 1997) to be $5 billion in 1996 increased to almost $6 billion in 1997 and was predicted to reach $19 billion in 2003. While the U.S. market for all fresh produce was $63 billion in 1996 and estimated to reach $66.2 billion at the end of 1997 and $85.1 billion in 2003 (FIND/SVP 1997). Growth in the fresh-cut produce segment has exceeded that of the uncut produce segment, resulting in an increased share of the produce market from 9% in 1997 to 22% in 2003 for fresh-cut (FIND/SVP 1997). Within the fresh-cut produce segment, there is a saturation of competitors and products in packaged salads, whilst the fresh-cut fruit segment with significant unrealized growth potential is predicted to experience increased growth when technology to extend shelf life is developed (FIND/SVP 1997).

5.1.1.2 Shelf life of fresh-cut products

Fresh-cut produce has a maximum shelf life of two to three weeks. Limits to shelf life include browning, microbial growth, senescence and the production of undesirable anaerobic fermentation volatile compounds such as acetaldehyde, ethanol and ethyl acetate. Modified atmosphere packaging which is associated with low O_2 and high carbon dioxide levels reduces respiration rates and is beneficial for extending the shelf life of fresh-cut produce (Watkins 2000). The reduced O_2 levels however create anaerobic conditions within the package, resulting in anaerobic fermentation.

5.1.1.3 Fresh-cut produce losses and waste

Results from a FreshTrack 2001 survey of produce retailers indicated that 7% of produce sales in 2000 was lost to shrink (spoilage and waste) (Perosio et al. 2001). With $38 billion in U.S. retail sales of fresh produce in 2000, almost $2.7 billion was

lost in retail produce shrink alone however, estimates of shrink in the $39.2 billion food service segment of the market were not available (Perosio et al. 2001).

Supermarket retail shrink increased from 2.32% of total sales in 2002 to 2.77% in 2005 (Miller and Allen 2005). In 2005 the perishable department (meat, seafood, produce, floral, deli and bakery) was responsible for 64% of total shrink in the average supermarket, with produce accounting for 16.4% (Miller and Allen 2005). Thus approximately 0.45% of total shrink loss was due to produce. The average U.S supermarket reported 2005 annual sales of almost $22 million, of which about $99,000 was lost to produce shrink (Miller and Allen 2005). U.S. 2005 supermarket sales figures were almost $464 billion of which about $2.1 billion was lost to produce shrink (Food Institute Report 2006). This figure may have increased in subsequent years with the rise in supermarket sales to $482 and $509 billion in 2006 and 2007 respectively (Food Institute Report 2008). Total produce shrink is worth at least $2 billion and could be as high as $4 billion if shrink in the food service segment is comparable to that observed in retail.

5.2 Value Proposition

Fresh-cut fruits and vegetables have a short shelf life of two to three weeks. This short shelf life has led to high levels of shrinkage (waste) in the retail and food service industries. We propose providing food processors with innovative technology that will double the current shelf life of fresh-cut produce.

5.2.1 Technology to inhibit anaerobic respiration in fresh-cut produce

Nitric oxide has been shown to inhibit the conversion of ethanol to acetaldehyde by alcohol dehydrogenase enzyme in rat and equine liver (Gergel and Cederbaum 1996).

Our research has shown that treatment with nitric oxide or nitrite inhibits the conversion of acetaldehyde to ethanol by yeast and plant alcohol dehydrogenase enzyme. In the presence of acid, nitrite is converted to nitric oxide and nitrate (Stohr and Ullrich 2002). Thus treating fresh-cut fruits and vegetables with nitric oxide or nitrite inhibits the accumulation of ethanol (a product of anaerobic fermentation) and extends the current shelf life by two to three weeks.

5.2.1.1 Benefits to food processors

Food processors will be able to locate their processing operations close to the main U.S. produce growing regions and achieve economies of scale. Currently, most fresh-cut produce (60%) is processed and sold locally within 100 miles (FIND/SVP 1995). This is inefficient as it requires transporting whole produce to numerous processing plants even though only about 50% of whole produce is useful post processing (Watada et al. 1996). Doubling the current 2 – 3 week shelf life of fresh-cut fruits and vegetables will enable processors to consolidate their operations to a few processing plants with a centralized distribution system and reduce their transportation and cooling costs. Increased shelf life may also result in increased sales from product innovation and new opportunities in international markets. Consumers are already paying a premium for fresh-cut produce thus processors are less likely to pursue a price differentiation strategy. By increasing the variety and quantity of their product mix, processors benefit from being able to access a broad market, whilst exploring new niches for their products.

5.2.1.2 Benefits to retailers and food service operators

Retailers and food service operators' main benefit from extended shelf life would be a reduction in the $2 - $4 billion annual shrinkage. Additional benefits identified by

(FIND/SVP 1995) include the elimination of on-site (retail store, food service outlet) processing with a reduction in labor and utility costs (water, refrigeration and waste disposal). There will also be a reduction in the costs associated with slip and fall, back injuries (from lifting heavy boxes of produce) and injuries to workers from sharp processing equipment (knives). Retailers can also practice Just in Time delivery of fresh-cut produce to manage their produce inventories and reduce the time spent sorting through the produce-display for wilted, shriveled and expired fresh-cut produce. Elimination of on-site processing also ensures consistent product quality throughout the supermarket and food service chains as well as improved food safety. Fresh-cut produce will also occupy less cooler space and require less energy for cooling compared with an equivalent amount of un-cut produce (FIND/SVP 1995).

5.3 Cost Benefit Analysis

The main cost to food processors associated with this invention is the purchase and use of sodium nitrite to extend the shelf life of fresh-cut produce. A pound (453.6 g) of food grade sodium nitrite costs a food processor $0.80 to $0.83. This value was obtained in October 2008 from an industry person who prefers to remain anonymous. The cost of food grade sodium nitrite quoted above, includes the cost of delivery to the processing plant. A liter of 2 mM sodium nitrite which contains 0.138 g (costs 0.025 cents) can be used to process 500 g (1.1 lb) of fresh-cut produce. This cost may turn out to be even cheaper since several dips of fresh-cut produce are made before the dipping solution is replaced. When combined with 10 g/L ascorbic acid, 0.5 mM sodium nitrite may be used instead of 2 mM, further reducing the cost of sodium nitrite (used to process 500 g of fresh-cut produce) to 0.00625 cents.

Sodium nitrite in acidic solution produces nitric oxide gas which is responsible for inhibiting alcohol dehydrogenase activity and the accumulation of ethanol in fresh-cut produce. Using sodium nitrite presents a cheaper alternative to nitric oxide gas as food processors can integrate it into their existing processing methods without the installation of costly equipment and gas tanks. Sodium nitrite would be incorporated into the antibrowning solution (ascorbic acid) dip, post-slicing, with the retention time (in the dip) adjusted to ensure that the desired inhibition of ethanol accumulation has been attained. An additional cost to food processors would be licensing fees to utilize the technology when Cornell University obtains a patent for this discovery.

The use of nitric oxide gas on the other hand would result in significant additional cost to food processors. Gas tanks must be purchased and installed outside the processing facility. Gas tight pipelines are required to transport nitric oxide into the processing facility. Treatment with nitric oxide may be done in either a continuous or batch process. In the continuous process, the fresh-cut fruits and vegetables will undergo an ascorbic acid dip to prevent browning and then packaged in plastic pouches with 1% nitric oxide (balance N_2) gas. Most processors flush their packages of fresh-cut fruits and vegetables with modified atmospheres. Nitric oxide is an inhalation hazard with serious implications for worker safety when allowed to accumulate in the processing environment. To ensure worker safety, standard operating procedures must be put in place to prevent and deal with gas leaks when they occur. Adequate ventilation must be provided and fume hoods installed above the gas flushing equipment, to prevent the accumulation of nitric oxide in the processing environment. In the batch process, fresh-cut fruits and vegetables will undergo an ascorbic acid dip after which they will be sealed in an anaerobic vessel. Nitric oxide 1% (balance, N_2) will be introduced into the vessel for one to twenty minutes depending on the requirements of the produce

being treated. The anaerobic vessel must be vented properly before opening to prevent nitric oxide from escaping into the processing environment. The treated fresh-cut fruits and vegetables will then be packaged in a 100% nitrogen atmosphere. Sodium nitrite presents a safe, cheap and convenient means for inhibiting anaerobic respiration in fresh-cut produce compared with nitric oxide gas.

5.4 Discussion of "necessary factors" for this product innovation to be successful as a business item

The main hurdle that has to be overcome to guarantee the success of this product innovation as a business item is successfully petitioning the Food and Drug Administration (FDA) to allow the use of nitric oxide (NO) and nitrite on fruits and vegetables including fresh-cut. At present, sodium nitrite (up to 200 ppm) may be used on cured meats (Code of Federal Regulations 2008), however its use is not approved for produce.

Consumer acceptance of sodium nitrite as a preservative on fruits and vegetables may be another hurdle to overcome. Since sodium nitrite is already approved for use in meats, consumer acceptance of its use on produce may not be difficult to obtain. The color, texture and taste of fresh-cut produce are also important to consumers, thus treatment with sodium nitrite must not adversely affect these sensory attributes. Fresh-cut fruit and vegetable processors are looking for technology to extend the shelf life of their current products and enable them to develop innovative products for the fresh-cut fruit segment. The use of sodium nitrite on fresh-cut fruits and vegetables will contribute minimal additional cost and does not require the purchase, installation and operation of additional processing equipment, thus no obstacles to processor acceptance of this technology are foreseen.

In the event that Cornell University is able to obtain a patent for the inhibition of anaerobic respiration in whole and fresh-cut produce using NO and $NaNO_2$, licensing fees may present a barrier to the adoption of this technology by food processors. These fees must be set so they are affordable for food processors, whilst enabling the University profit from developing the technology.

REFERENCES

Abat, J.K., Mattoo, A.K. and Deswal, R. (2008). S-nitrosylated proteins of a medicinal CAM plant *Kalanchoe pinnata* - ribulose-1,5-bisphosphate carboxylase/oxygenase activity targeted for inhibition. FEBS Journal **275**, 2862-2872.

Akeson, A. (1964). On zinc content of horse liver alcohol dehydrogenase. Biochemical and Biophysical Research Communications **17**, 211-214.

Al-Ati, T. and Hotchkiss, J.H. (2002). Application of packaging and modified atmosphere to fresh-cut fruits and vegetables. In: O. Lamikanra. Fresh-Cut Fruits and Vegetables Science, Technology, and Market. Boca Raton, CRC Press. 305-338.

Amissah, J.G.N., Hotchkiss, J.H. and Watkins, C.B. (2006). Diphenylamine and pre-slicing storage effects on the responses of apple slices to elevated CO_2 atmospheres. Postharvest Biology and Technology **39**, 178-184.

Badiyan, D., Wills, R.B.H. and Bowyer, M.C. (2004). Use of a nitric oxide donor compound to extend the vase life of cut flowers. HortScience **39**, 1371-1372.

Barroso, J.B., Corpas, F.J., Carreras, A., Sandalio, L.M., Valderrama, R., Palma, J.M., Lupianez, J.A. and del Rio, L.A. (1999). Localization of nitric-oxide synthase in plant peroxisomes. Journal of Biological Chemistry **274**, 36729-36733.

Bartley, I.M. and Hindley, S.J. (1980). Alcohol dehydrogenase of apple. Journal of Experimental Botany **31**, 449-459.

Beligni, M.V. and Lamattina, L. (2000). Nitric oxide stimulates seed germination and de-etiolation, and inhibits hypocotyl elongation, three light-inducible responses in plants. Planta **210**, 215-221.

Beligni, M.V. and Lamattina, L. (2001). Nitric oxide: a non-traditional regulator of plant growth. Trends in Plant Science **6**, 508-509.

Bourne, M.C. and Moyer, J.C. (1968). Extrusion principle in texture measurement of fresh peas. Food Technology **22**, 81-86.

Bowyer, M.C., Wills, R.B.H., Badiyan, D. and Ku, V.V.V. (2003). Extending the postharvest life of carnations with nitric oxide - comparison of fumigation and in vivo delivery. Postharvest Biology and Technology **30**, 281-286.

Brecht, J.K. (1995). Physiology of lightly processed fruits and vegetables. HortScience **30**, 18-22.

Brown, G.C. (2001). Regulation of mitochondrial respiration by nitric oxide inhibition of cytochrome c oxidase. Biochimica Et Biophysica Acta-Bioenergetics **1504**, 46-57.

Brown, G.C. and Cooper, C.E. (1994). Nanomolar concentrations of nitric oxide reversibly inhibit synaptosomal respiration by competing with oxygen at cytochrome oxidase. FEBS Letters **356**, 295-298.

Brune, B. and Lapetina, E.G. (1989). Activation of a cytosolic ADP-ribosyl transferase by nitric oxide generating agents. Journal of Biological Chemistry **264**, 8455-8458.

Chan, J.W.Y. and Burton, R.S. (1992). Variation in alcohol dehydrogenase activity and flood tolerance in white clover, *Trifolium repens*. Evolution **46**, 721-734.

Cheng, G.P., Yang, E., Lu, W.J., Jia, Y.X., Jiang, Y.M. and Duan, X.W. (2009). Effect of nitric oxide on ethylene synthesis and softening of banana fruit slice during ripening. Journal of Agricultural and Food Chemistry **57**, 5799-5804.

Cleeter, M.W.J., Cooper, J.M., Darleyusmar, V.M., Moncada, S. and Schapira, A.H.V. (1994). Reversible inhibition of cytochrome c oxidase, the terminal enzyme of the mitochondrial respiratory chain, by nitric oxide: Implications for neurodegenerative diseases. FEBS Letters **345**, 50-54.

Code of Federal Regulations (2008). 9CFR381.147 Code of Federal Regulations.

Cooney, R.V., Harwood, P.J., Custer, L.J. and Franke, A.A. (1994). Light-mediated conversion of nitrogen dioxide to nitric oxide by carotenoids. Environmental Health Perspectives **102**, 460-462.

Cooper, C.E. (1999). Nitric oxide and iron proteins. Biochimica Et Biophysica Acta-Bioenergetics **1411**, 290-309.

Dean, J.V. and Harper, J.E. (1986). Nitric oxide and nitrous oxide production by soybean and winged bean during the *in vivo* nitrate reductase assay. Plant Physiology **82**, 718-723.

Dean, J.V. and Harper, J.E. (1988). The conversion of nitrite to nitrogen oxides(s) by the constitutive NAD(P)H-nitrate reductase enzyme from soybean. Plant Physiology **88**, 389-395.

Dimmeler, S. and Brune, B. (1993). Nitric oxide preferentially stimulates auto-ADP-ribosylation of glyceraldehyde-3-phosphate dehydrogenase compared to alcohol or lactate-dehydrogenase. FEBS Letters **315**, 21-24.

Dimmeler, S., Lottspeich, F. and Brune, B. (1992). Nitric oxide causes ADP-ribosylation and inhibition of glyceraldehyde-3-phosphate dehydrogenase. Journal of Biological Chemistry **267**, 16771-16774.

Drum, D.E., Harrison, J.H., Li, T.K., Bethune, J.L. and Vallee, B.L. (1967). Structural and functional zinc in horse liver alcohol dehydrogenase. Proceedings of the National Academy of Sciences of the United States of America **57**, 1434-1440.

Duan, X.W., Su, X.G., You, Y.L., Qu, H.X., Li, Y.B. and Jiang, Y.M. (2007). Effect of nitric oxide on pericarp browning of harvested longan fruit in relation to phenolic metabolism. Food Chemistry **104**, 571-576.

Eklund, H., Nordstrom, B., Zeppezauer, E., Soderlund, G., Ohlsson, I., Boiwe, T. and Branden, C.I. (1974). The structure of horse liver alcohol dehydrogenase. FEBS Letters **44**, 200-204.

Feldman, P.L., Griffith, O.W. and Stuehr, D.J. (1993). The Surprising Life of Nitric-Oxide. Chemical & Engineering News **71**, 26-38.

FIND/SVP (1995). Value-added produce. Retrieved August 22, 2008 from MarketResearch.com Database.

FIND/SVP (1997). The market for value-added fresh produce, a market intelligence report. Retrieved August 22, 2008 from MarketResearch.com Database.

Food Institute Report: Supermarket sales increase in May (2006). Retrieved August 29, 2008 from Factiva.com Database.

Food Institute Report: Supermarket size rose 5.5% last year (2008). Retrieved August 29, 2008 from Factiva.com Database.

Furchgott, R.F. (1988). Studies on relaxation of rabbit aorta by sodium nitrite: the basis for the proposal that the acid-activable inhibitory factor from retractor penis is inorganic nitrite and the endothelium-derived relaxing factor is nitric oxide. In: P.M. Vanhoutte. Vasodilation: vascular smooth muscle, peptides, autonomic nerves and endothelium. New York, Raven Press. 401-414.

Garcia Mata, C., Gay, R., Sokolovski, S., Hills, A., Lamattina, L. and Blatt, M.R. (2003). Nitric oxide regulates K^+ and Cl^- channels in guard cells through a subset of abscisic acid-evoked signaling pathways. Proceedings of the National Academy of Sciences of the United States of America **100**, 11116-11121.

Garcia Mata, C. and Lamattina, L. (2001). Nitric oxide induces stomatal closure and enhances the adaptive plant responses against drought stress. Plant Physiology **126**, 1196-1204.

Gergel, D. and Cederbaum, A.I. (1996). Inhibition of the catalytic activity of alcohol dehydrogenase by nitric oxide is associated with S nitrosylation and the release of zinc. Biochemistry **35**, 16186-16194.

Giba, Z., Grubisic, D., Todorovic, S., Sajc, L., Stojakovic, D. and Konjevic, R. (1998). Effect of nitric oxide - releasing compounds on phytochrome - controlled germination of Empress tree seeds. Plant Growth Regulation **26**, 175-181.

Gow, A.J., Farkouh, C.R., Munson, D.A., Posencheg, M.A. and Ischiropoulos, H. (2004). Biological significance of nitric oxide-mediated protein modifications. American Journal of Physiology-Lung Cellular and Molecular Physiology **287**, L262-L268.

Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S. and Tannenbaum, S.R. (1982). Analysis of nitrate, nitrite, and N-15 -labeled nitrate in biological-fluids. Analytical Biochemistry **126**, 131-138.

Gunes, G., Watkins, C.B. and Hotchkiss, J.H. (2001). Physiological responses of fresh-cut apple slices under high CO_2 and low O_2 partial pressures. Postharvest Biology and Technology **22**, 197-204.

Gunes, G.G. and Hotchkiss, J.H. (2002). Growth and survival of *Escherichia coli* O157:H7 on fresh-cut apples in modified atmospheres at abusive temperatures. Journal of Food Protection **65**, 1641-1645.

Harper, J.E. (1981). Evolution of nitrogen oxide(s) during in vivo nitrate reductase assay of soybean leaves. Plant Physiology **68**, 1488-1493.

Hayes, J.E. and Velick, S.F. (1954). Yeast alcohol dehydrogenase: Molecular weight, coenzyme binding, and reaction equilibria. Journal of Biological Chemistry **207**, 225-244.

Hoch, F.L. and Vallee, B.L. (1956). Kinetic studies on the role of zinc and diphosph-pyridine nucleotide in the activity of yeast alcohol dehydrogenase. Journal of Biological Chemistry **221**, 491-500.

Hotchkiss, J.H. (1989). Relative exposure to nitrite, nitrate, and N-nitroso compounds from endogenous and exogenous sources. In: S.L. Taylor and R.A. Scanlan. Food Toxicology: A perspective on the relative risks. New York, Marcel Dekker. 57-100.

Ignarro, L.J. (1990). Biosynthesis and metabolism of endothelium-derived nitric-oxide. Annual Review of Pharmacology and Toxicology **30**, 535-560.

Ignarro, L.J., Byrns, R.E. and Wood, K.S. (1988). Biochemical and pharmacological properties of endothelium-derived relaxing factor and its similarity to nitric oxide radical. In: P.M. Vanhoutte. Vasodilation: vascular smooth muscle,

peptides, autonomic nerves and endothelium. New York, Raven Press. 427-436.

Jornvall, H., Woenckhaus, C. and Johnscher, G. (1975). Modification of alcohol dehydrogenases with a reactive coenzyme analog - Identification of labeled residues in horse-liver and yeast enzymes after treatment with nicotinamide-5-bromoacetly-4-methyl-imidazole dinucleotide. European Journal of Biochemistry **53**, 71-81.

Kader, A.A., Zagory, D. and Kerbel, E.L. (1989). Modified atmosphere packaging of fruits and vegetables. Critical Reviews in Food Science and Nutrition **28**, 1-30.

Kagi, J.H.R. and Vallee, B.L. (1960). The role of zinc in alcohol dehydrogenase V. The effect of metal-binding agents on the structure of the yeast alcohol dehydrogenase molecule. Journal of Biological Chemistry **235**, 3188-3192.

Kato-Noguchi, H. and Watada, A.E. (1997). Effects of low-oxygen atmosphere on ethanolic fermentation in fresh-cut carrots. Journal of the American Society for Horticultural Science **122**, 107-111.

Ke, D., Rodriguez-Sinobas, L. and Kader, A.A. (1991). Physiology and prediction of fruit tolerance to low oxygen atmospheres. Journal of the American Society for Horticultural Science **116**, 253-260.

Ke, D.Y., Yahia, E., Mateos, M. and Kader, A.A. (1994). Ethanolic fermentation of bartlett pears as influenced by ripening stage and atmospheric composition. Journal of the American Society for Horticultural Science **119**, 976-982.

Klinman, J.P. and Welsh, K. (1976). The zinc content of yeast alcohol dehydrogenase. Biochemical and Biophysical Research Communications **70**, 878-884.

Ku, V.V.V., Wills, R.B.H. and Leshem, Y.Y. (2000). Use of nitric oxide to reduce postharvest water loss from horticultural produce. Journal of Horticultural Science & Biotechnology **75**, 268-270.

Lakakul, R., Beaudry, R.M. and Hernandez, R.J. (1999). Modeling respiration of apple slices in modified-atmosphere packages. Journal of Food Science **64**, 105-110.

Leshem, Y.Y. (2000). Nitric oxide in plants occurrence, function and use. Dordrecht, Kluwer Academic Publishers.

Leshem, Y.Y. and Haramaty, E. (1996). The characterization and contrasting effects of the nitric oxide free radical in vegetative stress and senescence of *Pisum sativum* Linn foliage. Journal of Plant Physiology **148**, 258-263.

Leshem, Y.Y., Wills, R.B.H. and Ku, V.V.V. (1998). Evidence for the function of the free radical gas - nitric oxide ($NO^{\cdot}$) - as an endogenous maturation and

senescence regulating factor in higher plants. Plant Physiology and Biochemistry **36**, 825-833.

Mateo, R.B., Reichner, J.S., Mastrofrancesco, B., Kraft-Stolar, D. and Albina, J.E. (1995). Impact of nitric oxide on macrophage glucose metabolism and glyceraldehyde-3-phosphate dehydrogenase activity. American Journal of Physiology-Cell Physiology **268**, C669-C675.

Maxwell, D.P., Wang, Y. and McIntosh, L. (1999). The alternative oxidase lowers mitochondrial reactive oxygen production in plant cells. Proceedings of the National Academy of Sciences of the United States of America **96**, 8271-8276.

Mezey, E. and Holt, P.R. (1971). Inhibitory effect of ethanol on retinol oxidation by human liver and cattle retina. Experimental and Molecular Pathology **15**, 148-156.

Millar, A.H. and Day, D.A. (1996). Nitric oxide inhibits the cytochrome oxidase but not the alternative oxidase of plant mitochondria. FEBS Letters **398**, 155-158.

Miller, L. and Allen, J. (2005). 2005 Supermarket shrink report. The National Supermarket Research Group. Retrieved August 28, 2008 from http://www.paracon.com/pdfs/Supermarket_Survey_Executive_Summary.pdf.

Mohr, S., Stamler, J.S. and Brune, B. (1996). Posttranslational modification of glyceraldehyde-3-phosphate dehydrogenase by S-nitrosylation and subsequent NADH attachment. Journal of Biological Chemistry **271**, 4209-4214.

Molina y Vedia, L., McDonald, B., Reep, B., Brune, B., Disilvio, M., Billiar, T.R. and Lapetina, E.G. (1992). Nitric oxide-induced S-nitrosylation of glyceraldehyde-3-phosphate dehydrogenase inhibits enzymatic activity and increases endogenous ADP-ribosylation. Journal of Biological Chemistry **267**, 24929-24932.

Moncada, S., Palmer, R.M.J. and Higgs, E.A. (1991). Nitric oxide: physiology, pathophysiology and pharmacology. Pharmacological Reviews **43**, 109-142.

Moon, K.H., Kim, B.J. and Song, B.J. (2005). Inhibition of mitochondrial aldehyde dehydrogenase by nitric oxide-mediated S-nitrosylation. FEBS Letters **579**, 6115-6120.

Palmer, R.M.J., Ferrige, A.G. and Moncada, S. (1987). Nitric oxide release accounts for the biological activity of endothelium derived relaxing factor. Nature **327**, 524-526.

Perosio, D.J., McLaughlin, E.W., Cuellar, S. and Park, J. (2001). FreshTrack 2001: Supply chain management in the produce industry. Agricultural, Resource and Managerial Economics, Cornell University. Ithaca, NY., R.B. 2001-2005.

Pristijono, P., Wills, R.B.H. and Golding, J.B. (2006). Inhibition of browning on the surface of apple slices by short term exposure to nitric oxide (NO) gas. Postharvest Biology and Technology **42**, 256-259.

Pristijono, P., Wills, R.B.H. and Golding, J.B. (2008). Use of the nitric oxide-donor compound, diethylenetriamine-nitric oxide (DETANO), as an inhibitor of browning in apple slices. Journal of Horticultural Science & Biotechnology **83**, 555-558.

Rhodes, M.J.C. (1973). Co-factor specificity of plant alcohol-dehydrogenase. Phytochemistry **12**, 307-314.

Ribeiro, E.A., Cunha, F.Q., Tamashiro, W. and Martins, I.S. (1999). Growth phase-dependent subcellular localization of nitric oxide synthase in maize cells. FEBS Letters **445**, 283-286.

Rolle, R.S. and Chism, G.W. (1987). Physiological consequences of minimally processed fruits and vegetables. Journal of Food Quality **10**, 157-177.

Scandalios, J.G. (1977). Isozymes: Genetic and biochemical regulation of alcohol dehydrogenase. In: H. Smith. Regulation of enzyme synthesis and activity in higher plants. London, Academic Press. 129 -153.

Siman-Tov, R. and Ankri, S. (2003). Nitric oxide inhibits cysteine proteinases and alcohol dehydrogenase 2 of Entamoeba histolytica. Parasitology Research **89**, 146-149.

Snyder, S.H. (1992). Nitric Oxide: First in a new class of neurotransmitters. Science **257**, 494-496.

Soegiarto, L. and Wills, R.B.H. (2004). Short term fumigation with nitric oxide gas in air to extend the postharvest life of broccoli, green bean, and bok choy. HortTechnology **14**, 538-540.

Soegiarto, L. and Wills, R.B.H. (2006). Effect of nitric oxide, reduced oxygen and elevated carbon dioxide levels on the postharvest life of strawberries and lettuce. Australian Journal of Experimental Agriculture **46**, 1097-1100.

Soegiarto, L., Wills, R.B.H., Seberry, J.A. and Leshem, Y.Y. (2003). Nitric oxide degradation in oxygen atmospheres and rate of uptake by horticultural produce. Postharvest Biology and Technology **28**, 327-331.

Stadler, J., Billiar, T.R., Curran, R.D., Stuehr, D.J., Ochoa, J.B. and Simmons, R.L. (1991). Effect of exogenous and endogenous nitric oxide on mitochondrial respiration of rat hepatocytes. American Journal of Physiology **260**, C910-C916.

Stohr, C. and Ullrich, W.R. (2002). Generation and possible roles of NO in plant roots and their apoplastic space. Journal of Experimental Botany **53**, 2293-2303.

Sund, H. and Theorell, H. (1963). Alcohol Dehydrogenases. In: P.D. Boyer, H. Lardy and K. Myrback. The Enzymes. New York, Academic Press. 25 - 83.

Urios, A., Lopez-Gresa, M.P., Gonzalez, M.C., Primo, J., Martinez, A., Herrera, G., Escudero, J.C., O'Connor, J.E. and Blanco, M. (2003). Nitric oxide promotes strong cytotoxicity of phenolic compounds against *Escherichia coli*: The influence of antioxidant defenses. Free Radical Biology and Medicine **35**, 1373-1381.

Vallee, B.L. and Hoch, F.L. (1955). Zinc, a component of yeast alcohol dehydrogenase. Proceedings of the National Academy of Sciences of the United States of America **41**, 327-338.

van Eys, J., Ciotti, M.M. and Kaplan, N.O. (1957). Yeast alcohol dehydrogenase II. Properties of the catalytically active site. Biochimica Et Biophysica Acta **23**, 581-587.

Veillon, C. and Sytkowski, A.J. (1975). The intrinsic zinc atoms of yeast alcohol dehydrogenase. Biochemical and Biophysical Research Communications **67**, 1494-1500.

Watada, A.E., Abe, K. and Yamuchi, N. (1990). Physiological Activities of Partially Processed Fruits and Vegetables. Food Technology **44**, 116, 118, 120-122.

Watada, A.E., Ko, N.P. and Minott, D.A. (1996). Factors affecting quality of fresh-cut horticultural products. Postharvest Biology and Technology **9**, 115-125.

Watkins, C.B. (2000). Responses of horticultural commodities to high carbon dioxide as related to modified atmosphere packaging. HortTechnology **10**, 501-506.

Wills, R.B.H., Ku, V.V.V. and Leshem, Y.Y. (2000). Fumigation with nitric oxide to extend the postharvest life of strawberries. Postharvest Biology and Technology **18**, 75-79.

Wills, R.B.H., Pristijono, P. and Golding, J.B. (2007). Use of nitric oxide to reduce surface browning of fresh cut lettuce and apple slices. Acta Horticulturae **746**, 237-244.

Wills, R.B.H., Pristijono, P. and Golding, J.B. (2008). Browning on the surface of cut lettuce slices inhibited by short term exposure to nitric oxide (NO). Food Chemistry **107**, 1387-1392.

Wink, D.A. and Mitchell, J.B. (1998). Chemical biology of nitric oxide: Insights into regulatory, cytotoxic, and cytoprotective mechanisms of nitric oxide. Free Radical Biology and Medicine **25**, 434-456.

Wojtaszek, P. (2000). Nitric oxide in plants - To NO or not to NO. Phytochemistry **54**, 1-4.

Yamasaki, H. and Sakihama, Y. (2000). Simultaneous production of nitric oxide and peroxynitrite by plant nitrate reductase: in vitro evidence for the NR-dependent formation of active nitrogen species. FEBS Letters **468**, 89-92.

Yamasaki, H., Sakihama, Y. and Takahashi, S. (1999). An alternative pathway for nitric oxide production in plants: new features of an old enzyme. Trends in Plant Science **4**, 128-129.

Yamasaki, H., Shimoji, H., Ohshiro, Y. and Sakihama, Y. (2001). Inhibitory effects of nitric oxide on oxidative phosphorylation in plant mitochondria. Nitric Oxide-Biology and Chemistry **5**, 261-270.

Zamzami, N., Marchetti, P., Castedo, M., Zanin, C., Vayssiere, J.L., Petit, P.X. and Kroemer, G. (1995). Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death in-vivo. Journal of Experimental Medicine **181**, 1661-1672.

Zhang, J. and Snyder, S.H. (1992). Nitric oxide stimulates auto ADP ribosylation of glyceraldehyde-3-phosphate dehydrogenase. Proceedings of the National Academy of Sciences of the United States of America **89**, 9382-9385.

Zhu, L.Q., Zhou, J., Zhu, S.H. and Guo, L.H. (2009). Inhibition of browning on the surface of peach slices by short-term exposure to nitric oxide and ascorbic acid. Food Chemistry **114**, 174-179.

Zhu, S.H., Liu, M.C. and Zhou, J. (2006). Inhibition by nitric oxide of ethylene biosynthesis and lipoxygenase activity in peach fruit during storage. Postharvest Biology and Technology **42**, 41-48.

Zhu, S.H., Sun, L., Liu, M.C. and Zhou, J. (2008). Effect of nitric oxide on reactive oxygen species and antioxidant enzymes in kiwifruit during storage. Journal of the Science of Food and Agriculture **88**, 2324-2331.

Zhu, S.H. and Zhou, J. (2007). Effect of nitric oxide on ethylene production in strawberry fruit during storage. Food Chemistry **100**, 1517-1522.

Zottini, M., Formentin, E., Scattolin, M., Carimi, F., Lo Schiavo, F. and Terzi, M. (2002). Nitric oxide affects plant mitochondrial functionality in vivo. FEBS Letters **515**, 75-78.