AN ABSTRACT OF THE THESIS OF

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Title: Differential Storage Temperature and Duration Effects on Ethylene Synthesis and Firmness of 'Anjou' Pears.

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Abstract. 'Anjou' pears were sprayed with 2 concentrations of $CaCl_2$ during fruit development and harvested at 70 N flesh firmness. Pears were held at -1.1° , 5° , 10° , or 20° C for up to 100 days then ripened at 20° C for 11 days. Unexpectently, fruits stored at 20° C lost firmness and chlorophyll after 20 days without ethylene exceeding 0.3 ul/l. Only after 70 days did ethylene begin to rise above 1 ul/l and be sustainable. Fruits stored at -1.1° C produced climacteric ethylene after 55 days and firmness decreased in response to climacteric ethylene. Fruits stored at 5° or 10° C required only 40 days to produce climacteric ethylene. Calcium-treated pears had significantly lower internal ethylene and greater firmness in all treatments, but calcium had only a small effect on ripening parameters of 10° C stored fruits.

Both linoleic acid, the major fatty acid of 'Anjou' pear total lipids, and linolenic acid increased 100% during storage at -1.1° , and about 50% during storage at 5° or 10° C but did not increase during 20° C storage. Calcium treatments did not affect on fatty acid profiles. While linoleic acid simultaneously increased with chilling satisfaction, the significance of this

relationship is not yet known.

Fruits held at -1.1° C retained firmness and chlorophyll with little change in total proteins, amino acids, or soluble polyuronides. However, when fruit chilling requirements were satisfied (ie, after 70 days), internal ethylene peaked to 1.8 ul/l, ACC to 0.9 nmoles/g, and ethylene-forming enzyme(s) (EFE) activity to 71 nl C_2H_4/g/h, while titratable acidity decreased and sensitivity to propylene maximized. Pears stored at 20° C first softened, lost chlorophyll and titratable acidity and 15% of the cell wall polyuronides became soluble. Ethylene-forming enzyme(s) activity peaked then before ethylene, ACC, and total proteins increased. Sensitivity to propylene accelerated after the firmness decreased.

DIFFERENTIAL STORAGE TEMPERATURE AND DURATION EFFECTS ON ETHYLENE SYNTHESIS AND FIRMNESS OF 'ANJOU' PEARS

By

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"The unexamined life is not worth living"
Socrates.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF PLATES

DIFFERENTIAL STORAGE TEMPERATURE AND DURATION EFFECTS ON ETHYLENE SYNTHESIS AND FIRMNESS OF 'ANJOU' PEARS

CHAPTER I

INTRODUCTION

Ethylene, a plant hormone produced by climacteric fruits, is known to regulate fruit ripening. Many factors can influence storage life of fruits. Susceptibility to decay, mechanical injury, and rapid softening are but a few factors in addition to the normal senescent processes which limit storage life. Storage life of pear fruits (Pyrus communis) is often related to the initiation of fruit ethylene production. Differences in timing of ethylene production and the effect of other factors which modify it, have been observed in different types of fruits. Generally, fruits which produce ethylene early in storage have short storage lifes. Conversely, if ethylene production can be delayed, storage life can generally be increased.

'Anjou' pears harvested at normal maturity usually require about 60 days of cold storage to initiate ripening. However, additional calcium from orchard sprays or other factors can increase the amount of chilling requirement up to 50%. Knowledge of 'Anjou' fruit physiology is important not only from a scientific point of view but also because it may provide a tool for better control of ripening, and may have value in predicting useful storage life of the fruits. Knowing the storage behavior

of lots of fruit may improve marketing decisions.

The objectives of this thesis are to examine and describe certain effects of storage temperature, fruit calcium, and propylene on chilling requirements for ripening of 'Anjou' pear fruits.丙烯

Chapter II develops the literary background pertinent to the thesis problem.

The first manuscript (Chapter III) describes the effects of storage temperature and fruit calcium levels on chilling requirements and ripening of 'Anjou' pear.

The second manuscript (Chapter IV) describes changes in fatty acid composition of 'Anjou' pears in relation to storage temperature, fruit calcium and chilling requirements.

The third manuscript (Chapter V) describes the ripening behavior of 'Anjou' pear fruits ripened directly at 20° C as compared to fruits stored at -1.1° C for various amounts of time and then ripened at 20° C, as expressed by the changes of various ethylene synthesis parameters and other biochemical constituents such as protein, soluble pectins, etc., associated with ripening.

The fourth manuscript (Chapter VI) investigates changes in chilling requirements and sensitivity to exogenous propylene of 'Anjou' pears during storage at 20° C or -1.1° C.

Since these chapters are assembled in journal manuscript format, several figures redundant to the manuscript have been relegated to the appendix section.

CHAPTER II

LITERATURE REVIEW

Ethylene is a simple hydrocarbon gas, a natural product of metabolism which regulates or influences numerous plant growth, development, and senescence processes, including ripening (1).

Among the physiological effects attributed to ethylene are: stimulation of ripening of fleshy fruits (1,45,48), stimulation of leaf abscission (1,6,116), inhibition or promotion of root growth (55), and stimulation of membrane permeability (225). The interactions of ethylene and other kinds of plant hormones are numerous and complex (50,130,133,154,166,205,211) and will not be reviewed here.

A. FRUIT RIPENING

Fruits have been classified as either climacteric or non-climacteric depending on their respiratory behavior during ripening after the fruit matures (29).

Non-climacteric fruits exhibit a fairly steady respiration rate during ripening (1) and often change slowly as they ripen. Treatment of non-climacteric fruits with ethylene causes an unnatural climacteric-like respiratory CO_2 increase, (which subsides on removal of ethylene) but not an increase in endogenous ethylene. Climacteric fruits show a decrease in respiration rate as the fruits mature (the preclimacteric minimum)

followed by a large increase during ripening. This is accompanied by marked changes in composition and texture, and finally a decrease in respiration rate as the fruits enter a senescent decline. Ripening of climacteric-type fruits is associated with, and usually preceded by large increases in ethylene production. Treatment with sufficient ethylene concentration in the pre-climacteric stage induces the climacteric response and ethylene production and this ripening process is irreversible after the endogenous ethylene increases beyond a certain threshold level of about 1 10^{-6} ul/l. Aminoethoxyvinylglycine (AVG), a methionine homologue, and an ethylene synthesis inhibitor, vacuum infiltrated into 'Bartlett' pears retarded ripening (167). AVG sprayed on tree- attached apples delayed fruit ripening, reduced fruit drop, and increased fruit removal force (22). Under hypobaric conditions, removing ethylene retarded ripening and senescence (72). Mc Murckie, et al (158), found that propylene, an ethylene homologue, induced ethylene production in bananas (climacteric type) but not in citrus fruits (non-climacteric type). The authors suggested that biogenesis of ethylene in climacteric fruits is regulated by two systems: System 1, which is involved in the regulation of aging processes and is associated with the low rate of ethylene production during growth, and system 2, which is responsible for the autocatalytic increase in ethylene production which accompanies ripening. System 2 is missing in non-climacteric fruits.

Burg and others (45,46) have shown that for several

climacteric fruits, there is a rise in intercellular content of ethylene to a level which stimulates these fruits to ripen, and that this rise occurs well in advance of the respiratory CO_2 climacteric. Prior to induction of the respiratory climacteric, ethylene concentrations in excess of 3 ul/l in the central cavity of melon, is sufficient to induce the climacteric (190). Internal ethylene (0.02 ul/l) initiates ripening in apples although other factors may control the action of ethylene (186). The rise in internal ethylene concentrations appears promising as a maturity index for predicting ripening ability of apples (200). Fruits attached to the tree are less sensitive to ethylene than are harvested fruits, indicating that an inhibitor(s) may be translocated from other parts of the tree (207). Avocado fruits fail to ripen while still attached (238). Also, in 'Fuerte' avocado and 'Chaffey' cherimoya, the amount of internal ethylene, was believed to be insufficient to stimulate ripening at the beginning of the respiratory climacteric (116). Tomatoes show a different pattern. Immature tomatoes have a respiratory CO_2 climacteric but do not show ethylene production or other aspects of ripening. A respiratory climacteric and ethylene production occur together during ripening of more mature tomatoes (211). Immature tomatoes exhibit a climacteric increase in respiration when treated continuously with ethylene (137). However, substituting propylene for ethylene there was not a change in endogenous ethylene production until other symptoms of ripening (color) appeared (155). Pratt and Goeschl (183), have proposed

that the various fruit ripening reactions are triggered by ethylene but subsequently proceed independently of one another.

Tissue softening generally accompanies fruit ripening. Softening is due to the dissolution of cell walls (mainly the pectins of the middle lamella) which results in ripening associated changes in wall polysaccharides. A reduction in cell wall pectin content has been reported for virtually every ripening fruit (172,184). These observations have led to a great many studies which have documented the involvement of pectolytic and cellulolytic enzymes (polygalacturonase, pectinesterase and cellulase) in wall degradation (183). The degradation of pectates mediated by polygalacturonase is substantially inhibited by calcium (64). The cell-wall-bound calcium fraction in storage tissue of apple fruits constitutes up to 90% of the total calcium (76). Calcium strengthens the cell wall by bridging the carboxylic groups of pectates (151). High fruit calcium from preharvest spraying or postharvest dipping with calcium chloride is related to flesh firmness retention during storage (143,144,189) and ripening (86,189). In calcium deficient cucumber root tissue, polygalacturonase activity increases with the appearance of the typical symptoms of calcium deficiency in ripening fruits: the disintegration of cell walls and collapse of the affected tissues such as the petioles and upper parts of the stems (116). Decreases in cell wall neutral sugar content (mainly galactose and/or arabinose) have also been described for ripening fruits (106,111,228). Losses of neutral sugars are generally

analyzed by hydrolyzing cell walls prepared from fruits at different stages of ripeness and by determination of monosaccharide content (106,228). Losses of galactose residues from apple cell walls was similar for applications of high or low ethylene concentrations (112).

The ground color of the skin is determined by the concentrations of chlorophyll and carotenoids (87). The color changes may be due to degradative or synthetic processes, or both. Chlorophyll usually degrades with fruit maturity and ripening and thus the carotenoids contribute more to the color (77,87).

There is also an increase in soluble solids during fruit ripening which can be influenced by the storage regime. This increase could be due to conversion of starch to soluble sugars and/or a release of cell wall bound neutral sugars (8,58,91). It has been suggested by Ulrich (218) that most of the fruit organic acids are localized in the vacuole of the pulp cells. Fruit acids have been shown to decrease with time after fruit set (95). Titratable acidity is known to decrease during fruit ripening and storage (56,99,129). The rate of the loss in total or individual organic acids has been reported to be retarded in apples (114), and pears (129) during controlled atmosphere storage.

The metabolism of fatty acids during fruit ripening has been little studied. 'Bartlett' pears which have been temporarily stored at 0^{o} C and then ripened at 20^{o} C appear to show a decrease in linoleic and linolenic acid. However, apples treated the same way showed an increase in linolenic acid, (196). An

increase in linoleic but a decrease in linolenic acid with ripening of apples was observed in another study (84). However, Lurie and Ben-Arie (136), working with 'Calville de San Sauveur' apples showed a decrease in linoleic acid which was more evident after the climacteric rise in ethylene. During ripening of banana pulp tissue, a decrease in the proportion of linoleic and an increase of linolenic acid was observed (225). Paulin, et al (170), working with carnation suggested that peroxidation of fatty acids plays a fundamental role in the senescence process and ethylene appears to be a by product rather than an initiating factor. In 'Bosc' and 'Bartlett' pears the onset of ripening was found to correlate with the peroxide content of the fruit tissues (41). In addition to that, inhibitor studies suggest the involvement of free radicals in the peroxide content of the fruit tissues (13).

It is clear that there is also an increase in soluble protein content during the climacteric (93). Cycloheximide, an inhibitor of protein synthesis by 80s ribosomes, when infiltrated into harvested, mature fruit, prevents ripening in pears (80), and bananas (40). Ribonucleic acid synthesis peaked just before the onset of ripening (181). RNA synthesis is required for the synthesis of new enzymes involved in ripening (182).

Polygalacturonase (PG) in tomatoes was produced de novo after the beginning of autocatalytic ethylene production (36,88). The activity of malic enzyme increased during ripening of pear and apple fruits in association with other biochemical changes,

including an increase in the respiratory quotient of intact fruits, increase in NADP and NADPH content of the fruit cortex (52,80,101,102,109), increased capacity of the fruit tissue slices to decarboxylate malate (177) and a decline in malic acid content of the fruit. Phosphofructokinase (PFK), pyruvate kinase (PK), glucose-6-phosphate dehydrogenase (G-6-PDH), and malic enzyme (ME), activity was accelerated by exogenous ethylene fruit firmness decreased and respiration and ATP formation even at low oxygen partial pressure and low temperatures increased (42).

B. ETHYLENE BIOSYNTHESIS

In 1966, Lieberman and Mapson (131) suggested methionine as a possible precursor of ethylene based on the observation that methionine readily converted to ethylene in a model system consisting of Cu and ascorbic acid. Application of methionine to post-climacteric apple fruit, gave a 100% increase in ethylene, but did not increase ethylene production of pre-climacteric or climacteric fruit (140). Methionine (1 mM) has little effect on ethylene production in a variety of vegetative tissue (54). Addition of methionine in Atropa belladona callus culture decreased ethylene production (221). L-methionine more recently has been shown to be the major precursor of ethylene in higher plants (25,51,134,203), whereas D-methionine often act as an inhibitor. The C-3 and C-4 of methionine were converted to ethylene, C-1 to carbon dioxide, and C-2 to formic acid (51),

while the sulfur atom and the methyl group were retained in the tissues (51,245) and later shown to be recycled. Bacteria and fungi can utilize 4-methylthio-2-oxobutanoate in addition to methionine to produce ethylene (30), and this was later shown to be part of the recycling pathway back to methionine (121). Alfa-ketoglutarate and glutamate can be utilized by Penicillium digitatum, depending on growing conditions, to produce ethylene by several pathways (148).

Inhibition of the conversion of methionine to ethylene (using 2,4-dinitrophenol, an oxidative phosphorylation electron transport inhibitor) suggested a requirement for ATP (44,167,243).

Murr and Yang (167) later showed that S-adenosylmethionine (SAM) is an intermediate in the conversion of methionine to ethylene. SAM was degraded into carbon dioxide, formic acid, ammonia, ethylene and 5'-methylthioadenosine (MTA) (167). The same researchers, later found that MTA was recycled to methionine in apple tissue. They postulated that MTA, possibly through SAM, is an intermediate in the conversion of methionine to ethylene (4). Adams and Yang later provided other indirect evidence that SAM may be an intermediate in the conversion of methionine to ethylene and that the conversion of methionine into MTA and MTR (5-S-methyl-thioribose) are closely related to ethylene biosynthesis, in apple tissue (4). Previously, MTA was shown to convert to methionine via MTR-1-P in tomatoes and avocadoes (120), in rat liver (16), and in Enterobacter aerogenes (209). Both the CH_3S-group and the ribose portion of MTR are

CH_2S-

incorporated into the new methionine (150). Kushad, et al, showed later that MTR-1-P converts to methionine via alpha-keto-gamma-methylthiobutyric acid (alpha-KMB) (121) which is identical to 4-methylthio-2-oxobutanoate cited in earlier work (30).

Experiments with ^{14}C-methionine fed to apple tissue, held in air, in nitrogen, and first in nitrogen and then in air, has shown that in the first treatment (air) ethylene was produced; in the second (N_2), 1-aminocyclopropane-1-carboxylic acid (ACC) was accumulated; and in the last case the accumulated ACC was converted to ethylene. This also proved that oxygen is required for the conversion of ACC to ethylene (5).

Treatment of a variety of plant tissues with ACC increased ethylene production (54). For several fruits, ACC synthase has been suggested to be the rate limiting step in ethylene biosynthesis (96,249). Endogenous ACC has been postulated to regulate ethylene production in most plant tissues and fruit tissues at the climacteric stage of maturity (96). ACC can also be converted to its conjugated form, N-malonyl ACC (9,99,100,102). In sections from hypocotyls of dark-grown mung-bean (Vigna radiata L.) seedlings malonylation of D-amino acids and of ACC are intimately interrelated (96). Malonyl-ACC occurs in peanut seeds but does not serve as the source of ethylene during germination (99). Thus malonyl-ACC appears to be a means of inactivating the potentially harmful ACC as an ethylene precursor. However, in most fruit and vegetable tissues, malonyl-ACC is low in concentration and appears to play an insignificant role.

C. REGULATION OF ETHYLENE BIOSYNTHESIS

1. Light. Light has been shown to regulate ethylene production in plants. Illumination of sorgum seedlings (66), cranberries (65), and lettuce seeds (3) has been observed to increase ethylene production. Exposure of mature green tomatoes to red light resulted in a 3-day advance in pigmentation, climacteric CO_2 rise and ethylene production (68). Also a blue/far red light spectrum increased ethylene in peach apices (74). After a red light pulse on etiolated pea seedlings, ACC content, ACC oxidase activity, and ethylene production were increased (68). However, the conversion of exogenously applied ACC to ethylene was reversibly inhibited by light in tobacco (68) and in water-stressed wheat leaves (240).

2. Stress. It has been shown that ethylene (wound or stress ethylene) is induced in various plant tissues by stress-inducing chemicals, temperature extremes, drought, water logging, radiation, insect damage, and disease or mechanical wounding (1,245).

Bradford and Yang (34) have shown that waterlogging in tomato plants not only blocks the aerobic conversion of ACC to ethylene in the root, but also causes increased synthesis of ACC which is subsequently transported through the xylem to the shoot where it is aerobically converted to ethylene, causing to petiole epinasty. Yu and Yang (249), working with mung bean hypocotyls, orange peel, and unripened green tomatoes, have shown that ACC synthase regulates stress and wound ethylene. ACC synthase and the

production of ethylene forming enzymes (EFE) are both stimulated by wounding in cantaloupe, and in addition, EFE are stimulated by ethylene (247).

3. Carbon dioxide. Carbon dioxide prevents or delays ethylene production. At 1% (1) and 8% (52) concentration, CO_2 inhibited ethylene production. The concentrations of CO_2 giving half-maximal inhibition of ACC-dependent ethylene production is only several-fold higher than the ambient level of CO_2 in the atmosphere (247). CO_2 is used in commercial controlled atmosphere storage of fruits to help delay the ripening action of ethylene, and also to reduce the respiration rate.

4. Oxygen. Low oxygen inhibits ethylene production. Propylene administered in 6.5% O_2 or less did not induce ethylene production by 'Red Delicious' apples. An anaerobic atmosphere was necessary to completely inhibit ethylene synthesis in fruits once autocatalysis began (207). Apple tissue ceased ethylene production soon after it was placed in nitrogen atmosphere. Ethylene production resumed when the tissue was again placed in air (5).

5. Plant hormones. Auxin (IAA) induces ethylene production especially in vegetative tissues. Abscisic acid (ABA) treatment of wheat leaves in a stress induced ethylene production experiment, reduced ethylene and ACC but treatment with benzyladenine (BA) or IAA stimulated them (165). Auxin regulates ethylene production in mung bean hypocotyls by acting on ACC synthase, a key enzyme that converts SAM to ACC (243,244,248,249).

Auxin inhibits ripening in 'Bartlett' pears at early stages of maturity, but promotes ripening if applied at late maturity (79). In several studies on the effect of cytokinin on leaf senescence, there was substantial agreement (119,225) that cytokinin maintained protein by retarding the rate of breakdown rather than enhancing the rate of synthesis. Reports on the effects of cytokinins on fruit ripening also indicate that they act as senescence retardants, particularly of the peel (198).

6. Organic compounds. Aminoethoxyvinylglycine (AVG), and amino-oxyacetic acid (AOA), both inhibit ACC synthase action (249), and other rhizobitoxine analogs have been shown to inhibit ethylene production (17,18,96,146,249). Sensitivity of the tissue to these compounds decreases with the age of the tissue (132,138). Preharvest applications of AVG inhibit ethylene in pears (169,197,235) and apples (38) during storage. A period of storage at 0^{o} C, which promotes uniform ripening in 'Bartlett' pears after transfer to 20^{o} C, counteracted the effects of preharvest applications of AVG (197). However, ethylene production was inhibited in pears removed from storage at 0^{o} C and vacuum infiltrated with AVG. Infiltration with AVG also resulted in delayed respiratory climacteric and retarded ripening changes. Treatment of inhibited fruits with ethylene overcame the effects of AVG (169).

Cyclohexamide inhibits chemically stress-induced ethylene (2,241) and actinomycin D inhibits auxin-induced ethylene (241). Uncouplers of oxidative phosphorylation (such as 2,4-

dinitrophenol) (12,13), free radical scavengers such as n-propyl gallate or sodium benzoate (12,13,17), and phenothiazines (115), also inhibit ethylene synthesis. Benzohydroxamic acid and 3-chlorobenzohydroxamic acid, however, promote ethylene synthesis in the presence or absence of ACC in cocklebur cotyledons (202).

Carbohydrates have also recently been shown to stimulate ethylene production via an auxin-induced increase in ACC synthase activity and, more specifically, galactose has increased ethylene production in tobacco leaf discs (173). In another study, galactose stimulated ethylene production and increased free ACC in aged tobacco leaf discs (174). Galactose at 400 to 800 ug/g of tomato fresh fruit weight stimulated a transient increase in ethylene within 25 hours after infiltration. Transient increases in ethylene were also caused by infiltrating galacturonic acid, dulcitol, and mannose (108).

Cell wall fragments obtained from the digestion of pear cells by macerase (a mix of polygalacturonases and cellulases) led to rapid and transient ethylene production when applied to pear suspension culture cells (215). Induction of ethylene biosynthesis has also been observed by cell wall digesting enzymes like cellulysin (11). Purification of cellulysin revealed that the ethylene inducing factor of this enzyme mixture is a protein different than the ethylene inducing factors of pectinase, pectinlyase, and rhozyme enzymes (82). Recently, tomato polygalacturonase has been reported to induce ethylene production in tomato fruits (19).

7. Inorganic compounds. Several metallic ions can play regulatory roles in ethylene production. Silver ion inhibits ethylene action in a wide variety of plant responses including growth inhibition, abscission, and change in sex expression of cucurbit flowers (28). In addition, silver ion inhibits ethylene production in fruits (104), and vegetative tissues (7,27). Silver ion has been commercially used in cut carnations to extend their vase life (223) and is usually formulated as silver thiosulfate. Cost and mammalian toxicity preclude its use in edible crops.

Cobalt reduced ethylene production in subcellular fractions of tomato (124), and effectively blocked the conversion of ACC to ethylene. While cobalt is quite effective, because it is a heavy metal and possesses mammalian toxicity at the concentrations needed to inhibit ethylene it will likely not be cleared for use on edible crops.

Copper chelators blocked ethylene synthesis in apple tissue slices (135).

The physiology of plants is profoundly influenced by the level of calcium during growth and development (107,175). Although calcium was reported to enhance ethylene production in excised mung bean hypocotyls (125,246), apple protoplasts (10), potato discs (15), and aged slices of postclimacteric apple (132), this effect seems related to stressed or wounded tissues. In the latter case the effects of calcium to preserve the ethylene forming system was attributed to the stabilization of membranes by calcium (127). In most naturally occuring systems, calcium seems

to suppress the ethylene synthesis systems.

Increasing the calcium content of apples has been reported to retard senescence, and to reduce several types of physiological disorders during storage (20,165, 179, 188, 204). Respiratory rate and ethylene production (20,75,132,205), were also reduced. Calcium deferred senescence in leaf discs (177). Low calcium content is also associated with rapid senescence of vegetative tissue (20).

An antagonistic interaction between calcium and ethylene was observed in storage breakdown of apples, indicating that an accurate index of storage potential of apples could be based on both calcium and ethylene levels (238). 'Anjou' pears affected with cork spot (a calcium deficiency disorder similar to apple bitter pit) produced more and earlier ethylene than healthy fruits (165). Low calcium in 'Anjou' pear fruit decreases the chilling requirement to induce ripening (222), indicating that calcium may be affecting the conversion of ACC to ethylene (222) or possibly other intermediates in ethylene synthesis. In tomatoes, the delay and reduction in intensity of the climacteric, in calcium infiltrated fruits is associated with reduced ethylene production (239). Similar results were observed with calcium infiltrated avocado treated with 500 ul/l propylene (73), compared to non-treated (73,223). Poovaiah and Leopold (177) reported a similar delay in the ripening of high calcium content bananas.

There is much evidence that calcium is of fundamental

importance for regulating and maintaining membrane permeability and the maintainance of cell integrity (169). Electron microprobe studies of Roland and Bessoles (202), have revealed that calcium is located especially in the border zone between the cytoplasm and cell walls indicating high calcium in the plasmalemma. Calcium can be removed from membranes by treatment with EDTA. This treatment increases membrane permeability to such an extent that inorganic and organic compounds diffuse out of the cell and considerable damage may result (219). Impairment of membrane permeability by calcium deficiency, like the effect of EDTA, reduces the retention of diffusible cellular compounds (70). Membranes become leaky and as the deficiency progresses there is a general disintegration of membrane structure (142). In whole plants the disorder commonly occurs in meristematic tissues such as root tips, growing points of the upper plant parts and storage organs. Brown melanin compounds resulting from polyphenol oxidation are associated with the deficient tissues (162). Addition of calcium can restore damaged membranes (21,53). Marshner (162) suggests that the low calcium storage organs have a high membrane permeability and allow solute diffusion in these tissues. This is obviously of importance in fruits and storage organs which accumulate large amounts of sugars from the phloem.

Calcium increases membrane stability by bridging the carboxylic groups of membrane proteins with the phosphate group of phospholipids and by interacting with adjacent phospholipid phosphate groups. Calcium literally "tightens" the membrane

phospholipids from an average area of 42 Å to 39 Å with high calcium.

Research within the last decade initially in animal tissues (141) and then more recently in plants (150,176,220) has revealed the calcium binding proteins, and in particular calmodulin. This protein interacts reversibly with calcium to form a protein-calcium complex, the activity of which is regulated by the cellular flux of calcium. Calcium binding proteins act as potential receptors of calcium thus mediating the effect of calcium in cellular reactions. Calmodulin has been shown to play a central role in cellular regulation in animals (by regulating the protein kinases and more recently by altering phosphatidyl inositol phosphates) and the same seems likely to be the case for plants (176) as suggested by current research.

8. Chilling. In contrast to summer pears which ripen readily without cold treatment, winter type pears usually require cold treatment for a period of time to produce ethylene and ripen (57,59,116,176,208). Wang, et al, (236) have shown that short cold treatment in the orchard of 'Bartlett' pears induced ethylene production and climacteric rise in respiration accompanied by fruit softening and an increase in soluble pectin and protein nitrogen. By using cool temperatures at night (45° F) and normal temperatures (65° F) during the day, he induced premature ripening, a physiological disorder of 'Bartlett' pears. Control of premature ripening could be attained by sprays of gibberellins or daminozide which delayed ripening. However, cold treatment

reversed this effect (138). 'Bosc' pears typically require 20 days at -1.1° C to be able to produce ethylene and ripen (59). However, 6 days at 5° C before transfer to 20° C resulted in the highest ethylene production and better ripening (208).

'Anjou' pears usually require 50 to 60 days cold storage at -1.1° C (56,60,180) in order to ripen. However, in some instances ethylene production has been reported to be initiated shortly after harvest (57), or even at harvest (236). High calcium content in the fruits prolonged the cold requirement of 'Anjou' pears (222). Preharvest exposure of 'Anjou' pears for 6 weeks at 17.2° C and 13.9° C daily hourly average (DHA) resulted in normal ripening, but 20° C and 11.7° C resulted in failure to properly ripen, and much lower quality, after long term storage (160). Preharvest temperatures early in the season are very important in determining harvest maturity date of 'Anjou' pears (159). Sensitivity of 'Anjou' pears to exogenous ethylene increased progressively during maturation. Flesh firmness decreased, while ethylene production, respiratory rate, and protein content increased (237). In many cases, however, when the pears were treated with 0.05 or 0.1 ul/l ethylene the pears softened before any autocatalytic ethylene was observed (237).

In another study (86), 'Anjou' pears treated with 500 or 50 ul/l propylene (these are the equivalent to 4 and 0.4 ulu/l ethylene) at harvest, softened from 6.8 to 2 Kg flesh firmness before autocatalytic ethylene was triggered. However, after 110 days in -1.1° C storage, the loss in firmness occured

significantly with the rise in ethylene. The trigger of autocatalytic ethylene by the 500 ul/l propylene at harvest required 9 days, but after 110 days in -1.1° C storage, only 2 days. This further suggests that the sensitivity of the fruits to propylene increases with storage time. A decrease in flesh firmness before the rise in climacteric ethylene occured within 10 days at 20° C ripening and after 60 days in -1.1° C storage of 85% mature 'Anjou' pears (232). This decrease in firmness prior to the climacteric rise in ethylene was also observed by Toumadje and Richardson (216) when 'Anjou' pears were ripened at 20° C after they had been stored from 1 to 5 weeks in -1.1° C.

In an earlier work, Porritt (180) investigated the effects of temperature on respiration, firmness, and other quality characteristics of 'Anjou' pears and concluded that a chilling requirement was necessary for ripening and that -1° C storage temperature compared to 0° C increased the storage life by 40%. By examining this work more carefully we can observe that when the pears were held at 15° or 20° C the pears lost their firmness before any respiratory climacteric rise occured.

Blankenship and Richardson (31), have shown that during the 46 days chilling required for 'Anjou' pear fruits to initiate ethylene production, the capacity to convert exogenous ACC to ethylene preceded the endogenous production of ACC and ethylene. In 'Conference'pears, ethylene synthesis and ACC synthase activity increased rapidly after slicing of pears held at -1° C but more slowly in discs cut from pears immediately after harvest. The

formation of the mRNA for ACC synthase is suggested to have also occured in -1° C storage (113).

Cold treatment (which actually causes chilling injury in cucumbers) stimulated ACC synthase, ACC, and ethylene production in cucumbers, but only after they were transferred to warmer temperatures (230). However, cucumbers chilled at 2.5° C showed an increased capacity to synthesize ACC accompanied by a diminished loss of galactose residues (cell walls) relative to those exposed to non-chilling temperature (12.5° C) (90).

Ethylene synthesis occurs even at 0° C at low levels until fruit are transferred to 15° C, suggesting that those ethylene levels are adequate to autocatalytically trigger further ethylene production (116).

9. Autocatalysis. Autocatalysis of ethylene production is a common phenomenon of climacteric fruit. Ethylene above a threshold concentration triggers its own production in many plants (92,237). Trewavas (217) suggested that the limiting factor in plant development is sensitivity of tissues to plant growth substances rather than the changes in the endogenous concentrations of growth substances. Sensitivity of fruit to ethylene is not constant throughout the life of the fruit. Most fruits become more sensitive with increasing time after anthesis (155). Fruits still attached to the tree are less sensitive to ethylene than harvested fruits (158), suggesting that a ripening inhibitor may be supplied by the parent plant and this is well known for avocadoes (43). Ethephon (233) and propylene

(154,160,207) can also induce ethylene production. Propylene, like ethylene, causes epinasty, inhibits elongation of pea subapical sections, and promotes ripening (67). Propylene was found to be the second most active compound (after ethylene) mimieing ethylene activity. Equivalent molecular concentration requirements of propylene to cause half maximal ethylene responses was found to be 130 times that of ethylene (49).

It has been suggested by Hackett, et al (92) that the action of ethylene as a ripening hormone may be distinguished by two processes: 1) the initiation of ethylene synthesis, and 2) the physiological response. ACC synthesis (33,96,244), as well as conversion of ACC to ethylene (97,222,244), is stimulated by propylene during fruit ripening. However, inhibition (i.e. auto-inhibition as opposed to auto-catalysis) of ethylene production by ethylene has also been reported for non-climacteric fruit tissue (193), developing (252) and mature (39, 224, 251) climacteric fruit, in wounded (194,201), and in IAA-treated (7) vegetative tissues. In wounded citrus peel tissue, this inhibition may be due to inhibition of ACC synthase formation and activity (192). The concentration and the time of exposure to ethylene appears to determine the extent of inhibition in all cases.

CHAPTER III

EFFECTS OF TEMPERATURE AND FRUIT CALCIUM ON RIPENING AND CHILLING REQUIREMENTS OF 'ANJOU' PEARS

Additional index words: Pyrus communis, postharvest physiology, ethylene synthesis, firmness, storage.

Abstract. 'Anjou' pears were sprayed before harvest with 2 concentrations of $CaCl_2$ at the Mid-Columbia Experiment Station, Hood River Oregon, and harvested at 68 N firmness. The fruits were held at -1.1° , 5° , 10° , and 20° C, for 0, 13, 25, 40, 55, 70, and 85 days, before evaluation for ability to ripen when held at 20° C for up to 11 days.

Control fruits stored at -1.1° required 55 days in order to be able to produce more than 1 ul/l internal ethylene during ripening at 20° C. A decrease in firmness was associated with increased ethylene. Calcium-sprayed fruits showed the same pattern, but were able to produce more than 1 ul/l ethylene only after 70 days of -1.1° C storage.

Fruits held at 20° C storage, required 70 days to produce more than 1 ul/l of ethylene. Climacteric ethylene appeared only after firmness decreased to about 20 N. Calcium-sprayed fruits showed the same pattern, but required 80 days at 20° C to produce more than 1 ul/l ethylene and resisted softening for about 50 days.

Untreated pears stored at 5° or 10° C both required 40 days

to produce internal ethylene concentrations greater than 1 ul/l, whereas the calcium-sprayed fruits at 5 or 10° C required 55 and 40 days, respectively. Fruit ripening at 5° or 10° C was intermediate between -1.1° and 20° C stored fruits in relation to softening and ethylene synthesis.

INTRODUCTION

Winter pears usually require a period of cold storage in order to ripen and produce ethylene. Summer pear cultivars such as 'Bartlett' require no or only a very short cold treatment of a few days in order to increase ethylene production (236) and induce ripening (138). Among the winter pears, in one study (59), mature 'Bosc' required 20 days at -1.1° C to initiate ripening, but in another study required only 6 days at 5° C to initiate ripening at 20° C (208). Storage at -1° C for a sufficient (but unspecified) period abolished the lag in ripening of 'Conference' pears (109). 'Eldorado' pears require about 40 days in 0° C to produce ethylene and ripen (229).

'Anjou' pears normally require 50 to 60 days (59,56,60) in cold storage at -1.1° C to ripen. However, depending upon the fruit calcium concentration, and possibly other factors, they may require as much as 90 days (59), as few as 30 days (59) or even zero days (237) in cold storage at -1.1° C to begin to ripen at 20° C. In an earlier study, Porritt (181) investigated the effects of temperature on respiration and firmness in 'Anjou' pears. A

more rapid loss in firmness with higher temperature was observed and in all cases this happened before the climacteric rise of CO_2. High calcium 'Anjou' pear fruits required 15 more days in cold storage in order to initiate ripening than did 'normal' calcium fruits (86,222).

Increased calcium levels in apples and pears were found to retard senescence and to reduce physiological disorders during storage (23,165,179,181,204). Increased calcium levels have been associated with reduced respiratory rate and ethylene production in apples and pears (23,75,132,199). The effects of calcium on postharvest physiology of fruits are also related to promotion of flesh firmness and delayed softening of pears (222) and apples (143) during cold storage.

Exogenous ethylene applied to immature and mature 'Anjou' pears can induce ripening and softening. 'Anjou' pear sensitivity to exogenous ethylene increases as fruits mature (237). Treatments at harvest with various concentrations of propylene, on both calcium-sprayed and control fruits, resulted first in a decrease in firmness followed by an increase in autocatalytic ethylene. While autocatalytic ethylene production in calcium-treated 'Anjou' pear fruit was not different than the control, calcium-treated pears softened less rapidly (86).

The purpose of this study was to investigate the extent to which 'Anjou' pear chilling requirements for ripening were affected by the storage temperature and fruit calcium concentration.

MATERIALS AND METHODS

Two groups of ten mature 'Anjou' trees were selected at the Mid-Columbia Experiment Station, Hood River, Oregon in 1985, and sprayed eight consecutive weeks (during fruit development) with 0 (control), or 32.3 mM calcium chloride plus B-1956 surfactant. All fruits were harvested based on 147 days from full bloom and 68 N flesh firmness of the control fruits. The fruits were transferred into perforated polyethylene film-lined 20 kg cardboard cartons and placed in -1.1°C, 5° C, 10° C, or 20° C storage rooms until they were used. All the fruits were screened for cork spot and only healthy fruits used.

Fruit samples were taken at harvest and later from each storage temperature treatment after 13, 25, 40, 55, 70, and 85 days and placed in a 20° C room to ripen for 11 days. Five replicates for each ripening day at 20° C, temperature and calcium rate were used. Internal ethylene and flesh firmness were measured every other day for fruits stored at different temperatures and then ripened at 20° C.

Ripening parameter determinations. A one ml internal atmosphere sample was pulled by syringe from the water-immersed pears and internal ethylene was measured with a Carle Model 311 flame ionization gas chromatograph equipped with a 2.0 m, 80/100 mesh, activated alumina column. For comparative purposes, internal ethylene at 1 ul/l was arbitrarily chosen as having physiologic activity. The same five fruits were used for flesh firmness measurements (two punches on each fruit) by a Hunter

force gauge model LKG-1 12 kg tester equipped with 8mm tip, in a UC Davis apparatus.

Calcium determinations: After each ripening series was over, all fruits of the first ripening day, were washed and used for calcium extraction by the method described by Perring (135), as modified in our lab (148). Ten grams of a 1:1 fruit-water suspension were transferred into a serum-capped test tube, 10 ml of concentrated HCl (37.8% A.R.) were added and tubes were capped. Capping considerably reduced the analytical variability due to uneven evaporation of HCl. Acid concentration must remain constant for accurate atomic absorption readings. The suspension was boiled for 20 min in a water bath and then cooled. The digested contents were then filtered through Whatman 41 paper. Strontium chloride was added to a final concentration of 3% $SrCl_2$ in all extracts in order to reduce mineral interferences. The filtrate was diluted to 25 ml with distilled water. Calcium was determined in a Perkin Elmer Model 303 Atomic Absorption spectrophotometer calibrated against $CaCl_2$ standards in acid.

Statistical analysis. LSD's for comparisons between treatments at a particular time during ripening were calculated with the NCSS "Number Cruncher" statistical system on an IBM PC computer. LSD's for comparing firmness and internal ethylene were obtained by the application of factorial analysis.

RESULTS

This study investigated the effects of storage temperatures (-1.1°, 5°, 10°, and 20° C) and fruit calcium on chilling requirements of 'Anjou' pear by measuring the ripening parameters (fruit firmness, and internal ethylene) at 20° C after the fruits were stored for various periods of time.

For the preharvest spray treatments of water plus surfactant (control), and 4.2 mg $CaCl_2$/1, the fruit calcium concentrations were found to be 5.59 and 6.61 mgCa/100gr F.W., respectively. The 32.3 mM $CaCl_2$ -sprayed fruits calcium concentrations were significantly different compared to the control (LSD.05: 0.92). The internal ethylene was found to be 0.16 and 0.08 ul/l (LSD.05: 0.12), and the flesh firmness was found to be 67.6 and 71.3 N (LSD.05: 6.2) for both control and calcium treated-pears respectively, at harvest.

I. Storage temperature effects on ripening parameters of control 'Anjou' pears (no calcium treatment).

A. FIRMNESS.

Fruit firmness during -1.1 ° C storage declined only about 7 N (ie, from 68 to 61 N after 85 days). However, fruits stored at 5° C retained their firmness for about 40 days followed by a rapid decrease to 17 N on the 70th day. Fruits stored at 10° or 20° C gradually softened to 15 N within 55 and 70 days, respectively (Fig.III.1.).

Storage at -1.1° C (Fig.III.2.). Fruit firmness at harvest

was 67.6 N, and only slightly decreased during the first 11 days when the fruits were subjected to 20° C ripening. From 25 through 40 days of -1.1° C storage, fruit firmness was 60 N and remained constant for the 11 days of attempted ripening at 20° C. After 55, 70, and 85 days of cold storage, fruit firmness had decreased to 25, 19, and 11 N, respectively, by the 11th day of 20° C ripening.

5° C storage temperature (Fig.III.3.). No decrease in the initial 68 N firmness was observed when the pears were subjected to ripening from harvest through 13 days of 5° C storage. However, after 25 days of 5° C storage, fruit firmness began to decrease, and thus after 40, 55, and 70 days substantial softening was evident on transfer to ripening temperatures.

10° C storage temperature (Fig.III.4). Marked decreases in firmness were observed only after the fruits had been stored at 10° C for 25 days or longer. Fruits ripened after 25 days in 10° C had decreased firmness to 38 N in 11 days at 20° C while fruits stored for 40 and 55 days softened to 10 N by the 7th day of ripening at 20° C.

20° C storage temperature (Fig.III.5.). Only slight firmness loss was observed when the fruits were stored at 20° C for 13 days. After the 13th day the fruits started softening and the firmness decreased to 45, 38, 20, and 10 N on the 25th, 40th, 55th, and 70th day of storage at 20° C, respectively.

B. INTERNAL ETHYLENE.

Fruits stored at -1.1° C showed a slight increase in internal ethylene which reached about 3 ul/l on the 85th day. The increase in internal ethylene that led to the climacteric rise for pears stored at 5° C or 10° C was observed only after the 25th day. However, fruits stored at 20° C showed little internal ethylene up to 40 days, and then an increase to 40 ul/l on the 70th day (Fig.III.6.).

-1.1° C storage temperature (Fig.III.7.). The first detectable rise of internal ethylene above 1 ul/l when transferred to 20° C, was observed in fruits after 55 days of -1.1° C storage. This would be considered the normal minimum chilling requirement and typical of this pear variety.

After 70 days of -1.1° C storage and 11 days ripening at 20° C, internal ethylene rose to 10 ul/l. After 85 days in cold storage the internal ethylene in fruits increased considerably, and reached 45 ul/l after 11 days.

5° C storage temperature (Fig.III.8.). Internal ethylene remained at very low levels from harvest through 25 days in storage. Internal ethylene rose above 1.0 ul/l only after the fruits were 40 days in cold storage followed by 9 days ripening at 20° C. Internal ethylene rose to 15 ul/l after 11 days ripening.

After 55 days in 5° C cold storage the fruits were capable of producing more ethylene. Ethylene production was markedly increased after the fruits were held for 55 and 70 days in cold

storage and the chilling requirements were completely satisfied. Internal ethylene peaked at 51 and 55 ul/l, respectively.

10° C storage temperature (Fig.III.9.). Internal ethylene remained low upon transfer to 20° C, until after the fruits were held for 25 days at 10° C. Increasing amounts of ethylene (above 1.0 ul/l) were found during ripening at 20° C after the fruits were held at 10° C for 40 days or more. When the fruits were held for 55 days in 10° C and then transfered to 20° C, internal ethylene appeared to decline, probably indicating that peak ethylene production had already occurred in 10° C storage and prior to 20° C ripening.

20° C storage temperature (Fig.III.10.). Immediately after harvest a small increase in internal ethylene occurred and a minor peak was reached on the 6th day at 0.6 ul/l. The internal ethylene remained low after 25 and 40 days at 20° C. Only after 55 days did ethylene rise above 1 ul/l, and peaked after 70 days in 20° C.

II. Fruit calcium effects on ripening parameters.

A. FIRMNESS.

While there may not always have been differences in firmness large enough to be statistically significant relative to fruit calcium treatments, fruits with high calcium were consistently firmer than the controls by 4 N at harvest and by 1-4 N during storage at -1.1°, 5°, or 10° C. Larger differences (+ 4 to + 30 N) in firmness of calcium-treated fruits compared to controls were only observed in fruits stored at 20° C (Fig.III.1.).

-1.1° C storage temperature (Fig.III.11.). Fruit firmness of calcium-treated pears remained fairly constant during the first 55 days in cold storage followed by ripening at 20° C. However, the firmness of control fruits remained constant for only 40 days in cold storage. After 70 and 85 days in cold storage, calcium-treated pears softened significantly by the 11th day at 20° C and the flesh firmness was 52 and 15 N, respectively.

5° C storage temperature (Fig.III.12). Attempts to ripen calcium-treated fruits at harvest or through 25 days of cold storage failed to elicit ripening responses contrary to what happened with the control fruits. Fruit softening response of calcium-treared pears at 20° C was first shown after the pears had remained in cold storage at 5° C for 40 days. After 55 or 70 days cold storage plus 11 days of ripening at 20° C, both control and calcium-treated fruits had softened to 10 N on the 7th day.

10° C storage temperature (Fig.III.13.). Calcium-treated pears retained most of their firmness when they were stored for 25 days in 10° C storage followed by ripening at 20° C. The control fruits however, showed a significant decrease in firmness after 25 days in storage. After 40 days in cold storage the flesh firmness of both the calcium-treared and control pears dramatically decreased to 10 N within 7 days in 20° C.

20° C storage temperature (Fig.III.5.). Calcium-sprayed 'Anjou' pears retained most of their initial firmness through 55 days in 20° C storage. The control retained firmness only for 20 days. The calcium-treated and control fruits softened to 20 N

at 90 and 55 days of storage, respectively.

B. INTERNAL ETHYLENE.

During storage at -1.1° , 5° , or 10° C calcium-treated fruits produce consistently less (although not statistically different) ethylene than the controls. Only calcium-treated pears stored at 20° C showed a difference of about 10 days later to produce climacteric ethylene than the controls (Fig.III.6.).

-1.1° C storage temperature (Fig.III.14.). The first detectable rise of internal ethylene above 1 ul/l at 20° C ripening was observed in calcium-treated fruits after 70 days - 1.1° C storage, and this was 15 days later than the controls. After 85 days of -1.1° C storage, internal ethylene rose calcium-treated pears to 28 ul/l after 11 days at 20° C.

5° C storage temperature (Fig.III.15). Calcium-treated pear internal ethylene remained at very low levels from harvest through 40 days in 5° C storage. Internal ethylene rose above 1.0 ul/l only after the fruits were stored for 55 days and then transferred to 20° C. However, the internal ethylene of control fruits rose above 1 ul/l after 40 days in 5° storage.

10° C storage temperature (Fig.III.16.). Internal ethylene rose above 1 ul/l only after the calcium-treated pears were stored for 40 days the same compared to 55 days for the control pears.

20° C storage temperature (Fig.III.10.). The internal ethylene of calcium-treated pears showed a slight increase with a minor peak of 0.4 ul/l on the 6th day after harvest. From the 13th day through the 68th day internal ethylene of calcium -

treated pears remained below 0.1 ul/l and rose above 1 ul/l only after the 80th day of storage at 20° C. This was 10 days later than for the control pears.

III. Chilling requirement.

Control (no calcium) spray. The chilling requirement was defined as the time that the fruits must remain in storage in order to produce 1 ul/l or more of internal ethylene during ripening at 20° C within 11 days. Accordingly, 55, 40, 40 and 70 days were the chilling requirements for the 'Anjou' pears that were stored at -1.1°, 5°, 10°, and 20° C, respectively (Fig.III.17.).

Calcium spray. The chilling requirements of calcium-treated pears were 70, 55, 55, and 85 days for -1.1° C, 5° C, 10° C, and 20° C, respectively (Fig.III.17.).

DISCUSSION

Control 'Anjou' pears in this study required 55 days at -1.1° C to satisfy their chilling requirement (Figs.III.2, III.17). This is agrees well with the 50 to 60 days at -1.1° C most commonly reported in previous research (31,59). In all ripening series, after the fruits had satisfied the chilling at -1.1° C, loss of firmness parallelled, and was synchronous with endogenous ethylene production (Figs.III.2, III.7).

'Anjou' pears stored at temperatures from -1.1° to 25° C revealed that temperatures between 5° and 10° C resulted in a

faster loss of firmness and an earlier rise in climacteric respiratory activity compared to fruits held at temperatures of 0^{o} or -1.1^{o} C (180). Cold storage of 'Anjou' pears has been reported to stimulate ethylene forming enzyme(s) activity (31), and to lead to accumulation of ACC (31,216). Also, the sensitivity of the fruits to propylene was maximized when the chilling requirement was satisfied (86).

Winter pears have always been thought to require chilling in order to ripen. This finding that the ability of the pears to eventually ripen at 20^{o} C with no chilling has been overlooked by many researchers who usually examine pear postharvest ripening ability at 20^{o} C for only 20 to 30 days (86,216,229). 'Anjou' pears stored at 20^{o} C produce ethylene above 1 ul/l after 70 days (Fig.III.5.). The minor peak in internal ethylene observed right after harvest for all of the storage temperatures might be attributed to stress due to harvest, handling, and transportation. However, the sequence of ripening events is different for non-chilled 'Anjou' pears compared to those that have satisfied their chilling requirement in cold storage. No rise in internal ethylene above 1 ul/l can be observed before 70 days at 20^{o} C storage, even though fruit firmness gradually decreases to about 20 N. The climacteric rise in ethylene appears to be related to the loss in firmness (Figs.III.5, III.10.).

Storage at 5 or 10^{o} C results in a decrease in chilling requirement to 40 days for both storage temperatures (Figs.III.8, III.9, III.17.). Temperatures of 7^{o} to 10^{o} C were reported to

decrease the chilling requirement of 'Bosc' pears from about 20 days (59) to 5 days (208). The pattern of 'Anjou' pear ripening after chilling at 5 or 10° C is intermediate between -1.1° and 20° C. Approximately 30% and 50% loss in firmness (Figs.III.3, III.4.) is observed during ripening at 20° C after 25 days of chilling at 5° and 10° C, respectively, despite the fact that internal ethylene only ranges up to 0.1 ul/l (Figs.III.8, III.9.). Fifty percent loss in firmness is even observed in 'Anjou' pears stored at 10° C for 40 days, when followed by ripening at 20° C. These fruits readily produce ethylene (Fig.III.9.) and lose most of the remaining 50% of their firmness (Fig.III.4.). Storage at temperatures near 10° C appears to stimulate ripening events faster than all the other temperatures, probably because of the combined effects of fruit softening and the earlier ethylene synthesis compared to 20° or -1.1° C storage.

Loss of firmness prior to ethylene production ethylene in ripening 'Anjou' pears has previously been reported (180,216). This seeming reversal of the order of ripening events, certainly raises question regarding the conventional views of the causal nature of ethylene in ripening. Researchers have recently given increasing attention to the importance of fruit softening in other aspects of fruit ripening. Several studies suggest that galactose residues liberated from cell walls may trigger ethylene production (108,89). In other studies, wall fragments obtained by the action of macerase enzymes triggered ethylene production in cultured pear cells (215). Treatment of 'Anjou' pears at harvest

with 50 ul/l propylene (equivalent to 0.4 to 0.5 ul/l ethylene) resulted in slow softening from 67 to 10 N within 30 days without a rise in climacteric ethylene (86), which suggests that this low concentration of propylene stimulates the activity and/or production of wall softening enzymes. Other studies on 'Anjou' pears suggest that softening is sensitive to as little as 0.08 ul/l ethylene (232). Thus very small or slightly increased amounts of ethylene which are always present in the fruit tissue, depending on the temperature, can stimulate softening which in turn may stimulate the climacteric rise in ethylene production.

Fruits sprayed with calcium during fruit development and then stored cold showed delayed softening, and required more about 15 days more in storage (in all cases except in 10° C storage) in order to produce 1 ul/l ethylene during ripening at 20 ° C compared to the water-sprayed controls. Fruit softening was found to be closely synchronized to the appearance of ethylene when the fruits were stored at -1.1° C. Calcium-sprayed fruits which were constantly stored at 20° C showed the same pattern of ripening as did control fruits except that they hold their firmness and green color for about 50 days (Fig.III.5). Only after a dramatic decrease in firmness did internal ethylene rise above 1 ul/l on the 80th day (Fig.III.10). Similar delays in softening (Fig.III.11.) and ethylene production (Fig.III.14.) were observed when the calcium-treated fruits were stored at 5° C. However, calcium-sprayed fruits stored at 10° C did not show much difference probably because of the rapid induction of ripening

(Fig.III.112, 15).

Several reports support a strong association of calcium with firmness in both apples (145) and pears (222). There are several possible roles for calcium in the postharvest behavior of fruits. Calcium interacts directly with the cell wall pectic substances, thus strengthening the cell walls (24). Also polygalacturonase activity is inhibited when calcium is added in the reaction medium for the assay (64). Similarly, cell membranes are affected by calcium, which improves their integrity and reduces their permeability (62). There may also be undiscovered direct and indirect effects of calcium on the ethylene biosynthetic pathway as suggested by these studies.

The ability of fruits to ripen with or without chilling raises the question of whether we should consider the chilling requirement as a normal part of the postharvest physiology of the fruits or instead consider it a manifestation of another temperature stress. If we consider storage and delayed ripening at 20 ^{o} C as normal then we are faced with the time separation of the rise in internal ethylene occuring after the losses in flesh firmness) and the respectively different threshold ethylene concentrations which may be unique in the winter pear fruit ripening. Does this happen because 'Anjou' pears are slow ripening fruits or because there are inhibitors of ethylene production and/or action which are overcome either by chilling or by loss in firmness? Those questions no doubt will continue to attract researcher's attention.

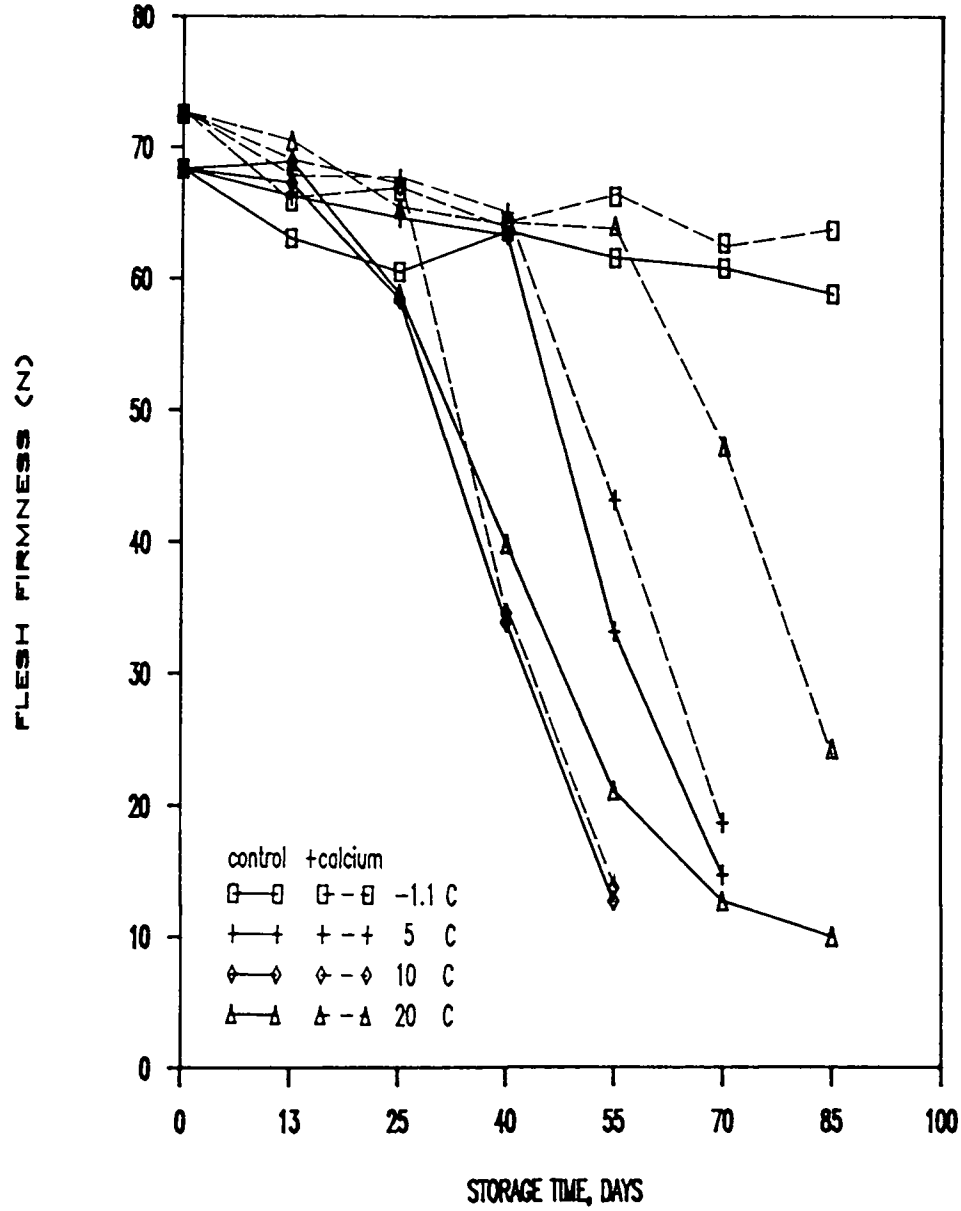


Fig.III.1. Effects of time at several storage temperatures on flesh firmness of preharvest calcium-treated or control 'Anjou' pear fruits. LSD.05: 11.2

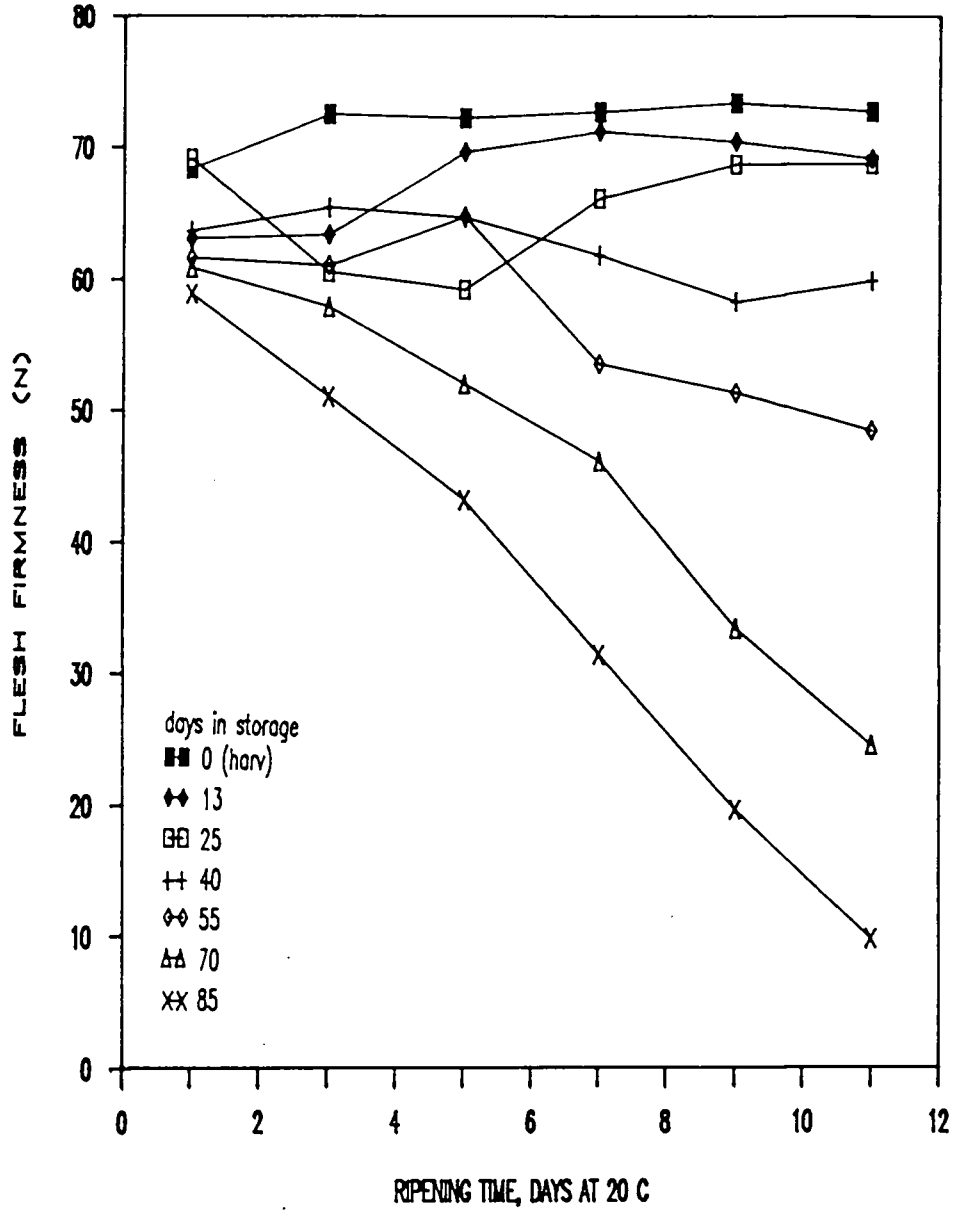


Fig.III.2. Effects of time (days) in -1.1°C storage on flesh firmness of 'Anjou' pears subjected to 20°C ripening.
LSD.05: 18.6

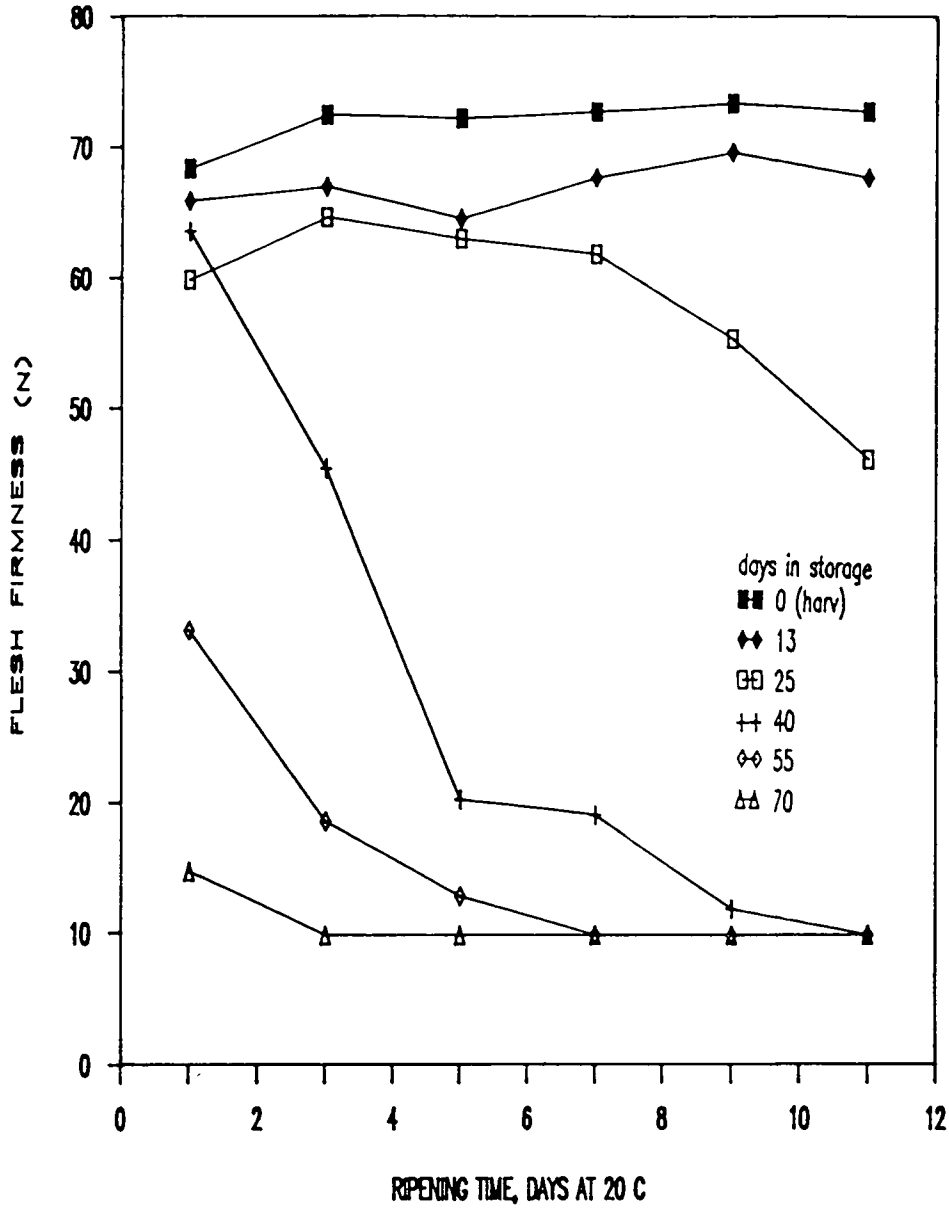


Fig.III.3. Effects of time (days) in 5°C storage on flesh firmness of 'Anjou' pears subjected to 20°C ripening.
LSD.05: 15.4

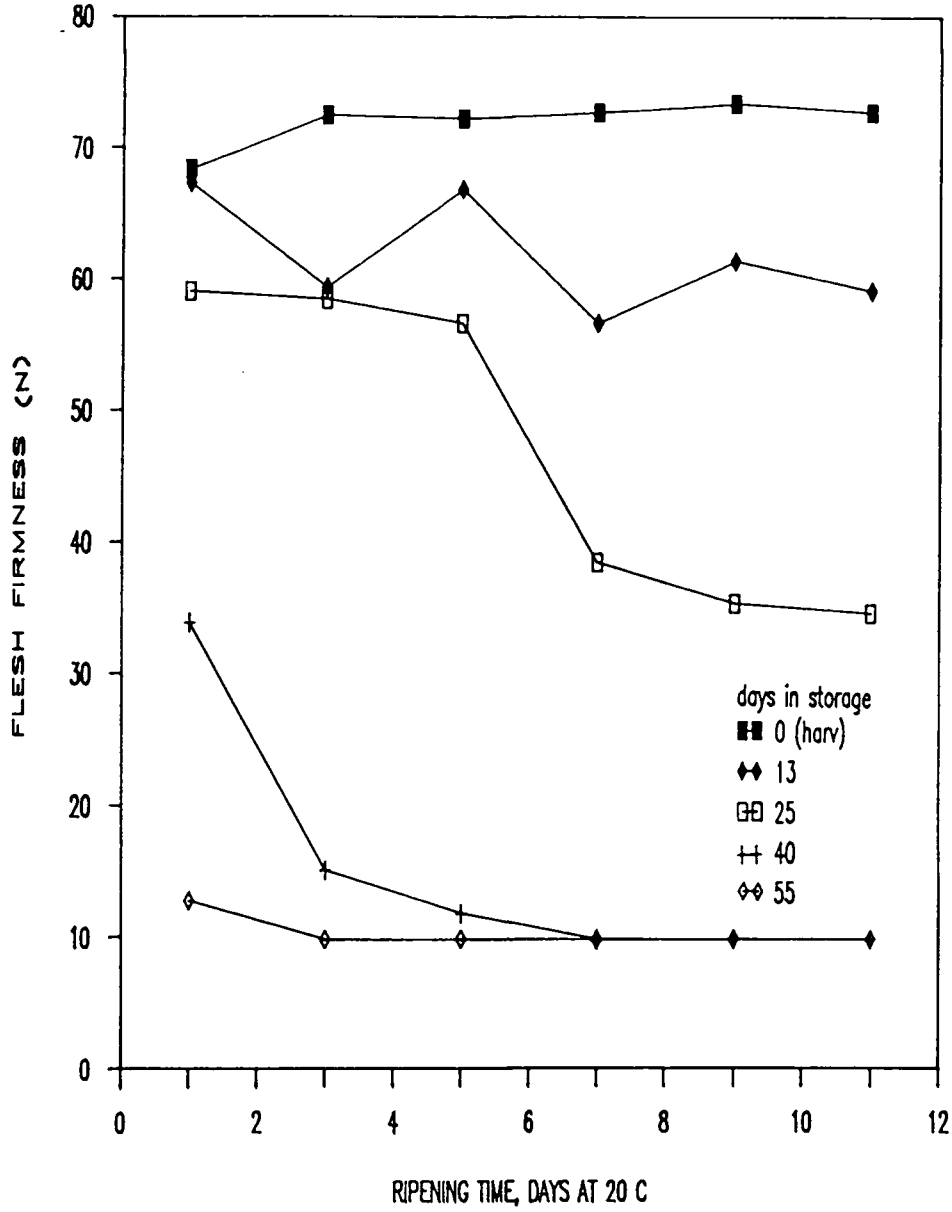


Fig.III.4. Effects of time (days) in 10°C storage on flesh firmness of 'Anjou' pears subjected to 20°C ripening.
LSD.05: 13.2

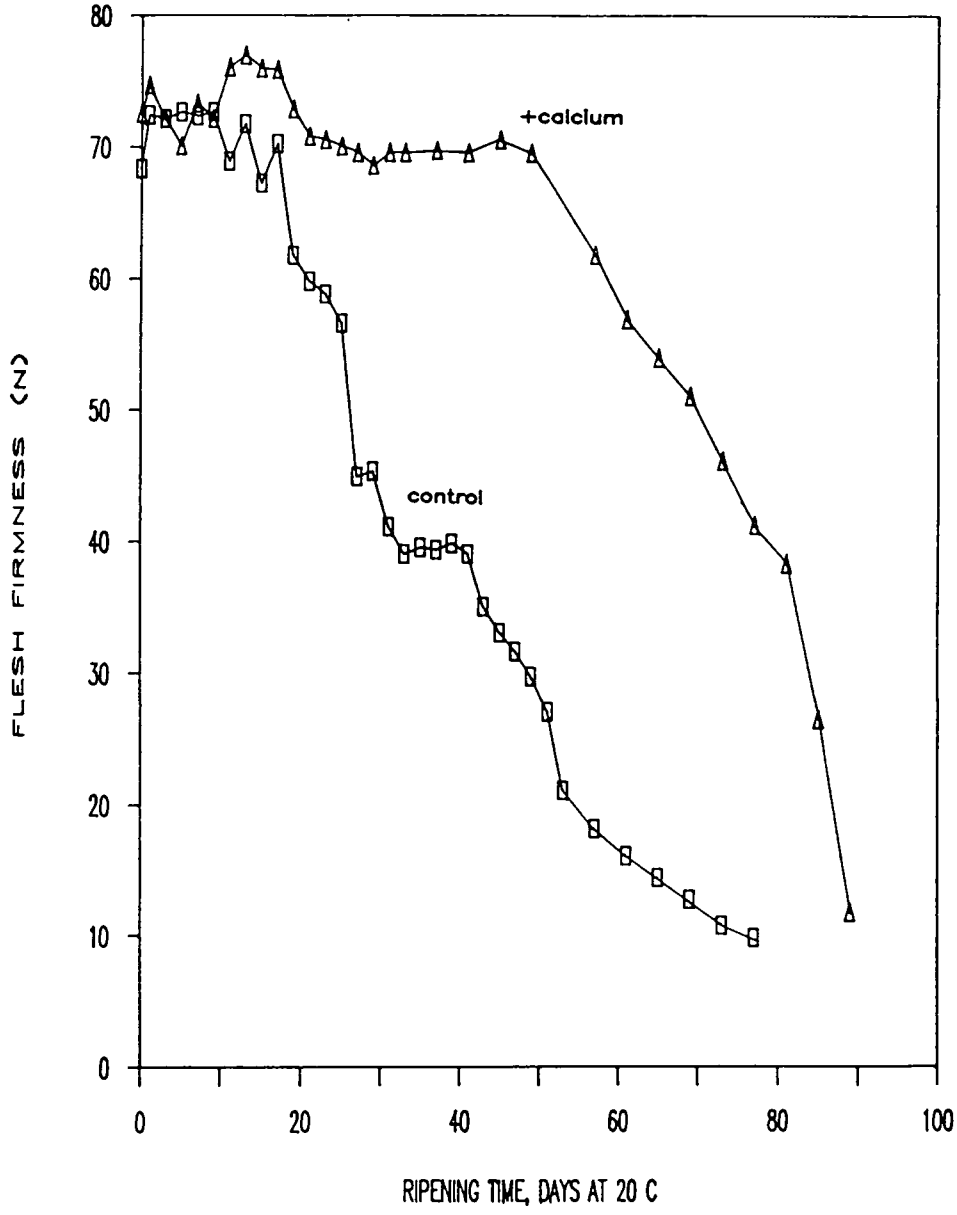


Fig.III.5. Flesh firmness of preharvest calcium-treated or control 'Anjou' pears as affected by time in 20°C storage. LSD.05: 12.1

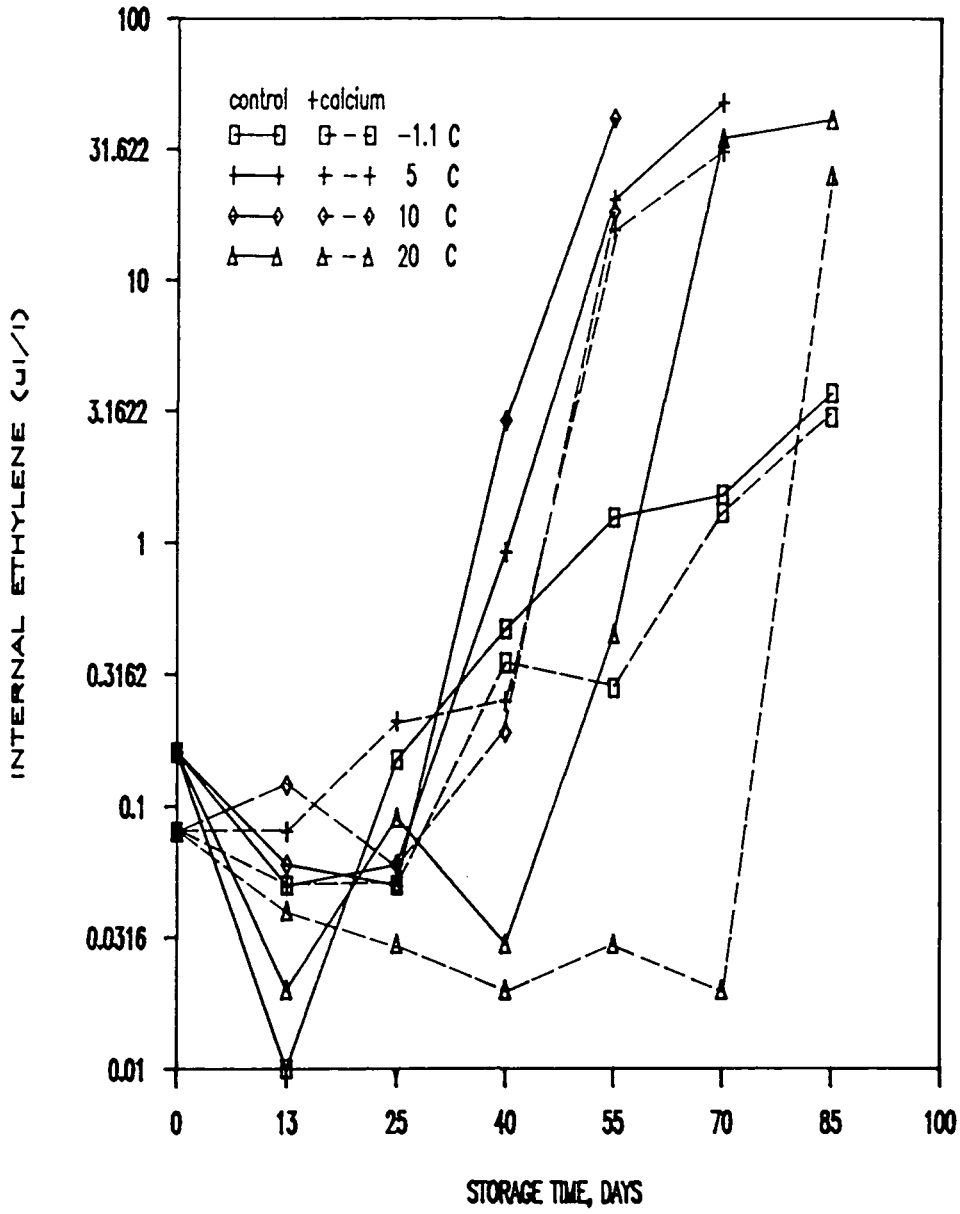


Fig.III.6. Effects of time at several storage temperatures on internal ethylene of preharvest calcium-treated or control 'Anjou' pear fruits. LSD.05: 6.3

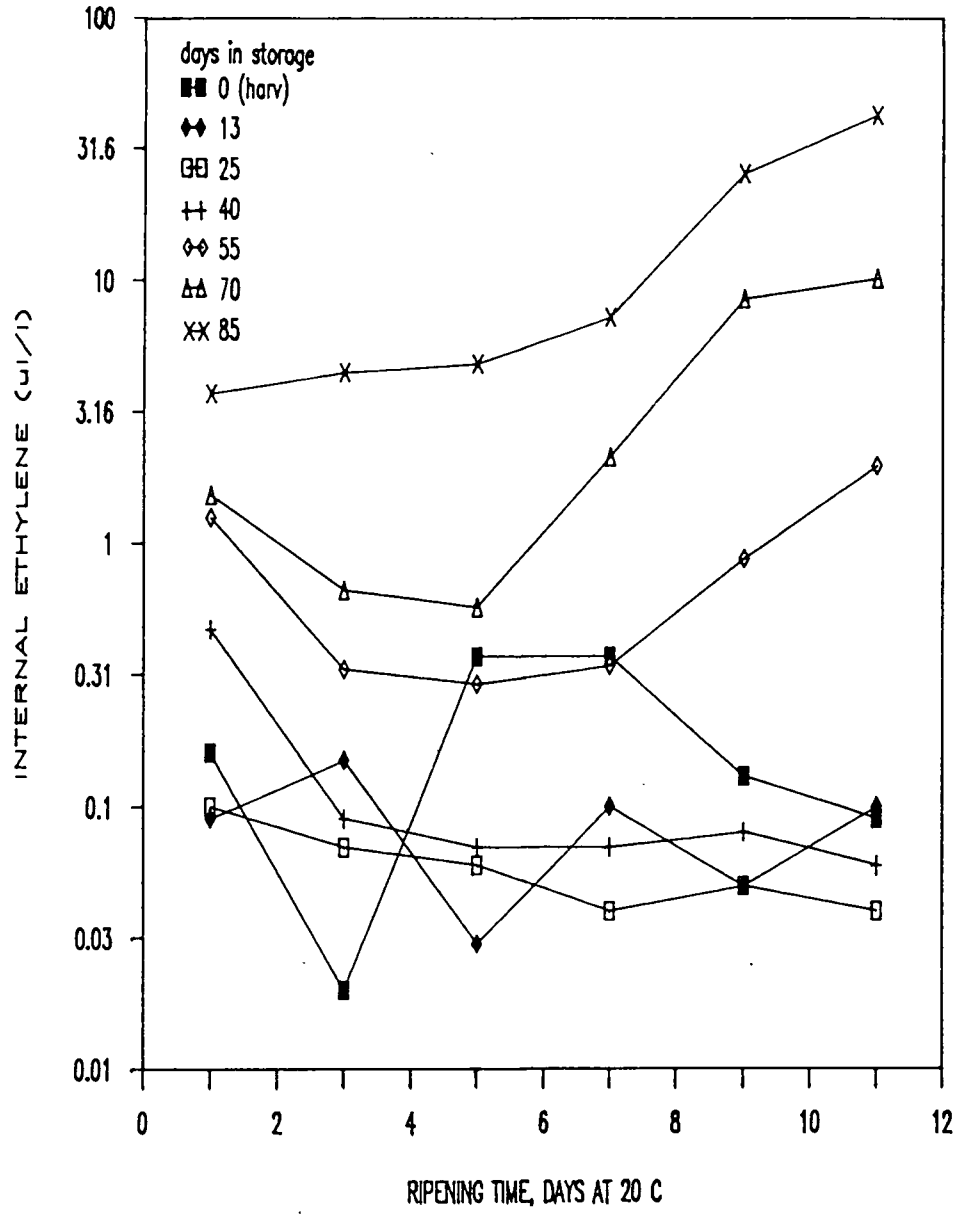


Fig.III.7. Effects of time (days) in -1.1 °C storage on internal ethylene of 'Anjou' pears subjected to 20°C ripening.
LSD.05:4.6

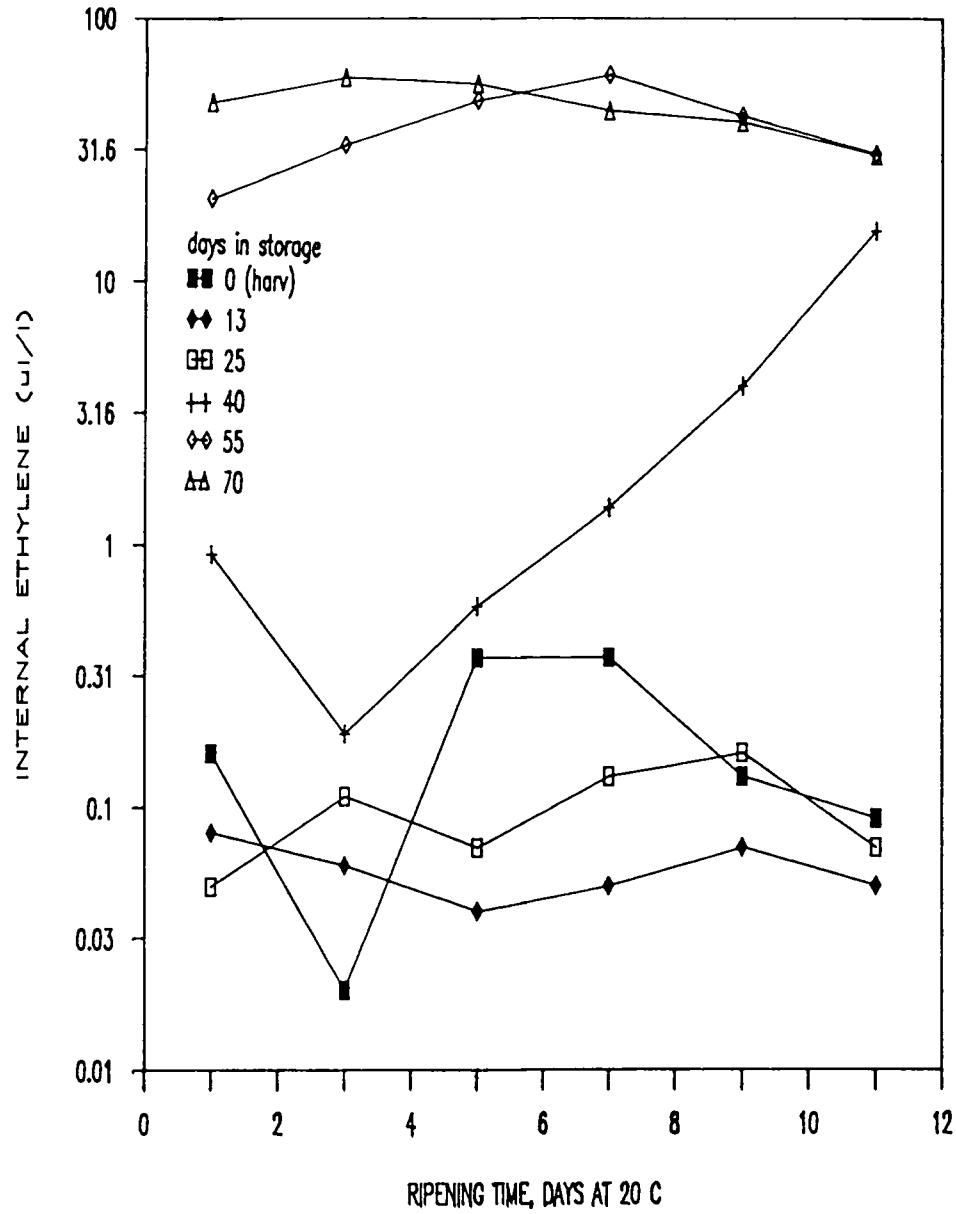


Fig.III.8. Effects of time (days) in 5 °C storage on internal ethylene of 'Anjou' pears subjected to 20°C ripening.
LSD.05: 5.5

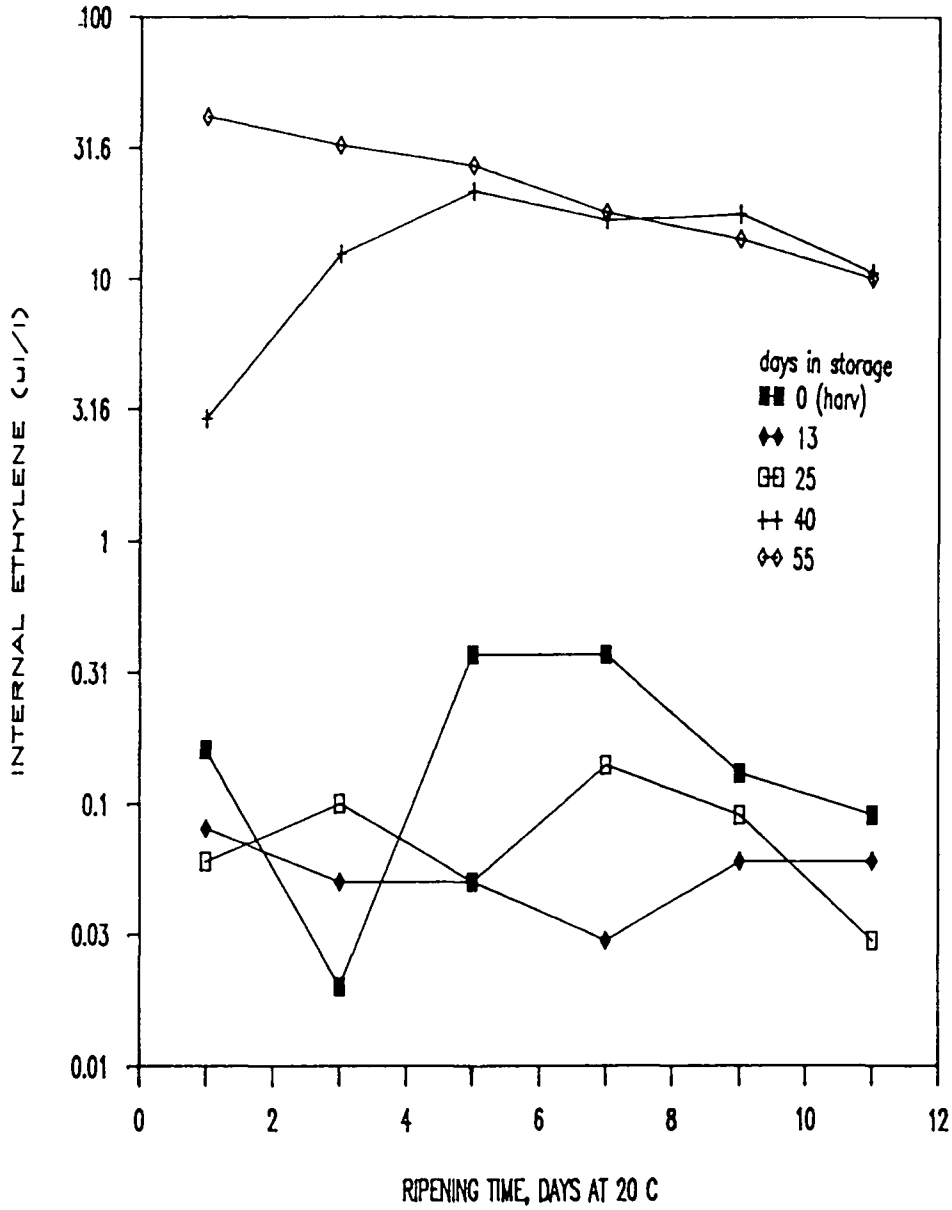


Fig.III.9. Effects of time (days) in 10°C storage on internal ethylene of 'Anjou' pears subjected to 20°C ripening. LSD.05:5.9

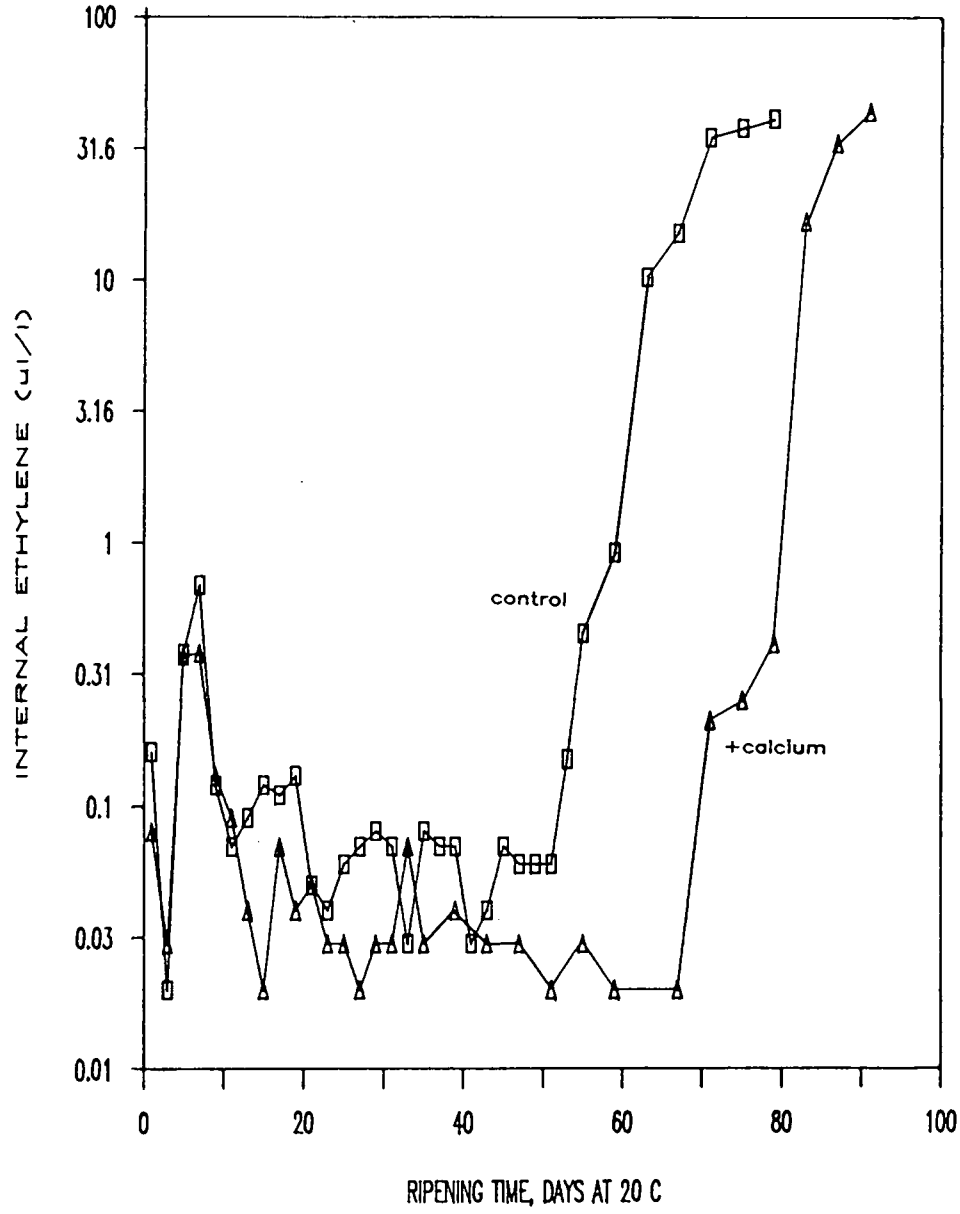


Fig.III.10. Internal ethylene of preharvest calcium-treated or control 'Anjou' pears as affected by time at 20°C storage. LSD.05:2.5

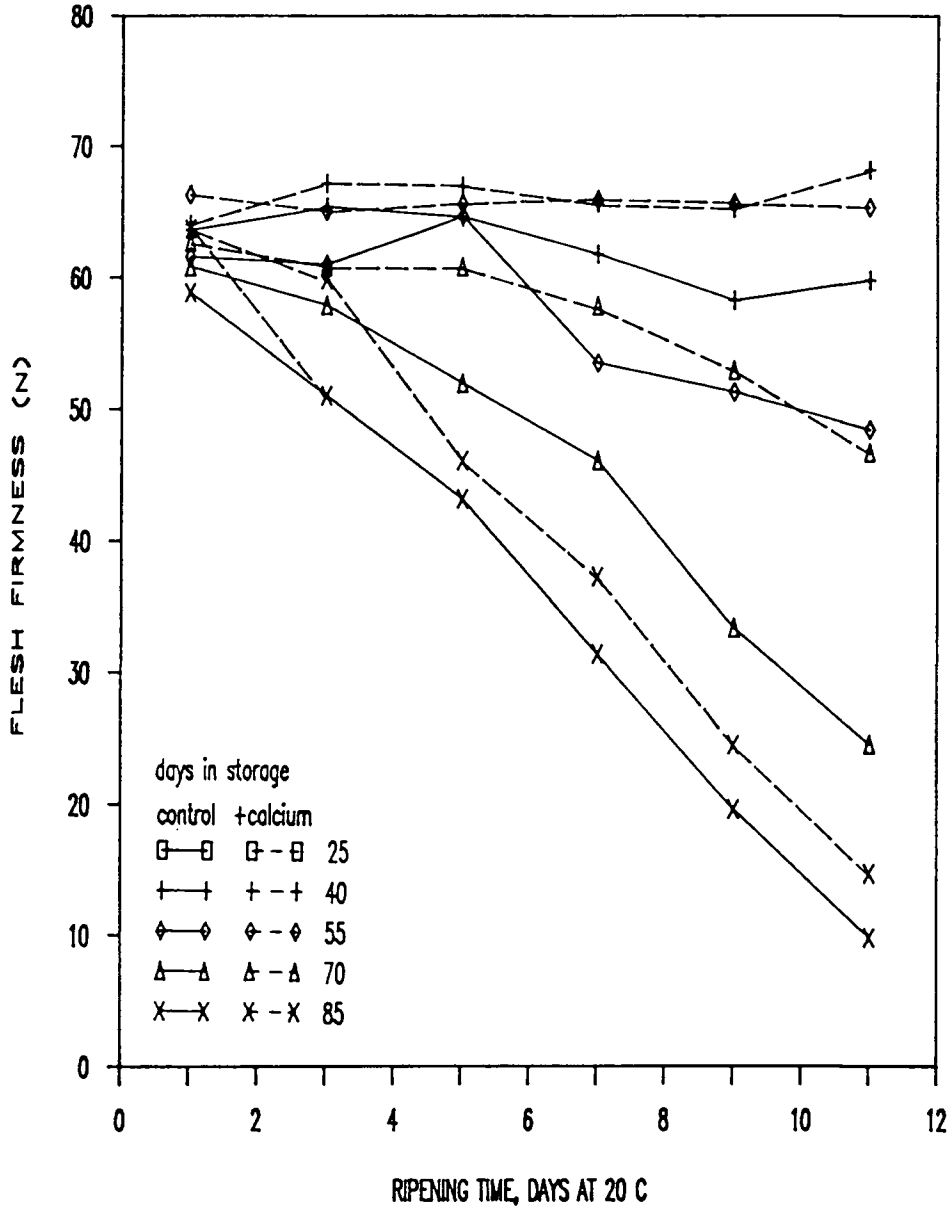


Fig.III.11. Flesh firmness of preharvest calcium-treated or control 'Anjou' pears as affected by time in -1.1 °C storage, followed by 20°C ripening regime. LSD.05: 16.7

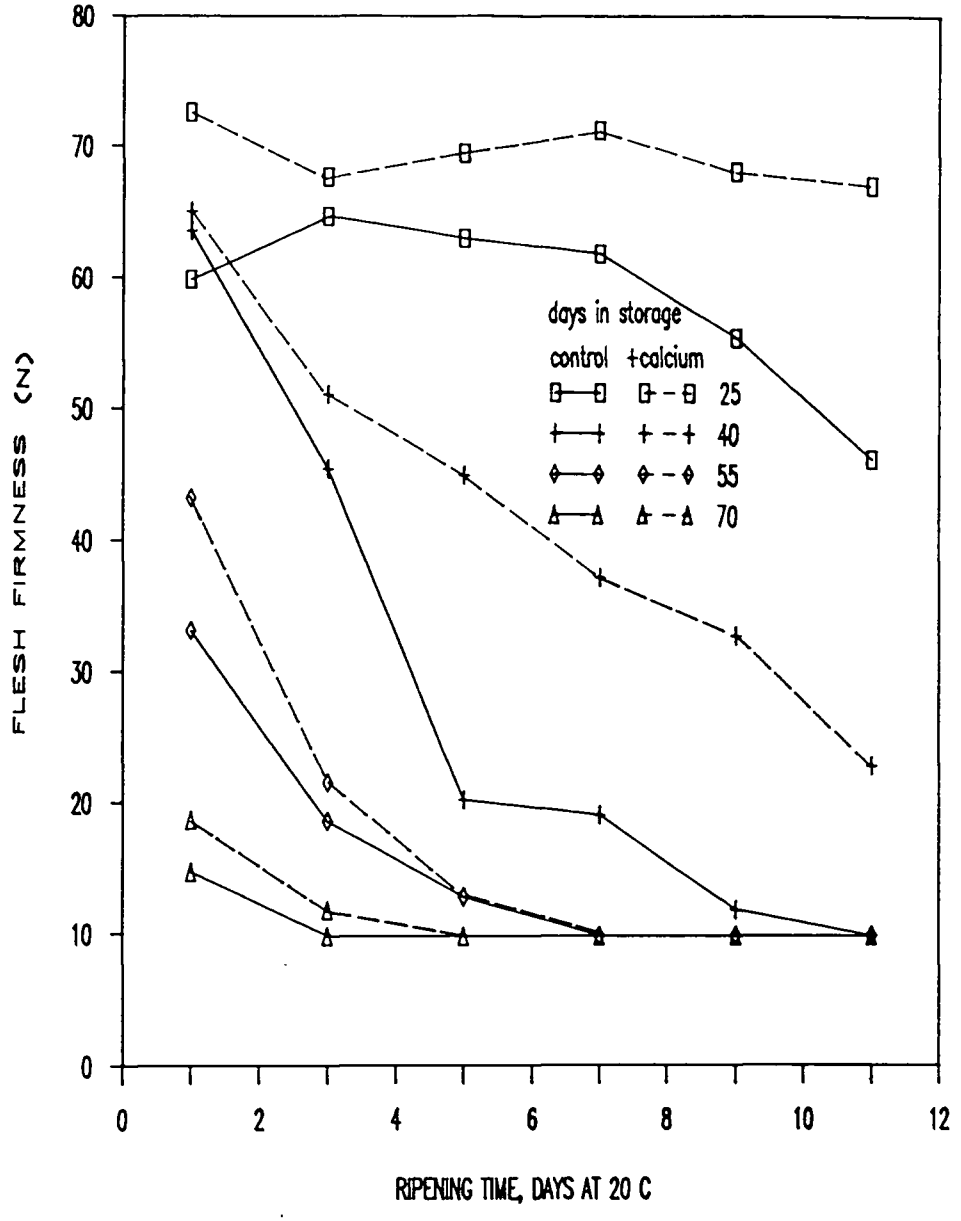


Fig.III.12. Flesh firmness of preharvest calcium-treated or control 'Anjou' pears as affected by time in 5°C storage, followed by 20°C ripening regime. LSD.05: 15.7

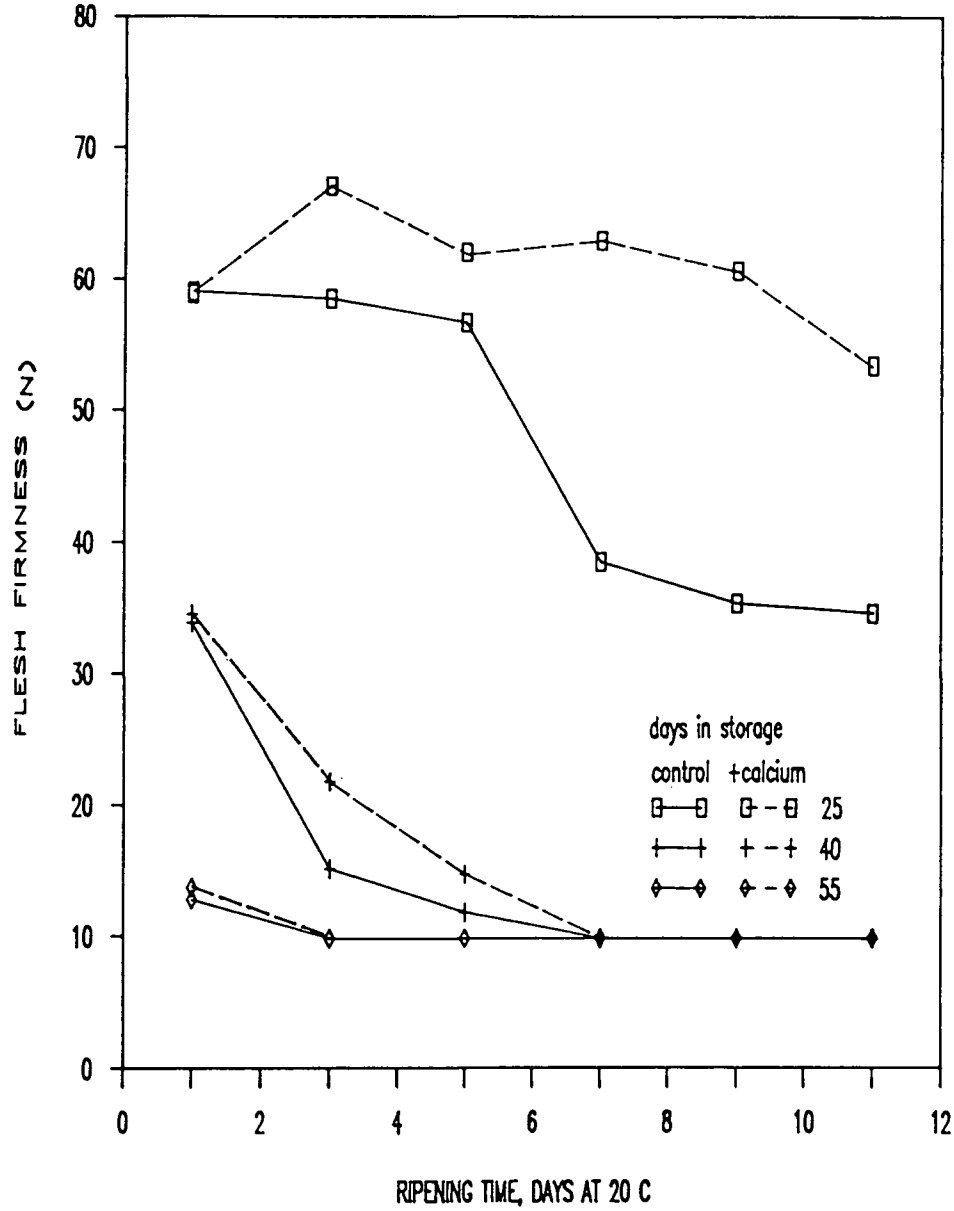


Fig.III.13. Flesh firmness of preharvest calcium-treated or control 'Anjou'pears as affected by time in 10°C storage, followed by 20°C ripening regime. LSD.05:15.4

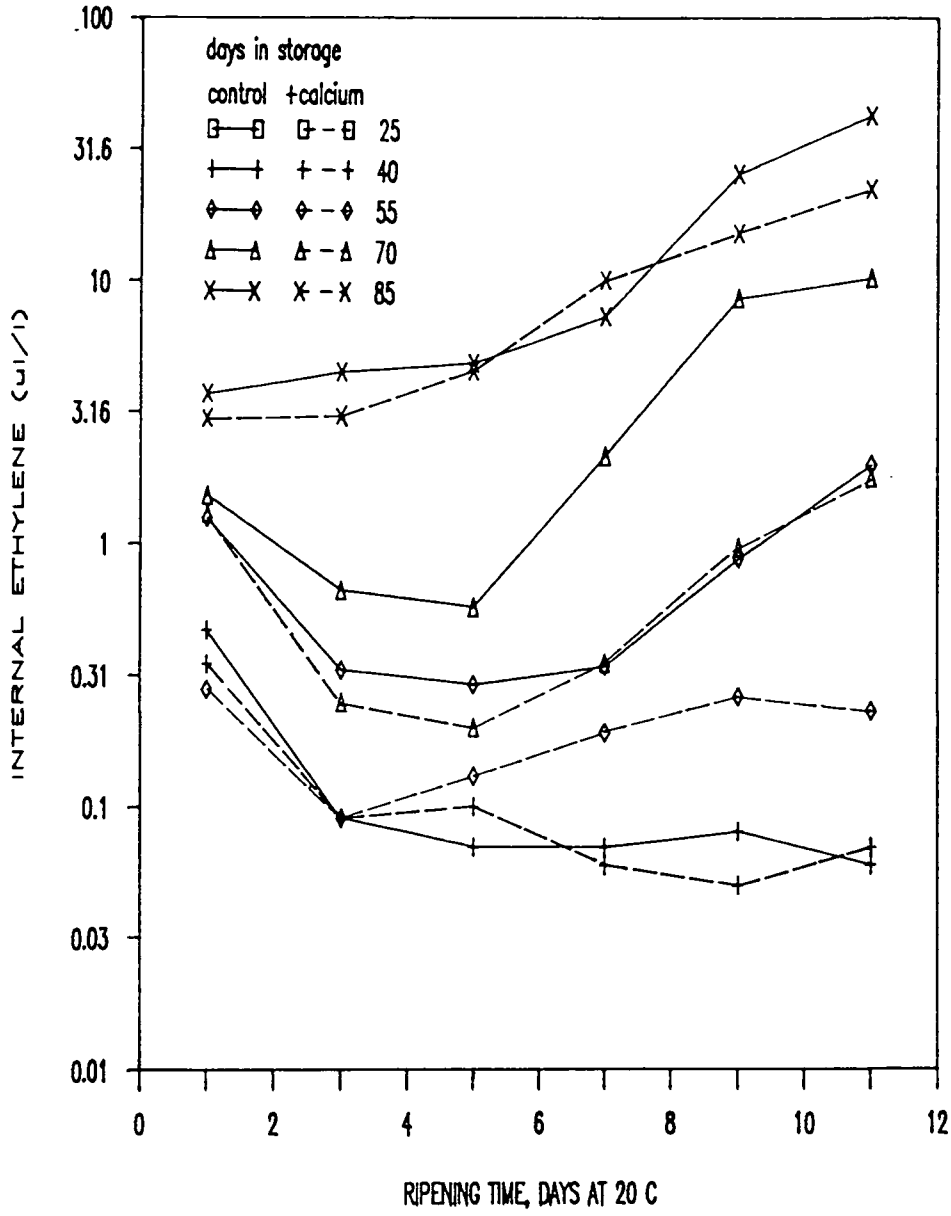


Fig.III.14. Internal ethylene of preharvest calcium-treated or control 'Anjou' pears as affected by time in -1.1 °C storage, followed by 20°C ripening regime. LSD.05: 4.5

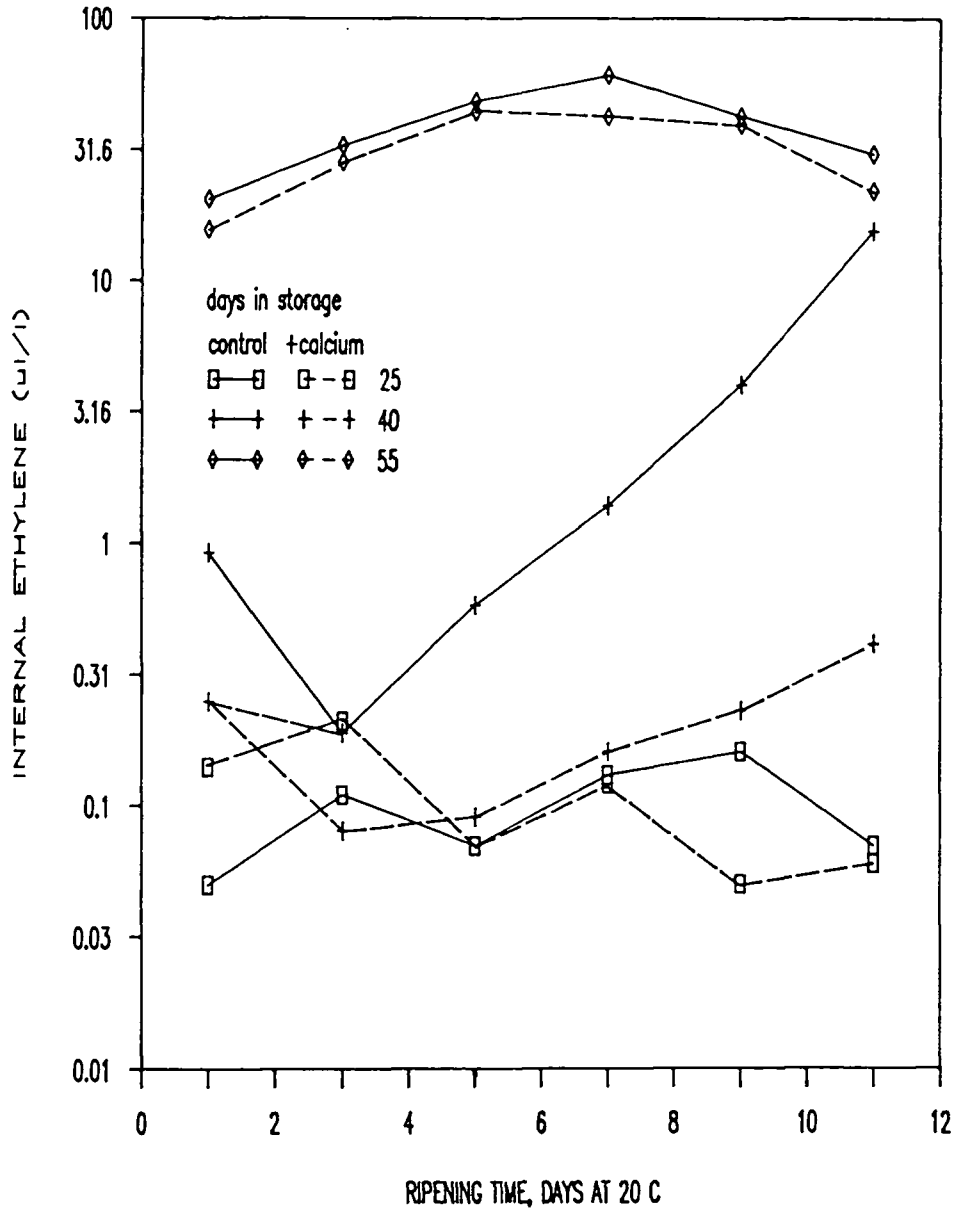


Fig.III.15. Internal ethylene of preharvest calcium-treated or control 'Anjou' pears as affected by time in 5 °C storage, followed by 20°C ripening regime. LSD.05: 5.2

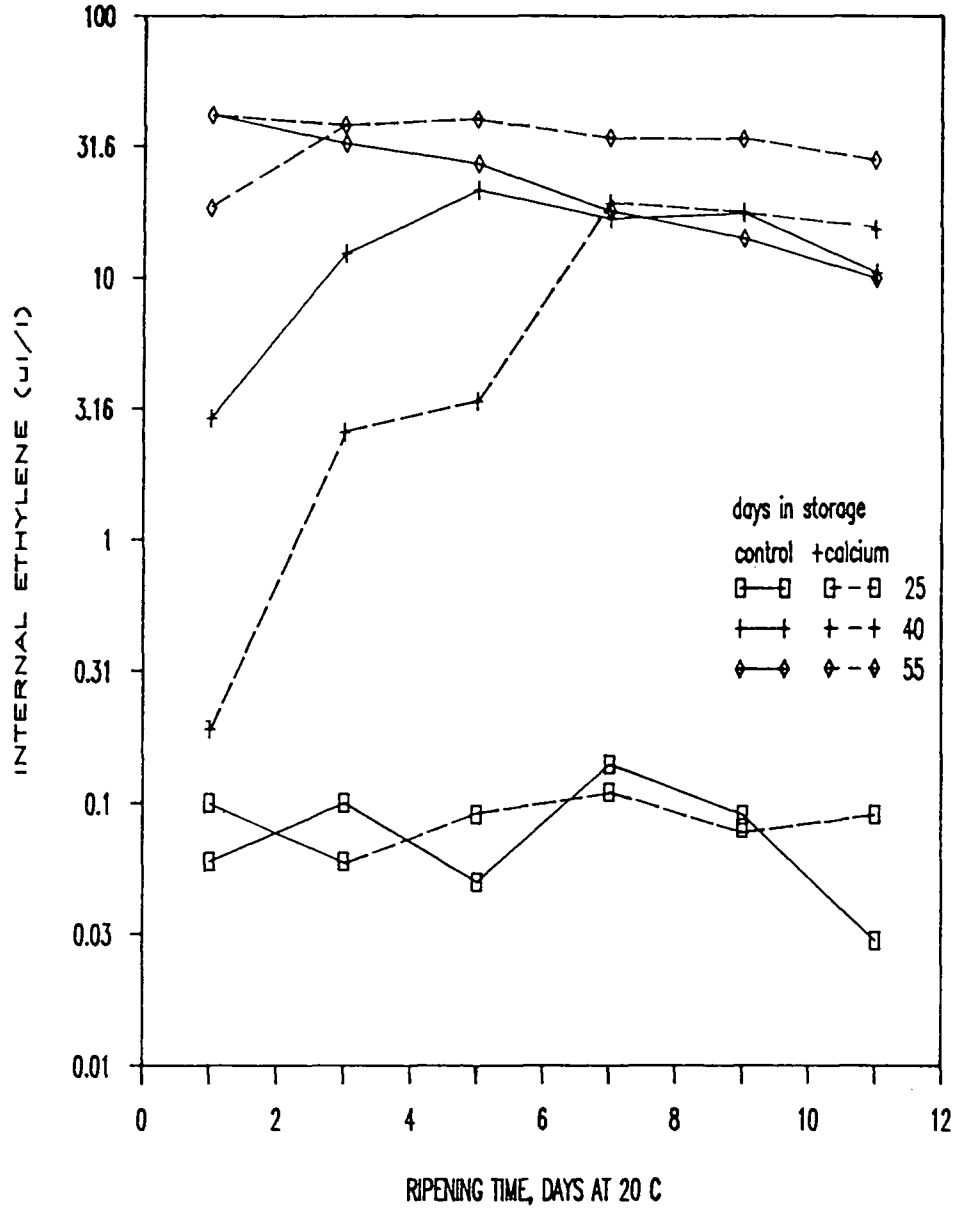


Fig.III.16. Internal ethylene of preharvest calcium-treated or control 'Anjou' pears as affected by time in 10 °C storage, followed by 20°C ripening regime. LSD.05: 5.4

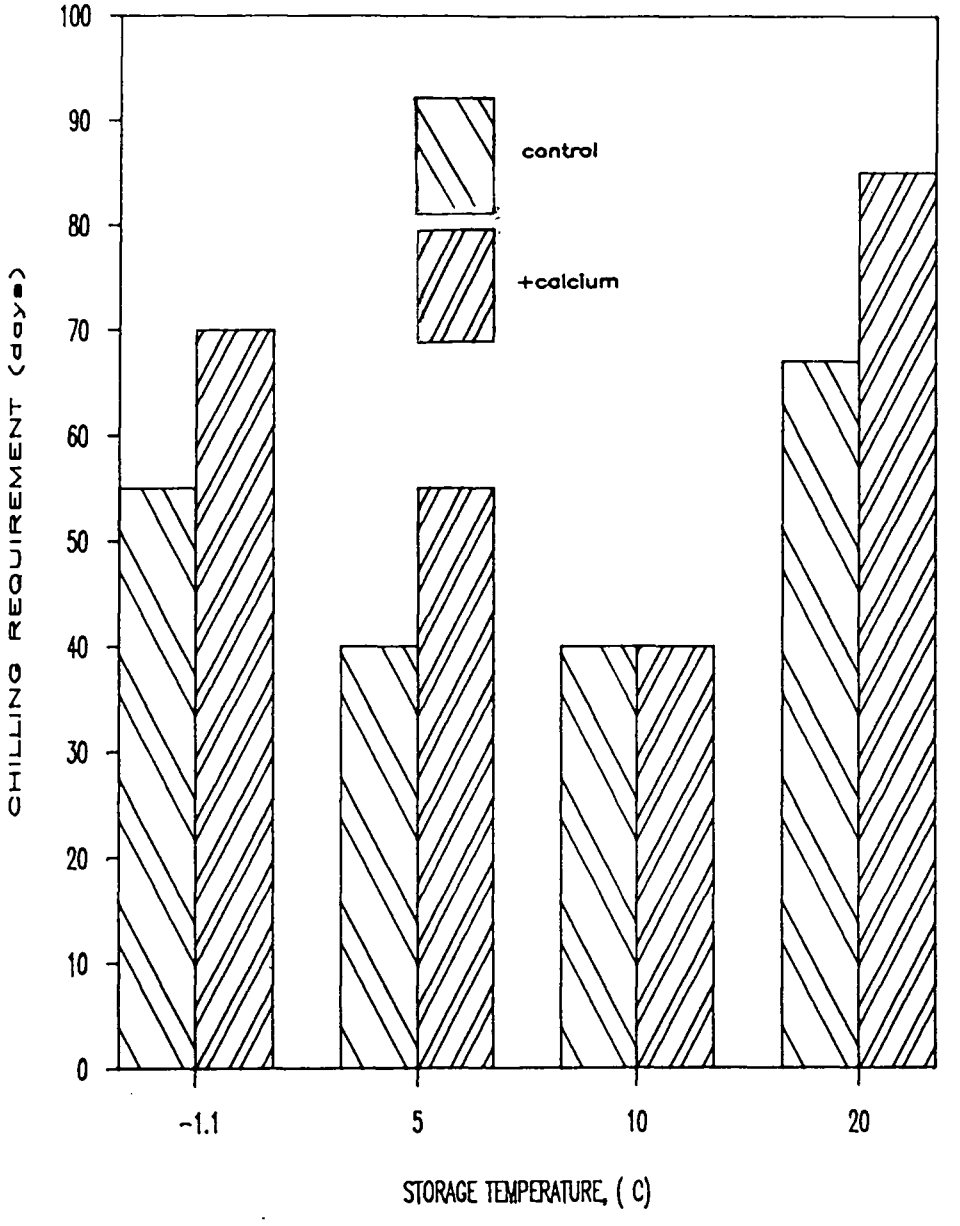


Fig.III.17. Interaction of fruit calcium and storage temperature on chilling requirements of 'Anjou' pears to begin ethylene synthesis (As determined by internal ethylene greater than 1 ul/l up to 11 days at 20°C).

CHAPTER IV

CHANGES IN FATTY ACIDS OF 'ANJOU' PEARS IN RELATION TO STORAGE TEMPERATURE AND CALCIUM

Additional index words: Pyrus communis, postharvest physiology, lipids, ethylene, firmness.

Abstract. 'Anjou' pears were treated with two concentrations of calcium chloride during fruit development. The fruits were collected at normal harvest maturity and stored -1.1° , 5° , 10° , and 20° C. Fruit flesh firmness, internal ethylene, and the total lipid fatty acid composition were examined after various storage times.

Fruits stored at -1.1° C remained firm and internal ethylene concentrations were initially around 0.1 ul/l and then leveled at about 3 ul/l after 100 days. Fruits stored at 5° C held their firmness for only 40 days followed by softening and a climacteric rise in ethylene. Fruits stored at 10° C and 20° C gradually lost their firmness and showed a climacteric rise in ethylene on the 40th and 55th day, respectively.

Linoleic acid, the main fatty acid, increased 100% during storage at -1.1° C but only 50% during storage at 5° or 10° C. No increase was observed during storage at 20° C. Linolenic acid increased only during -1.1° C storage.

Most other fatty acids did not change significantly during storage and showed little response to storage temperature

treatments. Preharvest-calcium-treated pears were not different than the control fruits in fatty acid profiles.

INTRODUCTION

Winter type pears usually require a period of cold storage in order to develop the ability to ripen and to produce ethylene. Short cold treatments of only a few days for 'Bartlett' pears (a summer type) increased ethylene production (236) and induced ripening (138). Fully mature 'Bosc' pears required 20 days at -1.1° C to initiate ripening (59). However, mature preclimacteric 'Bosc' pears required only 6 days at 5° C to initiate ripening at 20° C (208). Storage at -1° C for a sufficient (but unspecified) period abolished the lag in ripening of 'Conference' pears (109). 'Eldorado' pears required about 40 days in 0° C to produce ethylene and ripen (229).

'Anjou' pear fruits normally require 50 to 60 days (56,59,60) in cold storage at -1.1° C to ripen. However, depending on the fruit calcium concentration, and possibly other factors, they have been reported to require as much as 90 days (59) or as few as 30 days (59) or even zero days (237) in cold storage at -1.1° C to begin to ripen at 20 C. In an earlier study, Porritt (181) investigated the effects of temperature on respiration and firmness in 'Anjou' pears. More rapid loss in firmness was observed with higher storage temperature, occuring before the rise of climacteric CO_2 in all cases. High calcium 'Anjou' pear fruits required more time in cold storage in order to initiate

ripening than did low calcium fruits (222,86).

Increased calcium levels in apples and pears were found to retard senescence and to reduce physiological disorders during storage (23,165,179,181,204). Increased calcium levels have been associated with reduced respiration and ethylene production in apples and pears (23,75,132,199). The effects of calcium on postharvest physiology of fruits are also related to better retention of flesh firmness and delayed softening of pears (222) and apples (143) during cold storage.

Studies of a wide variety of organisms grown at low temperature and capable of adapting to low temperature have shown that there is a ubiquitous increase in lipid unsaturation. Generally, low temperatures have led to increased unsaturation of the esterified fatty acids, particularly linoleic (18:2) and linolenic (18:3) (14,69,115,186). Decrease in linoleic acid content was evident after the climacteric rise in ethylene of 'Calville de San Sauveur' apples (225). Fatty acid peroxidation is suggested to play a fundamental role in the senescence of carnations (170), and the peroxide content of 'Bosc' and 'Bartlett' pears was found to correlate with the onset of ripening (41).

The purpose of this study was to investigate the changes in fatty acid composition of 'Anjou' pears in relation to storage temperature, fruit calcium and the requirements for chilling.

MATERIALS AND METHODS

The orchard treatments, harvest maturity, storage temperature regimes, fruit sampling methods for ethylene, firmness etc, were previously described in Chapter III, pp 27.

Fruit samples from each temperature treatment (-1.1°, 5°, 10°, or 20° C) were taken at harvest or after storage for 13, 25, 40, 55, 70, 85 or 100 days and placed in a 20° C room to warm overnight. Five replicates were used for each treatment combination and sampling date. Internal ethylene concentration and flesh firmness readings were taken the following day and 25 g of 3 of those fruits were used for total lipids extraction. Fruit calcium was analyzed on the pooled quarter sectors of those five replicates.

Fatty acids analysis: Basically, the lipid extraction procedure of Folch, et al (78) was used. Twenty five g of pear tissue including the peel were washed, quartered and cored and homogenized in 100 ml of 2:1 chloroform/methanol solvent with a Brinkmann Polytron tissue homogenizer. The homogenate was filtered through Whatman #1 filter paper and the filtered residue was washed with 100 ml of the same solvent. Twenty five ml of 0.58% NaCl was added to the filtrate in a separatory funnel and the total lipids were obtained in the chloroform phase. The lipids were concentrated to oil at reduced pressure and 40° C in a rotary evaporator; dissolved in chloroform; transferred to pre-weighed teflon-lined screw cap test tubes and blown to dryness with nitrogen. Total lipids were weighed and the tubes were

filled with chloroform and stored at -5° C until they were analyzed for fatty acids. For the fatty acid analysis the tubes were brought to a constant volume of 10 ml under nitrogen. Four ml of the total lipid extract were evaporated in a N_2 stream to dryness, dissolved in 2 ml hexane and then 2 ml 14% BF_3/methanol (Pierce Chem. Co.) and 1 ml of internal fatty acid standard (21:0) were added to the tubes which were then capped and refluxed for 30 min. After cooling, 2 ml of deionized water were added to stop the reaction. The fatty acid methyl esters were recovered in the hexane phase, then blown down to dryness under nitrogen and dissolved in 0.5 ml of methylene chloride. Three ul of this extract were injected into an F&M gas chromatograph equipped with FID detector and a 2 m x 4 mm O.D. column packed with 3% SP-2310/2%SP-2300 on 100/200 mesh Chromosorb WAW (Supelco), run isothermally at 190° C and N_2 flow rate of 30 ml/min. The individual fatty acid methyl esters were identified by their retention times compared to authentic standards (Supelco). A typical 'Anjou' pear total lipid fatty acid methyl esters chromatogram is shown in the appendix (A.4.).

The determination of ripening parameters (fruit internal ethylene and flesh firmness), fruit calcium, and the statistical analysis are described in chapter III, page 27 this thesis.

RESULTS

Fruit calcium. Calcium levels were 5.59 for the control fruits and 6.61 mg calcium/100 g fresh weight for calcium-treated fruits.

Flesh firmness. (Fig.IV.1.) Fruit firmness at harvest was 68.6 N for the control and 72.1 N for the calcium-sprayed pears. During storage at -1.1° C, the pears retained nearly all of their initial firmness through 100 days. Fruits stored at 5° C retained their firmness for 40 days. Fruit softened to 15 N during the period from 40 to 70 days. Marked decreases in fruit firmness were observed for fruits stored at 10° and 20° C after the 13th day in storage at those temperatures. Flesh firmness reached 15 N on the 55th and 70th day of 10° and 20° C storage, respectively.

Internal ethylene. (Fig.IV.2.) Internal ethylene at harvest was 0.18 ul/l for the control but only 0.08 ul/l for the calcium-sprayed fruits. Both calcium-treated and control fruits stored at -1.1° C showed a slow increase in internal ethylene which leveled to about 3 ul/l at the end of this storage study (100th day). The control pears reached 1 ul/l in 55 days, whereas the calcium treated pears required nearly 70 days. The increase in internal ethylene that led to the climacteric rise for pears stored at 5° or 10° C was observed only after the 25th day. Control fruit stored at 20° C showed low internal ethylene levels up to the 40th day. Then the internal ethylene increased to 40 ul/l on the 70th day.

Changes in total lipids (Fig.IV.3.). The 'Anjou' pear total lipids slightly decreased and fluctuated within the 2.8 and 2.1 mg/g of fresh weight range for all treatments.

Changes in fatty acids. The analysis of 'Anjou' pear total lipid fatty acids (as methyl ester derivatives) showed major and minor components. Fatty acids are described in chemical nomenclature by the number of carbon atoms of the chain (n) and the number of the double bonds following the colon (n:#). Common names are also given (128).

Generally, as the major component, 34 to 48% consisted of linoleic acid (18:2) which exhibited large changes during storage (Fig.IV.7.). Palmitic acid (16:0) constituted 24 to 34%, linolenic acid (18:3) 9.0 to 14.5%, oleic acid (18:1) and (20:2) 5.4 to 6.8%, and arachidic acid (20:0) constituted of 0.7 to 1% of the total.

The minor individual fatty acids were all less than 0.4% and consisted of myristic acid (14:0), palmitoleic acid (16:1), and others of 14:1, 16:2, 20:1, 22:0, and 24:0 the latter two come most probably from the peel cuticular waxes.

Linoleic acid (18:2) levels remained constant during the first 25 days in -1.1^{o} C storage for both control and calcium-treated fruits. After 25 days, however, 18:2 increased by about 100 % on the 70th day at -1.1^{o} C followed by a 20 to 25 % decrease. Control fruits stored at 5^{o} C showed a peak of 18:2 on the 40th day, 40% above the initial amounts at harvest, followed by a slight decrease (Fig.IV.7). When the fruits were stored at

10° C, however, 18:2 again increased to 50 % above initial amounts on the 40th day (Fig.IV.6, 7.). Fruits stored at 20° C showed negligible change in 18:2 (Fig.IV.7.).

Linolenic acid (18:3) also showed a 70% increase during storage at -1.1° C with no difference between the control and calcium treatments (Fig.IV.8.). Storage at 10° C had almost no effect on linolenic acid content, although some increase was noted on the 55th day (Fig.IV.8.). The linolenic acid levels during 5° and 20° C storage appeared to decrease especially after the 40th day (Fig.IV.8.).

Most of the other fatty acids showed litle change during storage at -1.1° (Fig.IV.5.), 5°, 10° (Fig.IV.6.) or 20° C and the fluctuation that they do show was due to the dramatic change of 18:2. However, at 10° C, 14:0, 14:1, 16:1, 20:0, and 22:0 showed some increase after 40 days in storage (Fig.IV.6.).

Fruits stored at -1.1° C had an unsaturated/saturated fatty acid ratio with a peak on the 55th day for the control and on the 70th day for the calcium-treated fruits (Fig.IV.4.). Fruits stored at 5° C, and 10° C show a peak on the 40th day and 25th day (Fig.IV.4.). However, for fruits stored at 20° C, although there is not a distinctive peak, the ratio shows a net increase with time in storage (Fig.IV.4.). Interestingly, these peaks in 18:2 and the unsaturated/saturated ratio maxima do approximately coincide with chilling satisfaction, except for 20° C storage.

DISCUSSION

Two major factors affect the storage of 'Anjou' pears, the chilling temperature and the development of ripening. Fruits stored at -1.1° C satisfied their chilling requirement after 55 and 70 days for the control and calcium-treated fruits, respectively (See chapter III, page 35). The timing of the chilling requirement satisfaction correlates well with the increase in linoleic acid (18:2) (Fig.IV.7.), and with the unsaturated/saturated fatty acid ratio (Fig.IV.8.). Other changes during storage also occur. Although flesh firmness may remain essentially unchanged at -1.1° C (Fig.IV.1.), there are increases in internal ethylene (Fig.IV.2.), an accumulation of ACC (31,216), and increases in EFE(s) activity (31), and a net increase in the sensitivity of the fruit to propylene (86). 'Bosc' and 'Bartlett' pear fruit peroxide content was found to correlate with the onset of ripening (41). Inhibitor studies suggested the involvement of free radicals in the reaction sequence which converted ACC to ethylene (11). Paulin, et al (170), suggested that peroxidation of fatty acids plays a fundamental role in senescence processes in carnation and ethylene appears to be a by-product rather than an initiating factor. Perhaps along with the other changes during -1.1° C storage of 'Anjou' pears, the increase in linoleic (18:2) may be required, may facilitate and/or be associated with the onset of ripening once chilling requirement is satisfied by providing substrate for peroxide formation. Alternatively, the increased unsaturation may increase

membrane permeability, or activate membrane-bound enzymes such EFE(s), since the fatty acid composition reported here, derives almost exclusively from the membrane lipids (pears contain very little triglycerides).

Although there is no data on changes of the factors mentioned above, an increase in linoleic acid still occurs in pears stored at 5° and 10° C. (Fig.IV.7.). The peak of the increase in linoleic acid and the peak in the unsaturated/saturated fatty acid ratio corresponds well with satisfaction of the chilling requirement, of 40 days (see Chapter III, page 36) for both storage temperatures. After the chilling requirement is satisfied, the fruits slowly and partially ripen even low storage temperatures (Fig.IV.1, 2.), and a decrease in linoleic acid is later observed (Fig.IV.7.). It is intriguing to speculate whether this could be important later in storage when pears fail to ripen if held too long (160).

Storage at 20° C results in a decrease in firmness (Fig.IV.1.) which is considerably separated in time from the later climacteric rise in ethylene (Fig.IV.2.). Linoleic acid levels remain unchanged and there is only a slight 18:2 increase associated with the climacteric rise in ethylene (Fig.IV.7.). Linolenic acid (18:3) levels show a general decrease (Fig.IV.8.) however. 'Bartlett' pears which have been briefly stored at 0° C and then ripened at 20° C reportedly show a decrease in linoleic and linolenic acid. However, apples treated the same way increased in linoleic acid (196). An increase in linoleic and a

decrease in linolenic acid with ripening of apples was observed in another study (84). However, Lurie and Ben-Arie (136) working with 'Calville de San Sauveur' apples show a decrease in linoleic acid which was more evident after the climacteric rise in ethylene. During ripening of banana pulp tissue, a decrease in the proportion of linoleic and an increase of linolenic was observed (226).

Whether there are any distinctive changes of fatty acids upon ripening at 20° C it cannot be positively determined from these results and additional studies are required. However, it is clear that chilling leads to increased unsaturation of 'Anjou' pear esterified fatty acids, particularly of linoleic (18:2) and linolenic (18:3). This effect of temperature on fatty acid unsaturation has been shown for a wide variety of organisms (114,115,186) but little connection has been made with ethylene synthesis or the chilling requirement for ripening.

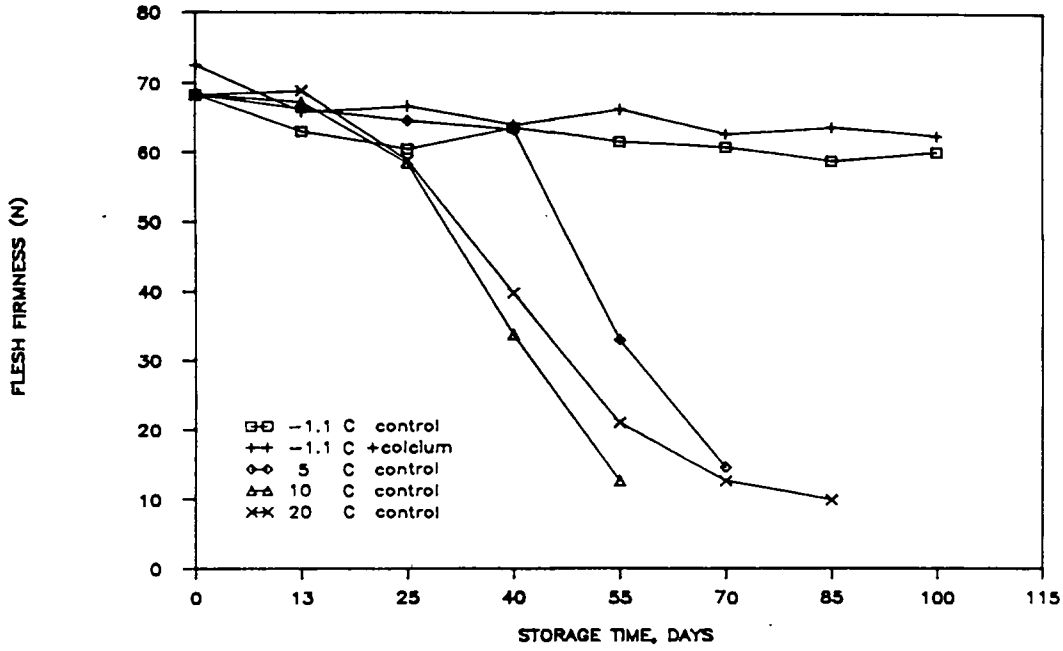


Fig.IV.1. Flesh firmness of control and calcium-treated 'Anjou' pears as affected by storage temperature. LSD.05: 11.2

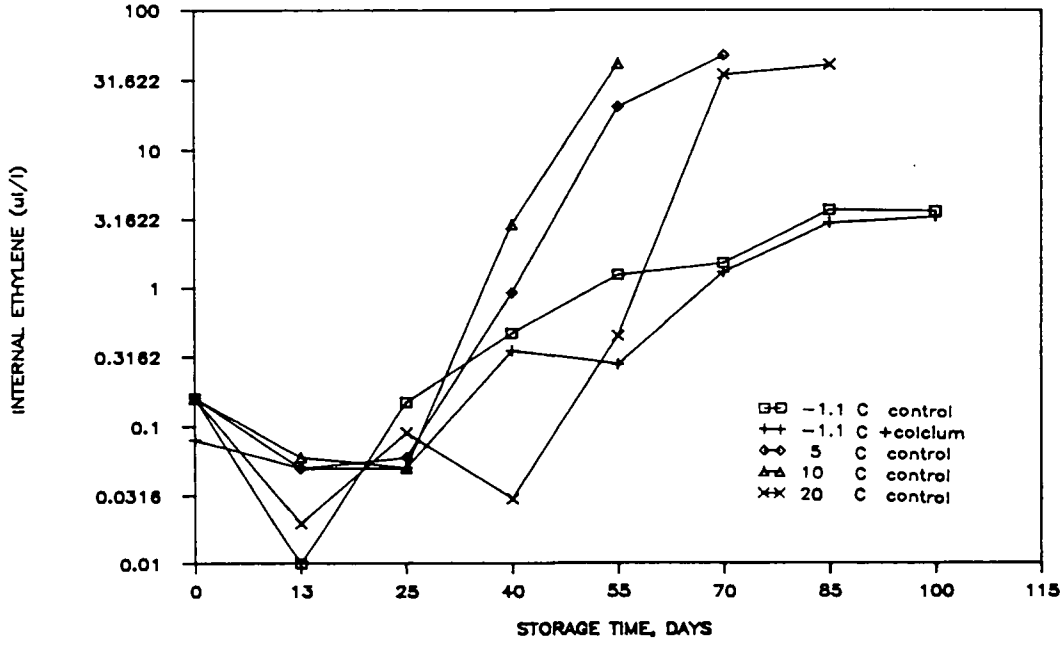


Fig.IV.2. Internal ethylene of control and calcium-treated 'Anjou' pears as affected by storage temperature. LSD.05: 4.3

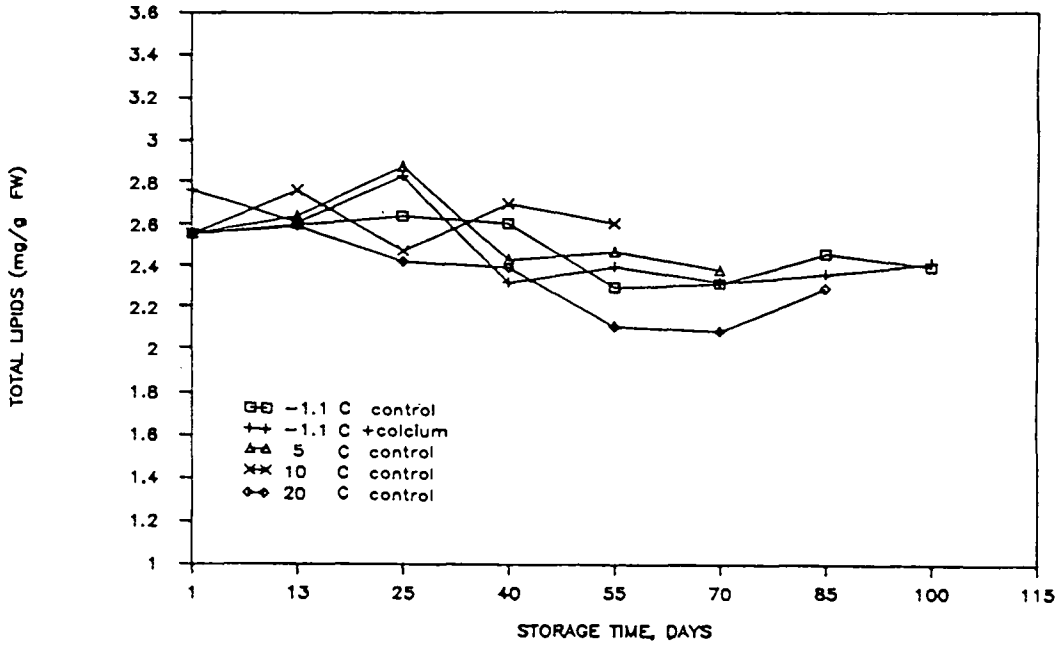


Fig.IV.3. Total lipids of control and calcium-treated 'Anjou' pears as affected by storage temperature. LSD.05: 0.41

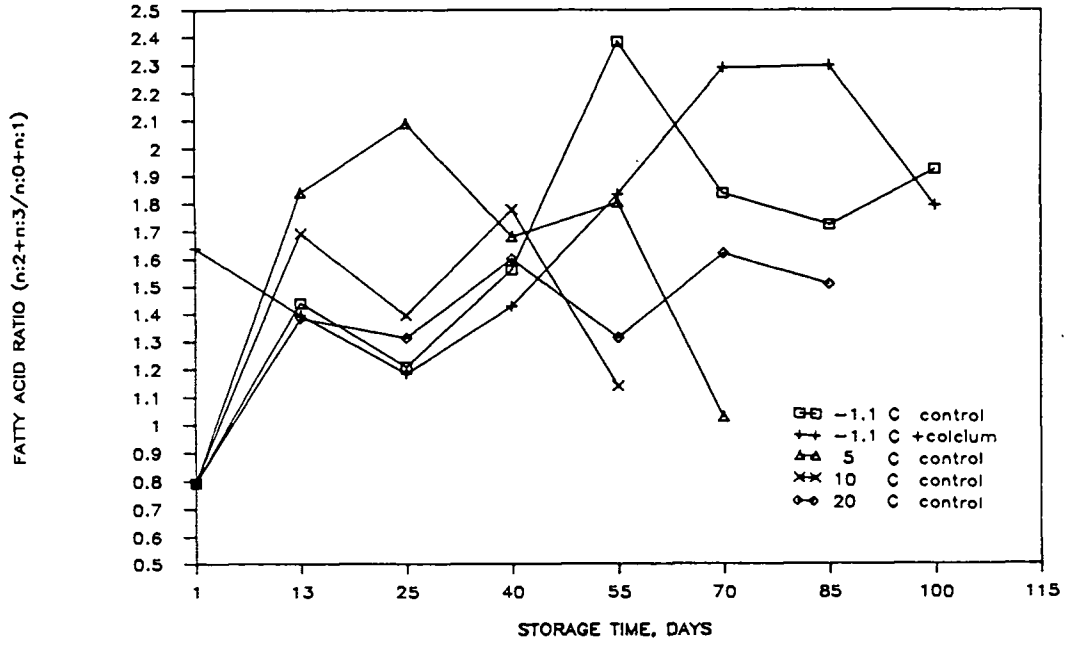


Fig.IV.4. Unsaturated/saturated fatty acid ratio of control and calcium-treated 'Anjou' pears as affected by storage temperature. LSD.05: 0.63

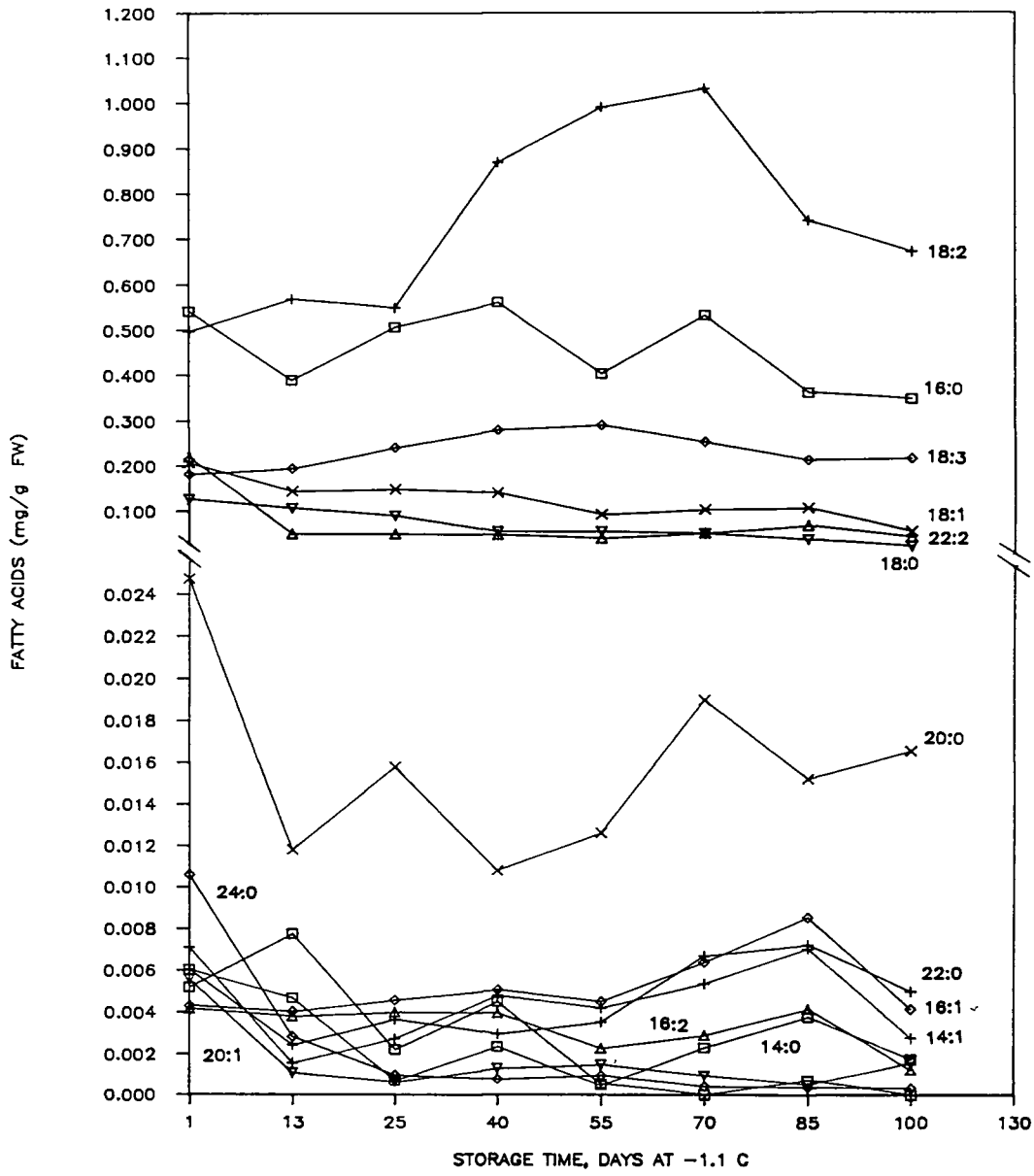


Fig.IV.5. Fatty acid composition of 'Anjou' pears stored at -1.1° C. LSD.05: 0.161

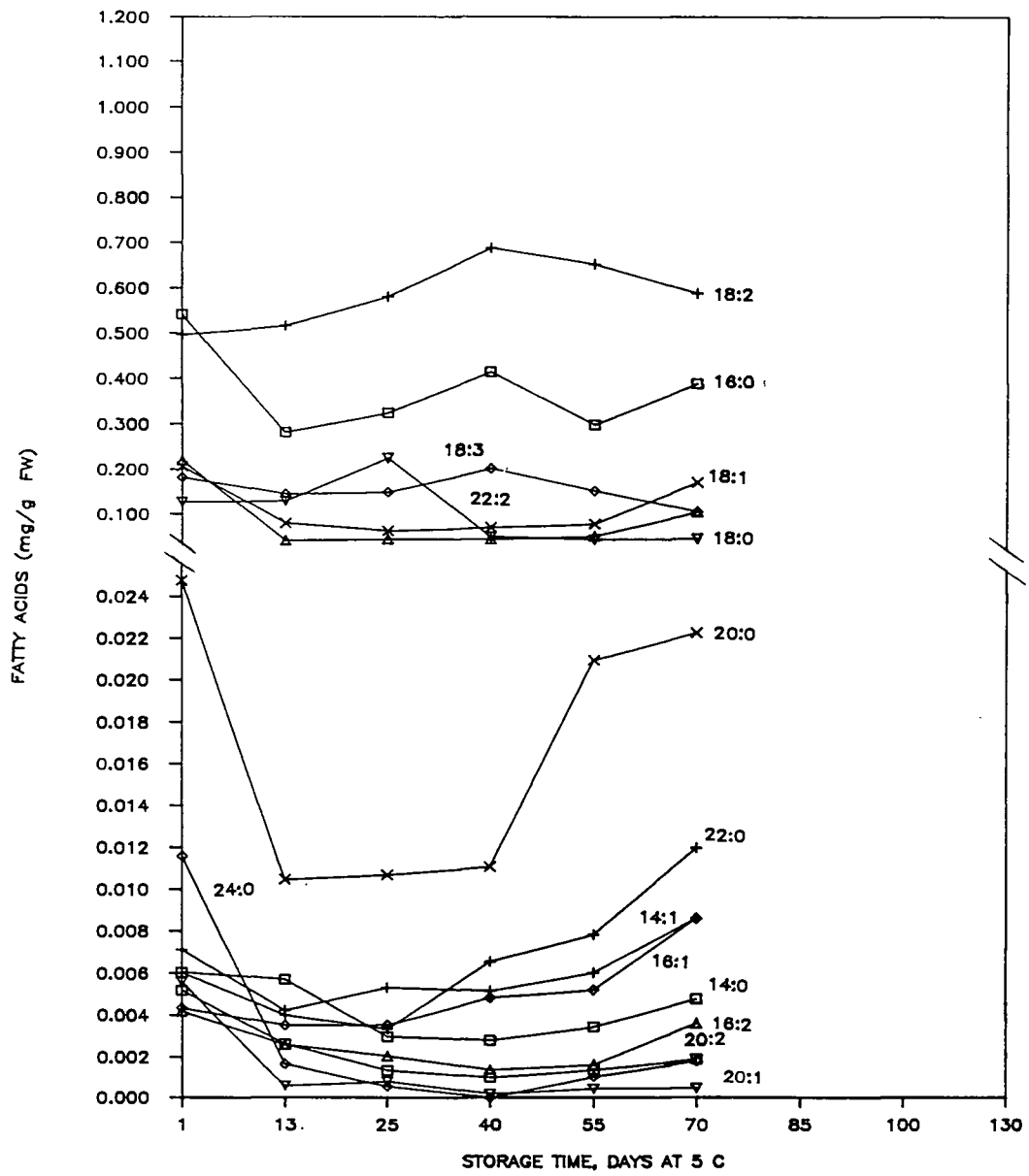


Fig.IV.6. Fatty acid composition of 'Anjou' pears stored at 10°C. LSD.05: 0.175

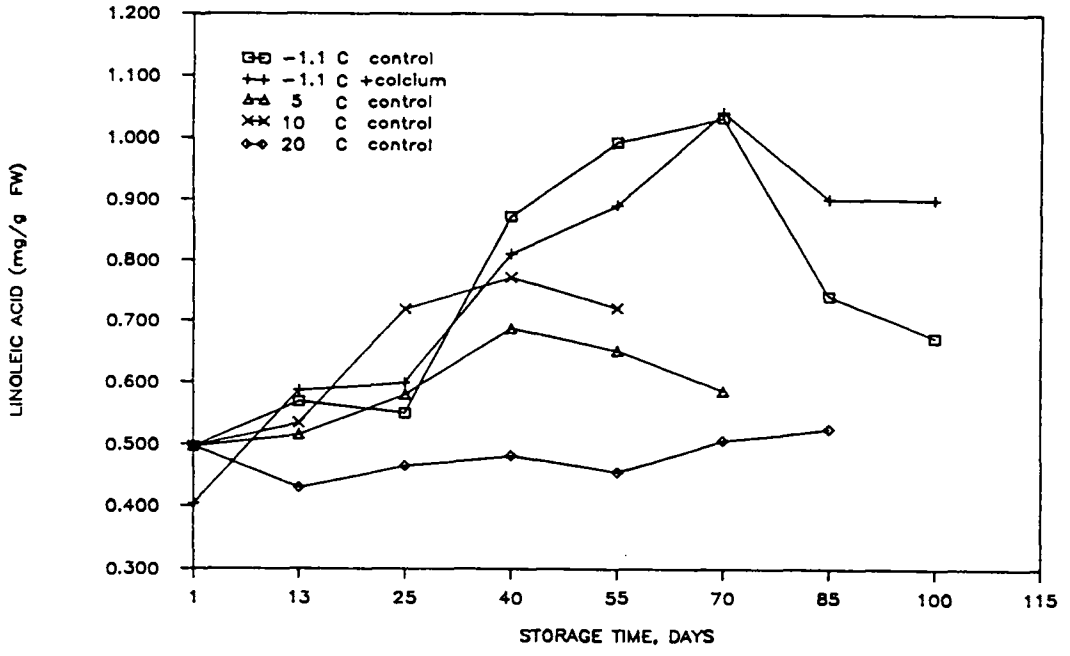


Fig.IV.7. Linoleic acid content of control and calcium-treated 'Anjou' pears as affected by storage temperature. LSD.05: .183

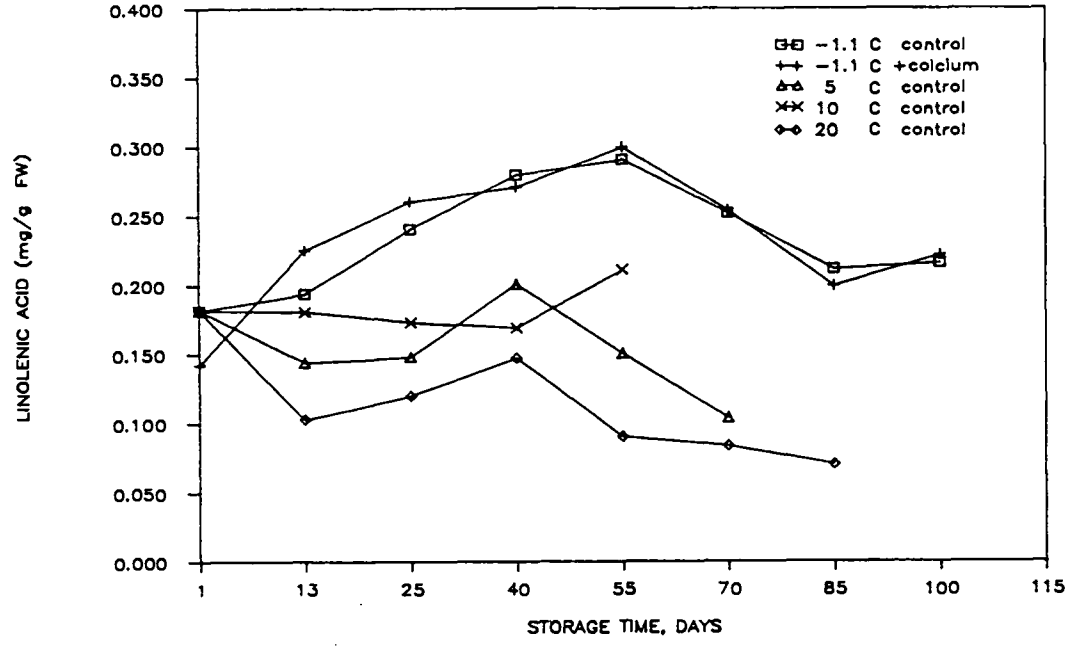


Fig.IV.8. Linolenic acid content of control and calcium-treated 'Anjou' pears as affected by storage temperature. LSD.05: 0.172

CHAPTER V

TIME SEPARATION OF CELL WALL SOFTENING AND CHLOROPHYLL LOSS FROM THE ETHYLENE PATHWAY AND OTHER RIPENING EVENTS OF 'ANJOU' PEARS WHEN HELD AT 20° C COMPARED TO -1.1° C.

Additional index words: Pyrus communis, postharvest physiology, firmness, pectins, proteins, chlorophyll, organic acids, amino acids, ACC.

Abstract. 'Anjou' pears were harvested at 68 N firmness commercial maturity from the Mid-Columbia Experiment Station, Hood River Oregon in 1987. Storage temperatures of 20° C or -1.1° C exhibited some surprising differences in the sequence of ripening events up to 100 days.

Pears held at 20° C continually from harvest showed little change in parameters for two weeks, but then chlorophyll began to degrade losing about 10% per week. Firmness held at initial values for the first 3 weeks, then began to lose about 4 N per week for the next 12 weeks. The 40% increase in soluble polyuronides paralleled the firmness loss, but with no change in total uronides. Titratable acidity decreased slowly the first 6 weeks, then more rapidly to 10 weeks, then leveled. During all of these changes, internal ethylene did not rise above 0.2 ul/l until after the 13th week. Interestingly, while ethylene-forming enzyme (EFE) activity exhibited slow increases 3 nl C_2H_4/g/h in the first

5 weeks, it was not until the 9th week that the EFE activity began to peak. Total protein paralelled the timing of EFE, ACC did not rise above 0.5 nmoles/g until after the 12th week. This slightly preceded the rise in internal ethylene which remained below 0.3 ul/l until after the 13th week at 20° C. Thus softening, loss of cholrophyll, and the increase in total protein and EFE activity preceded ACC accumulation and the rise in internal ethylene by several weeks when 'Anjou' pears were held continuously at 20° C.

Anjou pears held at -1.1° C showed no changes in chlorophyll, firmness, protein, amino acids, or total polyuronides for at least 12 weeks. Despite essentially no change in firmness, there was a slow, but steady increase by about 15% in soluble polyuronides at -1.1° C storage. Surprisingly, EFE activity increased steadily by 10-fold (up to 71 nl C_2H_4/g/h) during the first 4 weeks, levelled to 10 weeks, then rose again. ACC remained very low (less than 0.15 nmoles/g) for the first 5 weeks, followed by a rapid increase to almost 1.0 nmole/g by the 12th week. Internal ethylene showed a slow increase from less than 0.1 ul/l from the 5th week to the 8th week when it rose and levelled near 1 ul/l.

Thus 'Anjou' pears held at cold (ie, -1.1° C) storage temperatures, the softening and chlorophyll systems are held in check, whereas the becoming functional and increasing in advance of any sustainable rise in internal ethylene. Satisfaction of a chilling requirement thus appears to favor the development of ethylene synthesis capacity, which upon transfer from cold storage to warm temperatures results in enough internal ethylene to

rapidly drive of the associated ripening mechanism.

Clearly the softening that occurs with no chilling satisfaction is not dependent upon internal ethylene in excess of 0.1 ul/l. Either that softening at 20° C is not ethylene dependent or, alternatively, it is already maximally stimulated at very low ethylene levels, typically less than 0.03 ul/l.

Fruits with satisfied chilling requirements (70 or more days at -1.1° C) softened in response to accelerated ethylene production in one week at 20° C. However, these pears did not sustain the increased ACC levels or EFE activity. The changes in the biochemical constituents associated with ripening were accelerated in response to ethylene.

Fruits with partially satisfied chilling requirements ripened similarly to fruits that received no chilling. In all cases a decrease in firmness to about 25 N preceded the climacteric rise in ethylene or increases in ACC levels. EFE activity reached a maximum before ethylene in all cases for incompletely chilled 'Anjou' pears. Duration in -1.1° C storage was positively correlated with early, peaking of the parameters associated with ethylene biosynthesis; the changes were also more rapid for the other biochemical constituents associated with ripening.

INTRODUCTION

Winter type pears usually require a period of cold storage in order to ripen and produce ethylene. Pertinent literature on general aspects of chilling requirements were covered in Ch.III, pp.26 and will not be repeated here. 'Anjou' pears normally require 50 to 60 days (59,56,60) in cold storage at -1.1° C to ripen. However, depending on the fruit calcium concentration, and possibly other factors, they may require as much as 90 days (59) or as few as 30 days (59) or even zero days (237) in cold storage at -1.1° C to begin to ripen at 20 C. During storage at -1.1° C, the ACC concentration increased (31,216,222,229), along with increased activity of ethylene forming-enzyme(s) (EFE) (31) the latter increased before ethylene appeared. While there have been several citations in the literature (245) implying or stating outright that the conversion of ACC to ethylene is a one-step, single enzyme-mediated process, there is no supporting evidence for that hypothesis. In fact, despite numerous attempts to isolate the ethylene-forming enzyme, none have been succesful. It seems much more likely that several steps are needed to convert ACC to ethylene and that several enzymes would be necessary. We therefore have chosen to write and speak of EFE(s) rather than EFE alone to connote this alternate hypothesis. However, for the sake of clarity, the use of EFE as an abbreviation will be used, but with the understanding that in our minds, several enzymes are inferred as a system.

Linoleic and linolenic acids, predominantly as membrane

constituents, also increase with -1.1° C storage (Chapter IV). This may, in some as yet uncharacterized way, relate to chilling satisfaction and ripening.

'Anjou' pears are eventually able to ripen at 20° C even if they are not exposed to chilling (chapter III). Loss in firmness occurs first followed by climacteric ethylene. The two events can be separated by time at the 20° C temperature. Partial chilling satisfaction of 'Anjou' pears, ripened at 20° C resulted in low ethylene production (216), loss in firmness and depletion of the ACC pool. Softening is due to solubilization of polyuronide (229) and reduction in cell wall neutral sugar content (mainly galactose and/or arabinose) (106,111,172).

It is clear that there is also an increase in soluble protein content during the climacteric (93). Cycloheximide, an inhibitor of protein synthesis, when infiltrated into harvested mature fruits prevents ripening in pears (80), and bananas (40). RNA synthesis is required for the synthesis of new enzymes involved in ripening (182).

'Anjou' pears with high sugar and acid content are associated with high postharvest quality but these costituents are not related to storage duration whereas high protein at harvest results in short storage life (160). All these constituents fluctuate from season to season and do not appear to be closely associated with postharvest life (59). Titratable acidity is generally known to decrease during fruit ripening and storage (56,99,129). Chlorophyll usually degrades with fruit maturity and

ripening and thus the carotenoids contribute more to the color (77,87).

The purpose of this study was to investigate the time course of the changes in various parameters of ethylene biosynthesis and other biochemical constituents associated with no chilling or with chilling satisfaction and ripening of 'Anjou' pears at 20° C when they are subjected to -1.1° C storage for various amounts of time.

MATERIALS AND METHODS

Fruit material. 'Anjou' pears were purchased from the Mid-Columbia Experiment Station, Hood River Oregon in 1987. The fruits were harvested based on 147 days from full bloom and 67 N flesh firmness. After harvest the fruits were transferred into perforated polyethylene-film-lined 20 kg cardboard cartons and placed into a -1.1°C, storage room. All the fruits were screened for cork spot. Only healthy fruits were used.

Fruit samples, were taken at harvest or out of -1.1° C storage after 13, 25, 40, 55, 70, and 85 days and placed in a 20° C room to ripen. Five replicates were used for each sampling day. Internal ethylene and flesh firmness were measurment three times per week. The determination of ripening parameters such as internal ethylene, flesh firmness, and the statistical analysis are described in the methods of chapter III.

ACC analysis. ACC was extracted and prepared according to Miller, et al (163) with some modifications. Five grams of pear flesh tissue was homogenized in 15 ml 90% acetone with a Polytron tissue homogenizer (Brinkmann Instruments) and the homogenate was filtered through a Whatman #1 filter paper into a scintillation vial. The extract was then stored at -20° C until it was used. An aliquot equivalent to 1 g fresh weight of tissue was then passed through a 2 ml Dowex 50 column. The column was then washed with water, and ACC was eluted with 1 N NaOH and further assayed according to Lizada and Yang (134).

Ethylene Forming Enzyme activity determination. EFE activity was determined by adding known amounts of ACC to tissue plugs, allowing ACC to convert to ethylene which was measured by GLC according to Meadows and Richardson (31), with some modifications. Three replications consisting of 3 individual pears were used. For each pear, 12 plugs of flesh were cut with a 0.5 cm ID cork borer. Six of these plugs (about 3 g) were weighed and vacuum infiltrated (90 seconds, water aspirator) with 0.35 M mannitol (control). The excess liquid was drained, the plugs blotted dry, and a partial vacuum applied for 10 seconds to free trapped ethylene. The plugs were then placed in 25 ml erlenmeyer flasks, sealed with serum caps, and incubated for 30 min at 20° C. Then, 1 ml atmospheric gas samples were withdrawn by syringe and ethylene was measured by GLC as described earlier. The other six plugs from the same fruit were treated identically except they were vacuum infiltrated with 0.05 mM ACC in 0.35 mannitol. Three

replications (ie, three pears) were used for each time-temperature treatment. Control plugs (0.35 M mannitol-treated) ethylene was subtracted from that produced by ACC-treated plugs to calculate EFE activity, expressed as nl C_2H_4/g fresh weight /h.

Fruit total and water soluble polyuronide. Ten grams of peeled and cored pear flesh tissue of each of three individual fruits was homogenized in 90 ml of acetone. The homogenate was placed in -20° C for two days for precipitation of the insoluble material. After centrifugation (2000xg, 20 min), the pellet was washed twice with 50 ml acetone, dried under vacuum, and stored in glass vials until further analysis. Before analysis, the pellet of acetone insoluble solids was dried at 50° C overnight. Forty mg of the acetone insoluble powder was then dissolved in 100 ul concentrated sulfuric acid overnight under nitrogen at 4° C and then diluted to 1N sulfuric acid. Then, 25 ul was further diluted with 175 ul of 1N sulfuric acid and used for galacturonic acid determination with meta-hydroxydiphenyl reagent according to Blumenkrantz and Asboe-Hansen (32). Galacturonic acid in 1 N sulfuric acid was used as a standard. The total polyuronide content was calculated as mg galacturonic acid equivalents/g of acetone insoluble solids (AIS).

Forty mg of the AIS was dissolved in 10 ml of water and heated for 30 min at 80° C. The solution was then cooled to room temperature and the water insoluble material was precipitated by centrifugation. The hot water soluble extract (80 ul) was also analyzed for polyuronide residue according to Blumenkratz and

Asboe-Hansen (32). Galacturonic acid in water was used as a standard and the water soluble wall fraction was expressed as mg galacturonic acid equivalents/g AIS.

Peel chlorophyll detemination. The epidermis of three fruits was peeled and ten discs 1.1 cm diameter per fruit were cut with a cork borer. The peel tissue was immediately homogenized in 5 ml of 80% acetone and insoluble material was pelleted by centrifugation. The absorption at 665 nm of the extract was then determined by spectrometry (139).

Titratable acidity. An aliquot equivalent of 1 g of fresh pear tissue of the extract prepared for ACC analysis was titrated to pH 7.2 using 0.001 N NaOH prepared and standardized as suggested by Skoog and West (210). The results are expressed as ueq/g of fresh weight.

Amino acids analysis. The extract used for ACC analysis was also used for amino acid analysis with the ninhydrin reagent based on the method described by Moore and Stein (164) as modified by Yemm and Cocking (242) with leucine used as a standard.

Total protein determination. The AIS (40 mg) was dissolved in 5 ml 1 N NaOH. One hundred ul of this solution was neutralized with 100 ul 1 N HCl and the protein assayed with the Coomassie Blue reagent according to Bradford (35). Bovine serum albumin standard was treated identically.

RESULTS

Flesh firmness. (Fig.V.1.) Fruit firmness at harvest was 70 N. The fruit retained 94% of initial firmness throughout -1.1° C storage for 100 days. Fruits harvested and directly subjected to 20° C storage retained their firmness for about three weeks followed by a gradual decrease to 15 N on the 14th week. Fruits subjected to -1.1° storage for 25, 40, 55, 70, and 85 days and then transfered to 20° C to ripen showed a dramatic decrease in firmness to about 10 N on the 10th, 7th, 5th, 3rd, and 2nd week at 20° C, respectively.

Internal ethylene (Fig.V.2.). Internal ethylene at harvest was 0.08 ul/l. No increase was observed during the first 4 weeks of -1.1° C storage. However, after the 4th week, internal ethylene began to increase, reaching 1.85 ul/l by the 10th week and remained constand thereafter. Internal ethylene of fruits directly (no cold storage) held at 20° C showed a trancient small ethylene peak and then decreased to 0.01 ul/l from harvest to the 2nd week of ripening. A gradual increase in internal ethylene to 0.45 ul/l on the 13th week preceded the climacteric rise above 30 ul/l on the 14th week of ripening. Fruits stored at -1.1° C for 25 days and then transfered to 20° C had 0.25 ul/l internal ethylene after warming. Internal ethylene then decreased to 0.03 ul/l on the 2nd week of ripening at 20° C. A small increase to 0.29 ul/l was then observed on the 9th week which was then followed by the climacteric rise in ethylene. The internal ethylene of fruits stored for 40 days at -1.1° C and then warmed

to 20° C was 0.31 ul/l. Following a decrease to 0.05 ul/l in the 2nd week of ripening at 20° C, internal ethylene rose above 1 ul/l in the 5th week showing the climacteric rise in ethylene above 30 ul/l. Internal ethylene of fruit stored at -1.1° C for 55 days was 0.47 ul/l after warming at 20° C. Internal ethylene decreased to 0.11 ul/l in the 1st week of ripening at 20° C. An increase in ethylene above 1 ul/l in the 3rd week led to the climacteric rise with a peak of 37 ul/l during the 4th week. Fruit stored at -1.1° C for 70 or 85 days had internal ethylene concentration of about 1 ul/l which remained constant during the first week of ripening at 20° C. The climacteric rise in ethylene occured during the 2nd week with peaks of 47 and 53 ul/l for 70 and 85 days of -1.1° C storage, respectively.

ACC (Fig.V.3.). ACC, an ethylene precursor, was at harvest about 0.09 nmoles/g of fresh weight. ACC levels remained about the same up to the 5th week in -1.1° C storage, then increased to 0.9 nmoles/g on the 9th week, leveled for two more weeks and decreased on the 14th week of -1.1° C storage as climacteric ethylene increased (Fig.V.2).

ACC levels did not change through the 13th week when the fruits were held at 20° C. ACC increased to 0.38 nmoles/g during the 14th week during ripening at 20° C.

Fruit stored for 25 days at -1.1° C and then transferred to 20° C showed an increase to 0.19 nmoles ACC/g only in the 9th week. ACC levels of fruit stored for 40 and 55 days at -1.1° C and then warmed briefly to 20° C were 0.28 and 0.2 nmoles/g,

respectively. ACC then decreased to 0.02 nmoles/g on the 4th and 2nd week. Thereafter, ACC rose with a peak of 0.38 and 0.48 nmoles/g on the 6th and 4th week of ripening at 20° C, for the fruits stored at -1.1° for 40 and 55 days, respectively. ACC concentration of fruits stored for 70 days at -1.1° C then warmed to 20° C, was 0.35 nmoles/g. After one week at 20° C, ACC levels further declined to 0.13 nmoles/g and increased the following week to 0.32 nmoles/g. The pears stored for 85 days at -1.1° C had 0.21 nmoles/g ACC upon warming to 20° C. The levels of ACC declined after one week to 0.05 nmoles and increased after two weeks to 0.16 nmoles.

EFE activity (Fig.V.4.). EFE activity at harvest was 1.4 nl C_2H_4/g /h. During -1.1° C storage, the EFE activity (actually assayed at 20° C) gradually increased and peaked at about 71 nl C_2H_4/g /h on the 14th week.

The EFE activity of fruits ripened at 20° without any prior cold storage increased to 4.5 nl C_2H_4/g /h on the 5th week and remained unchanged up to the 9th week. After the 9th week at 20° C, EFE activity peaked to 13.4 nl C_2H_4/g /h on the 10th week and declined thereafter to 2.8 nl C_2H_4/g /hr on the 14th week.

Fruits stored for 25 days at -1.1° C showed an EFE activity of 17 nl C_2H_4/g /h after warming at 20° C. Following ripening at 20° C, EFE activity decreased to 4.2 nl C_2H_4/g /h in the 2nd week, and remained unchanged until the 6th week. After a small peak at 6.7 nl C_2H_4/g /h on the 7th week, EFE activity further decreased to 4.3 nl C_2H_4/g /h the 10th week of ripening at 20° C.

The EFE activity of fruits stored for 40 and 55 days at -1.1° C after transfer to 20° C was 23.5 and 51.8 nl C_2H_4/g /h, and decreased to 4.1 and 6.2 on the second week, respectively. The fruits held for 40 days at -1.1° C peaked in EFE activity the 5th week at 10 nl C_2H_4/g /h, and the fruits stored for 55 days (the 5th week) had EFE activity of 20.2 nl C_2H_4/g /h, during the 5th week of ripening at 20° C. The EFE activity of fruits stored for 70 and 85 days at -1.1° C was 50 nl C_2H_4/g /h after transfer to 20° C, and dramatically decreased to 4.6 and 9.8 nl C_2H_4/g /h the 3rd and 2nd week, respectively.

Total (Fig.V.5.), and soluble (Fig.V.6), polyuronides. Total polyuronides of fruits continually stored at -1.1° C, or directly ripened at 20° C, or held after various periods of -1.1° C storage and then ripened, was about the same (in the range of 107 to 118 mg/g AIS) and did not change throughout the experiment. The soluble fraction of the polyuronides behaved differently.

Soluble polyuronide concentration of 'Anjou' pears at harvest was 63 mg/g of AIS. During storage at -1.1° C, soluble polyuronides began to increase from the 6th week of storage and reached 72 mg/g on the 11th week. No further increases were observed. The soluble polyuronide of fruits not stored at -1.1° C but held directly in 20° C gradually increased to 84 mg/g AIS the 14th week of ripening. Fruits stored for 25 days at -1.1° C and then ripened at 20 ° C showed the same pattern as fruits not cold stored. However, the 10th week of 20° C ripening, the soluble polyuronide rose to 88 mg/g AIS. Fruits stored at -1.1° C

for 40, 55, 70, and 85 days had, respectively, 77, 74, 73, and 74 mg/g AIS. Soluble polyuronides increased to 90, 88, 95, and 100 mg/g AIS on the 7th, 5th, 2nd, and 2nd week of ripening at 20° C for 40, 55, 70, and 85 days for -1.1° C stored pears, respectively.

Chlorophyll (Fig.V.7.). Only a slight change in peel chlorophyll was observed during -1.1° C storage for 100 days. Fruits ripened at 20° C, without cold storage, had only slight chlorophyll loss the first 2 weeks, followed by a progessive degradation that resulted in nearly complete loss on the 14th week of ripening at 20° C.

Fruits stored for 25, 40, 55, 70 and 85 days at -1.1° C and then ripened at 20° C lost nearly all chlorophyll on the 10th, 7th, 5th, 3rd and 3rd week, respectively.

Titratable acidity (Fig.V.8.). Titratable acidity at harvest was 33 ueq/g of fresh weight. The titratable acidity of pears stored at -1.1° C slowly decreased to 25 ueq/g the 14th week of storage. However, for the fruits directly held at 20° C, the titratable acidity decreased even more to 20 ueq/g on the 14th week of storage.

Titratable acidity of fruits ripening at 20° C after -1.1° C also decreased. Fruits stored for 25, 40, 55, 70 and 85 days at -1.1° C had, respectively, 29, 28, 26.5, 25.5, and 24.8 ueq/g titratable acidity immediately after removal from storage. Titratable acidity during 20° C ripening after cold storage decreased to 21, 24, 23, 24, and 22.5 ueq/g, respectively on the

10th, 8th, 6th, 4th, and 3rd week of ripening.

Total amino acids (Fig.V.9.). The total free amino acid concentration of the fruits at harvest was 0.68 umoles/g. During storage at -1.1° C, the amino acid concentration declined to 0.55 umoles/g. The fruits that were placed directly in 20° C at harvest showed an increase in amino acids with a peak of 0.9 umoles/g the 2nd week, decreased to 0.6 umoles/g the 4th week, and finally decreased to 0.5 umoles/g the 14th week.

Fruits stored at -1.1° C for 25, 40, 55, 70, and 85 days had free amino acid concentrations of 0.56, 0.56, 0.54, 0.59, and 0.48 umoles/g, respectively. Fruits stored for 25, 40 and 55 days at -1.1° C and then ripened at 20° C showed peaks in the 1st to 3rd week to about 0.69 umoles/g followed by a decrease to about 0.5 umoles /g on the 5th week of ripening at 20° C, then the total amino acids rose again to 0.8, 0.7, and 0.6, respectively. However, the fruits stored at -1.1° C for 70 and 85 days showed only a decrease in free amino acid content on the 3rd and 2nd week of ripening at 20° C, respectively.

Total proteins (Fig.V.10.). Total proteins during -1.1° C storage decreased slightly from 0.35 mg/g at harvest to 0.30 mg/g the 2nd week followed by a very slight increase to 0.36 mg/g the 14th week. The fruits not stored at -1.1° C but held directly at 20° C also showed a decrease to 0.28 mg/g the 3rd week, increased to 0.38 mg/g the 6th week and again the 13th week to 0.45 mg/g.

The total protein of fruit stored at -1.1° C for 25, 40, 55,

70, and 85 days showed steady increases to 0.38, 0.39, 0.42, 0.46, and 0.49 mg/g, respectively. The pattern of changes in total protein was similar for all fruits. Fruit total protein decreased the first two weeks and then increased to around 0.45, mg/g.

DISCUSSION

'Anjou' pears usually do not soften (142) or soften only slightly (about 7%) during -1.1° C storage (Fig.V.1.) and slowly lose chlorophyll (about 7%) (Fig.V.7.). However, ethylene can build up to 1.5 ul/l (Fig.V.2.), ACC up to 0.9 nmoles/g (Fig.V.3.) and EFE activity up to 71 nl C_2H_4/g /h (Fig.V.4.). The soluble polyuronide fraction increases by about 15% (Fig.V.6.), titratable acidity decreases 20% (Fig.V.8.), accompanied by a small net decrease (7%) in protein (Fig.V.10.) and decrease (25%) in amino acids (Fig.V.9.) content.

Changes in ethylene synthesis parameters, as well as the other biochemical constituents with -1.1° C storage have been previously reported (31,216,222,229). As will be shown in chapter VI, the sensitivity of the fruit to propylene also increases during -1.1° C storage, and is maximized once the chilling requirements are satisfied.

Fruits placed at 20° C after harvest, with no cold treatment, ripen in a very characteristic way, by showing a much earlier loss in flesh firmness and chlorophyll, 6 to 7 weeks ahead of the

climacteric rise in ethylene (Fig.V.1,2). Climacteric ethylene production occurs on the 13th week of 20° C storage, well after the fruits become edibly ripe and soft to thumb pressure (about 20 N) (see also chapter III). Chlorophyll loss accompanies the loss in firmness (Fig.V.7.), and titratable acidity becomes half that at harvest (Fig.V.8.). Free amino acid concentration decreases (Fig.V.9.), and total protein increases by about 20% (Fig.V.10.) before climacteric ethylene initiates (Fig.V.1.), along with the rise in ACC (Fig.V.3.) in the 13th week at 20° C. EFE activity (Fig.V.4), however, begins to peak 4 weeks earlier (on the 9th week) and this is a bit unexpected.

Generally, the sequence of ripening events at 20° C (with no chilling) are; first the cell walls and chlorophyll degrade, then titratable acidity exhibits some stepwise decreases, EFE activity rises then falls, and later climacteric ethylene appears and is always associated with an increase in ACC and protein.

'Anjou' pears with partially satisfied chilling requirement (more than 70 days at -1.1° C) show a decrease in firmness upon transfer to 20° C as well as decreases in chlorophyll, amino and organic acids, and increases in protein and water soluble polyuronides. To simplify the discussion of other ripening events, it is most useful to use firmness and internal ethylene as major criteria for comparison. The rate of loss in firmness is determined by the extent to which the chilling requirement is satisfied. The more chilling that the fruits receive in -1.1° C storage, the faster is the loss in firmness at 20° C. Fruit

receiving only 25 days of chilling, require about 10 weeks at 20° C ripening temperature to soften to 10 N. However, pears stored at -1.1° C for 55 days and with about 75% of the chilling requirement satisfied, soften to 10 N in 5 weeks when ripened at 20° C. 'Anjou' pears with satisfied chilling requirement (70 or more days in -1.1° C) soften to 10 N in about a week at 20° C (Fig.V.1.). The initiation of climacteric ethylene (above 1 ul/l) in pears which have not fully satisfied the -1.1° C chilling requirement show a pattern similar to that of firmness once transferred to 20° C. The closer to chilling requirement satisfaction (ie, the longer held at -1.1° C) the sooner is climacteric ethylene synthesized during ripening at 20° C. The rise in ethylene for partially chilled pears occurs when firmness is in the range of 25 to 15 N (Fig.VI.1, 2.). When chilling is satisfied, climacteric ethylene begins to initiate when firmness has decreased to 40 to 25 N.

'Anjou' pears with increasingly satisfied chilling requirement, when exposed to warm temperatures (20° C for our experiment) usually show increasingly higher internal ethylene (Fig.V.2.), higher ACC content (Fig.V.4.), and higher EFE activity (Fig.V.3.), and are more sensitive to propylene (as will be seen in chapter VI), and probably have a high ratio of unsaturated/saturated fatty acids (chapter IV). The chilling requirement is satisfied in cold storage when all those factors peak. The fruit ripens in a very characteristic way with: losses in firmness, green color, organic and amino acids, and gains in

protein and water soluble polyuronides in response to rising climacteric ethylene (chapter IV).

Another point to consider is the fact that for incompletely chilled 'Anjou' pears after storage at -1.1° C followed by transfer to 20° C the fruit is usually not able to sustain the accumulated ACC (31) or the high EFE activity. Both ACC concentration and EFE activity decrease to a certain level and then peak before or during the climacteric rise in ethylene (Fig.V.2, 3, 4.). Apparently, from these data we can clearly answer the question that researchers often pose, ie, whether or not EFE activity, or ACC peaks before or after the climacteric rise in ethylene. Clearly EFE activity preceded ACC and the internal ethylene rise in all cases. For the 25 or 40 day -1.1° C chilling and for the non-chilled pears, EFE activity appears to increase well before ACC or climacteric ethylene. Once chilling is satisfied, this is readily apparent in the 70 and 85 days cold storage treatments where EFE activity starts high then decreases. There is another difficulty also in the handling of tissue for the assay of the EFE activity, especially when tissue is quite soft. ACC increases closely parallel the initiation of ethylene for nearly all treatments. The fact that ACC accumulates in cold-stored pears implies either that it is compartmentally segregated from the ethylene forming enzyme or, alternatively, that enzymes leading up to ACC are not as low-temperature-inhibited as are the enzymes converting ACC to ethylene. There is some evidence (Fig.V.3.) to support the latter alternative as ACC

can disappear quite rapidly once chilling is satisfied and pears are placed at 20° C.

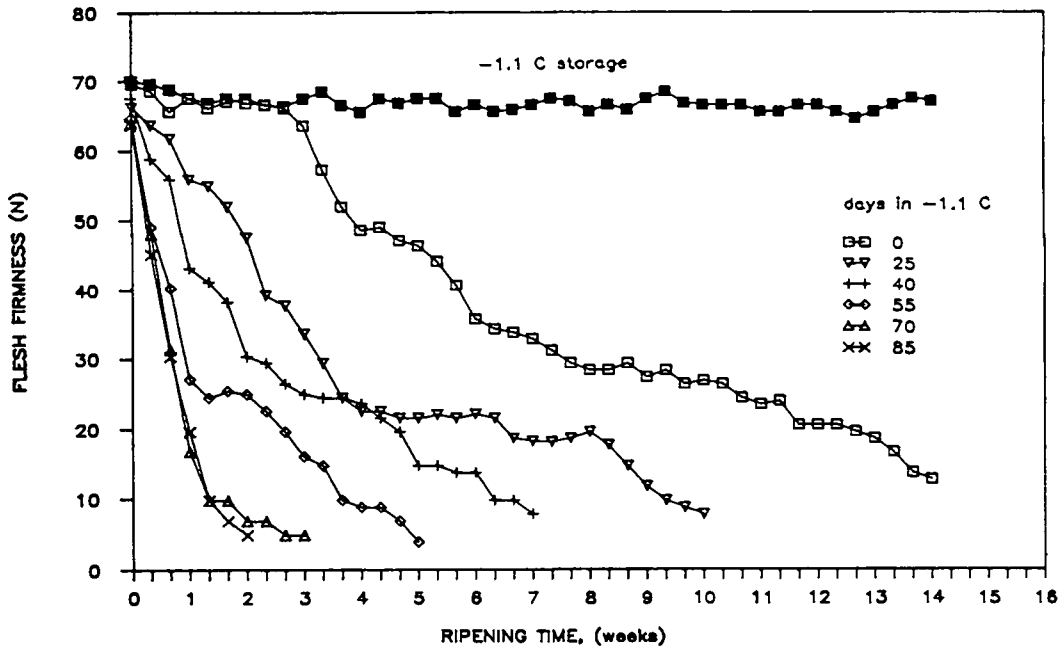


Fig.V.1. Flesh firmness of 'Anjou' pears during ripening at 20°C after storage at -1.1°C for various time durations. LSD.05: 18

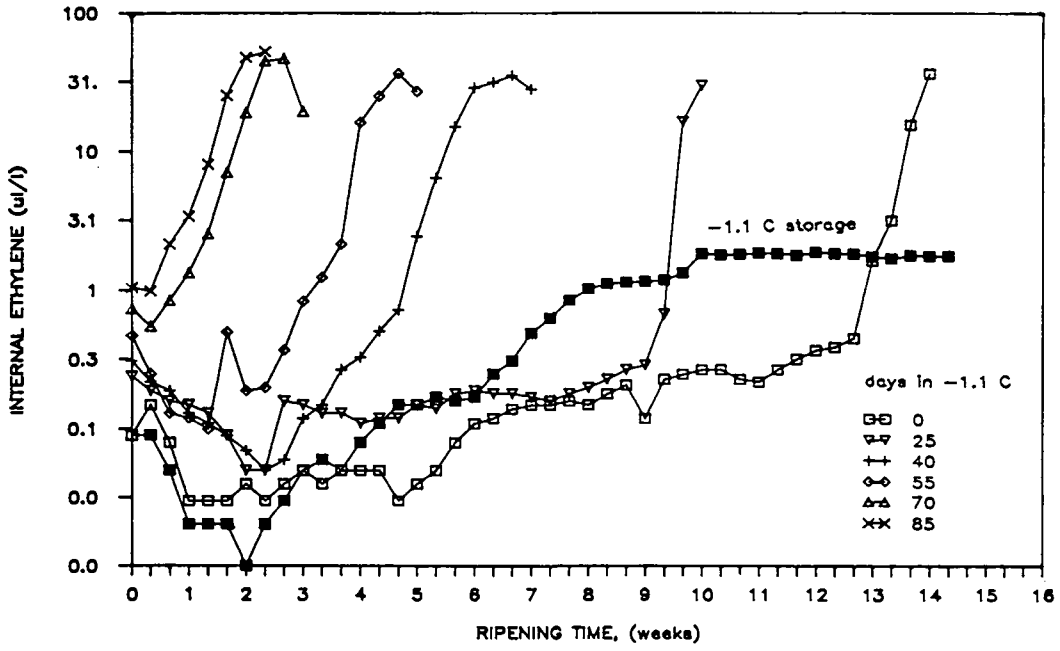


Fig.V.2. Internal ethylene of 'Anjou' pears during ripening at 20° C after storage at -1.1°C for various time durations. LSD.05: 4.6

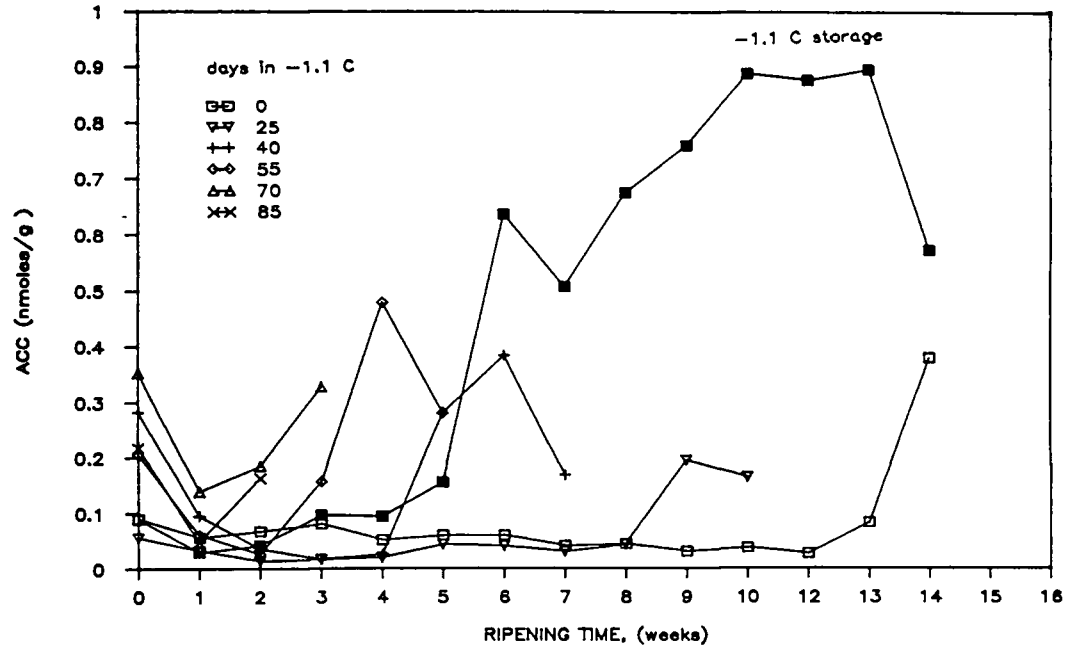


Fig.V.3. 1-amino-cyclopropane-1-carboxylic acid (ACC) content of 'Anjou' pears during ripening at 20°C after storage at -1.1° C for various time durations. LSD.05: 0.16

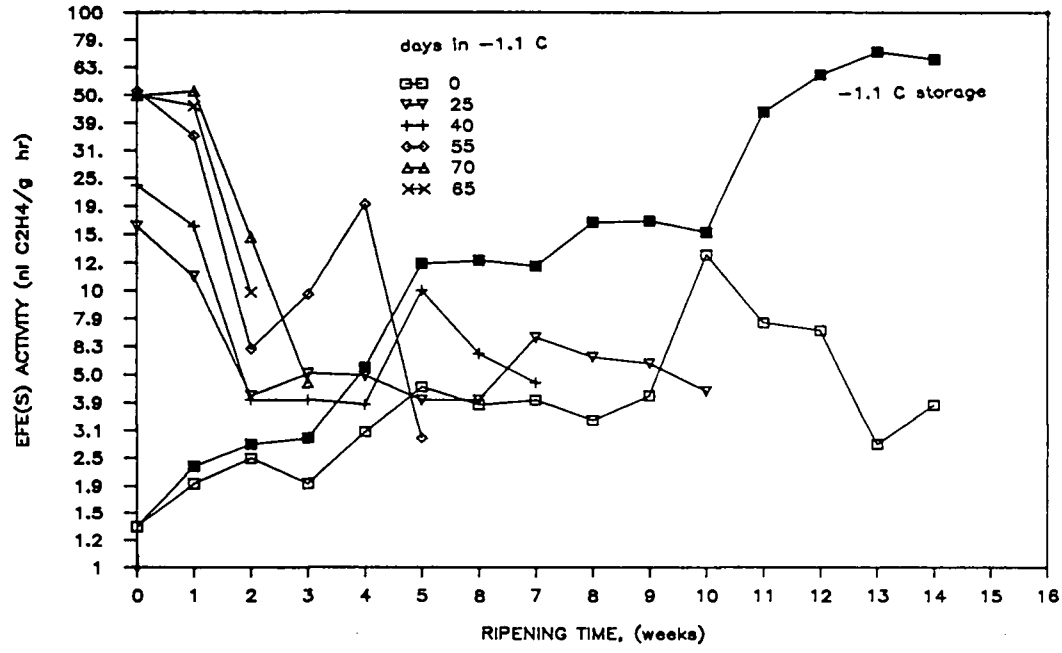


Fig.V.4. Ethylene-forming-enzyme (EFE) activity of 'Anjou' pears during ripening at 20°C after storage at -1.1°C for various time durations. LSD.05: 4.9

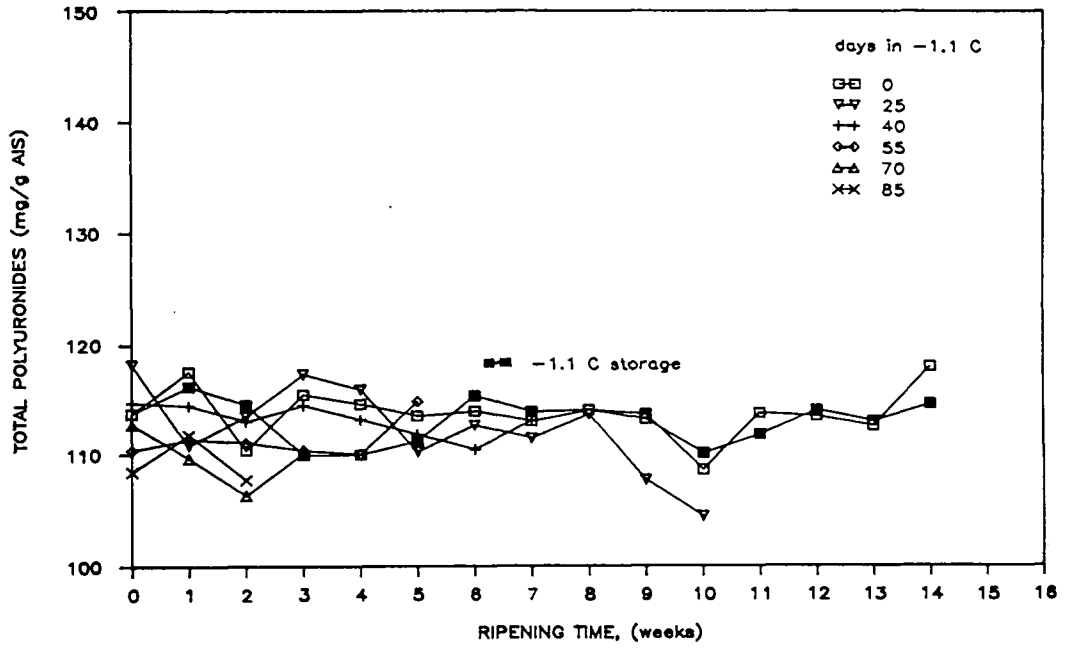


Fig.V.5. Total polyuronides of 'Anjou' pears during ripening at 20°C after storage at -1.1°C for various time durations.
LSD.05: 5.3

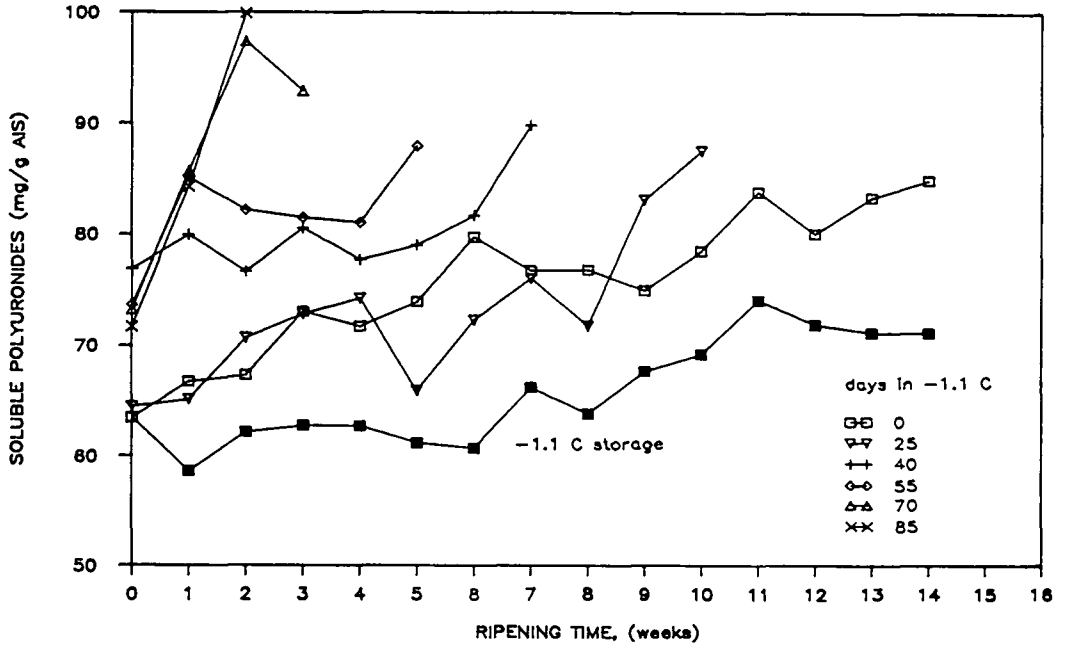


Fig.V.6. Soluble polyuronides of 'Anjou' pears during ripening at 20°C after storage at -1.1°C for various time durations.
LSD.05: 6.5

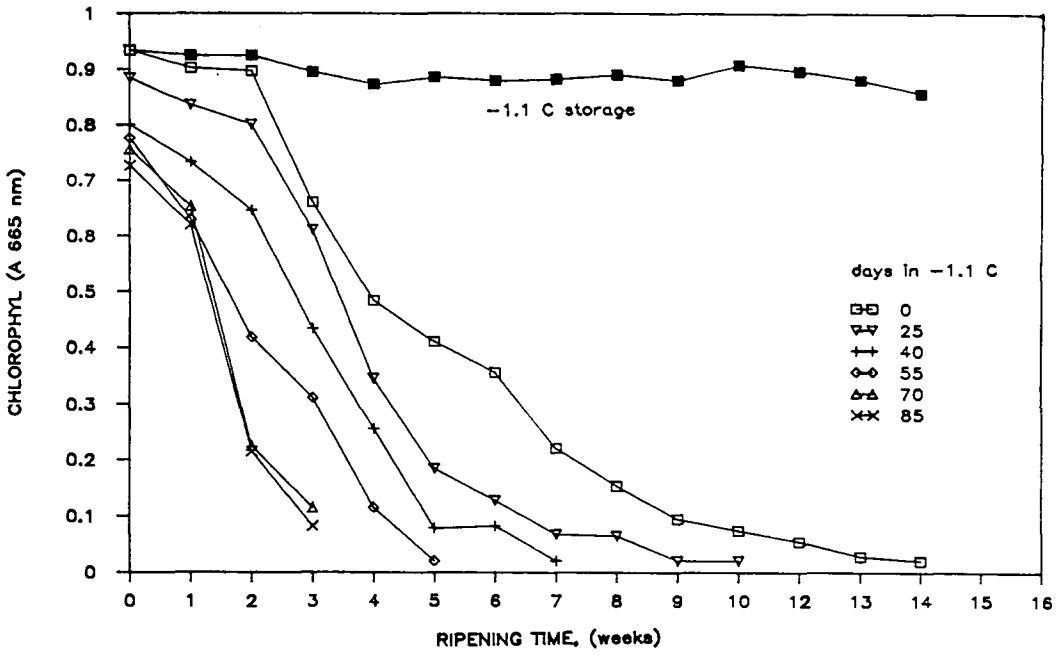


Fig.V.7. Chlorophyll of 'Anjou' pears during ripening at 20° C after storage at -1.1°C for various time durations. LSD.05: 0.17

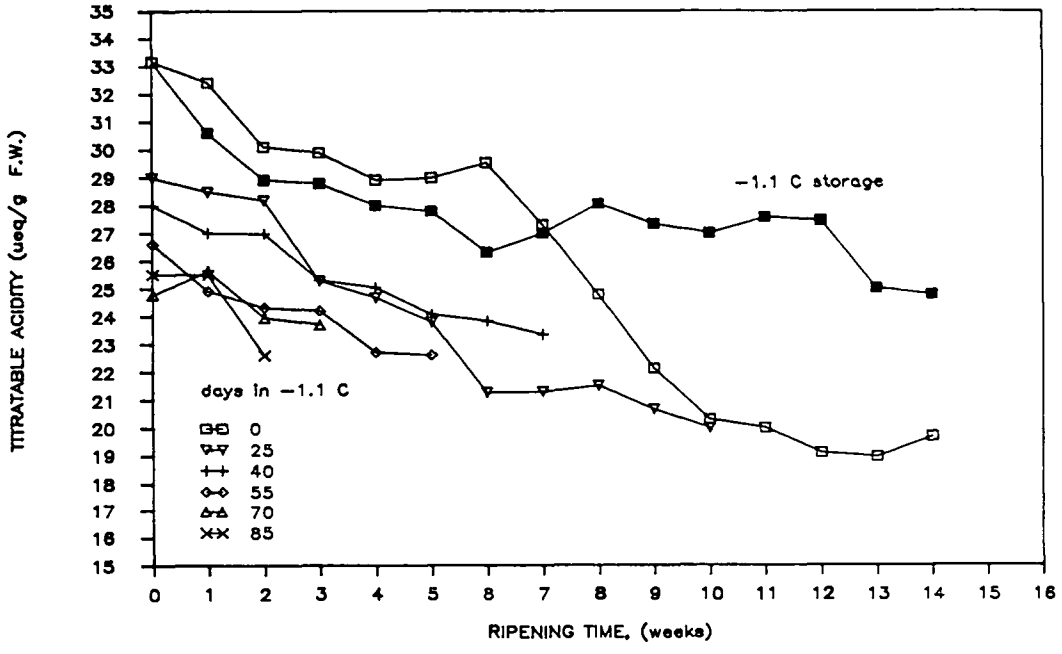


Fig.V.8. Titratable acidity of 'Anjou' pears during ripening at 20°C after storage at -1.1°C for various time durations.
LSD.05: 2.3

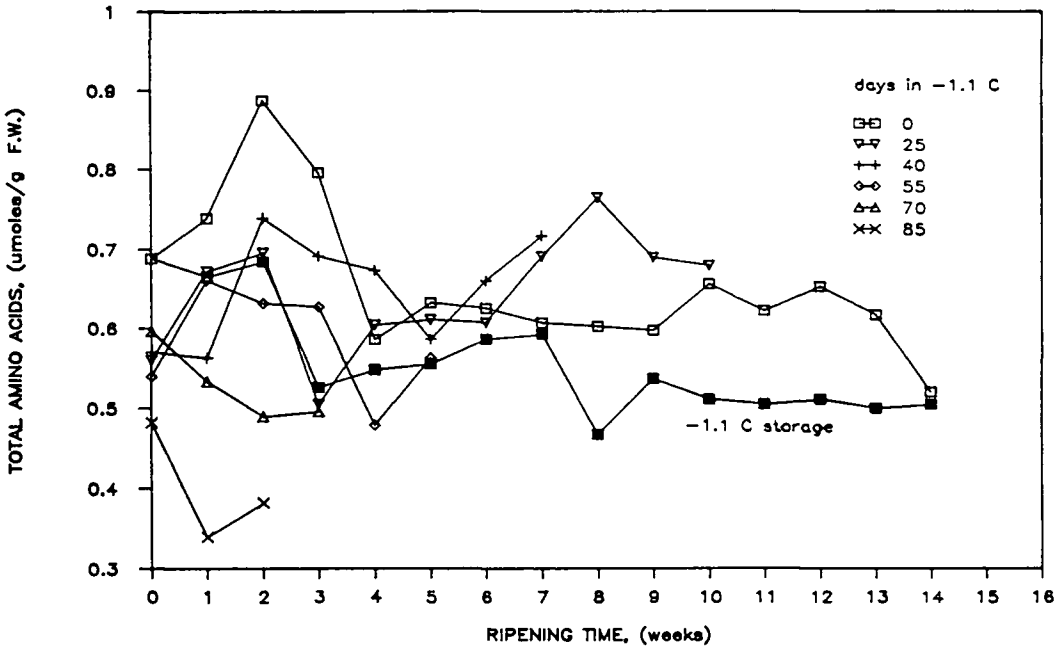


Fig.V.9. Total amino acids of 'Anjou' pears during ripening at 20°C after storage at -1.1°C for various time durations.
LSD.05: 0.12

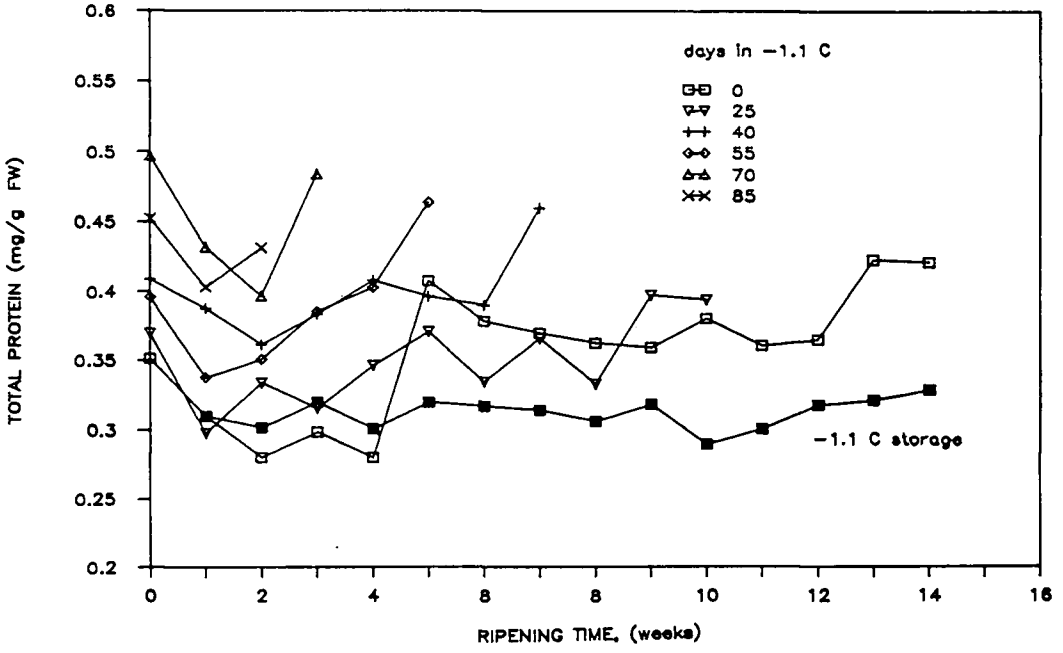


Fig.V.10. Total protein of 'Anjou' pears during ripening at 20°C after storage at -1.1° C for various time durations.
LSD.05: 0.6

CHAPTER VI

THE DEVELOPMENT OF AUTOCATALYTIC SUSTAINABLE ETHYLENE AND ALTERED SENSITIVITY TO PROPYLENE BY 'ANJOU' PEARS IN 20° C OR -1.1° C STORAGE AND WHEN WARM AND COLD STORAGE ARE ALTERNATED, FOLLOWED BY 20° C RIPENING TEMPERATURE

Additional index words: Pyrus communis, postharvest physiology, autocatalytic ethylene synthesis, firmness, ripening.

Abstract. 'Anjou' pears were harvested from the Mid-Columbia Experiment Station, Hood River Oregon, at 70 N firmness. The pears were stored at -1.1° or 20° C. Fruits stored for various amounts of time at -1.1° C were then transferred to 20° C to ripen. Conversely, fruits stored at 20° C for various amounts of time were transferred to -1.1° C for various durations, followed by transfer to 20° C to ripen. The ability to produce climacteric (greater than 1 ul/l) ethylene was measured and the elapsed time of the storage temperature was noted. In a parallel experiment, fruits removed from both -1.1° C and 20° C storage after predetermined times were treated with 500 ul/l propylene at 20° C, to determine when autocatalytic ethylene and softening were induced.

For pears stored at -1.1° C then transferred to 20° C, the time required to produce climacteric ethylene progressively decreased as time in cold storage increased. The total amount of time in -1.1° C storage plus the time at 20° C ripening

temperature remained nearly constant at 85 days. Similarly, the sensitivity of the fruit to exogenous propylene increased progressively with time in -1.1° C storage.

However, this was not the case with fruits initially stored at 20° C. The total amount of time to produce 1 ul/l ethylene in 20° C storage plus -1.1° C chilling treatment increased with time in storage and required more time than the -1.1° C storage treatment alone, but finally decreased to about the same. While the fruits stored at -1.1° C responded faster to propylene proportionate to storage time, the fruits stored at 20° C did so after 55 days out of 90 total when the firmness is decreased to about 25 N. Thus, the manner that 'Anjou' pears ripen in response to warm storage temperature is different than for cold storage also in respect to both the chilling requirement for autocatalytic ethylene and to the sensitivity of the fruit to exogenous propylene.

INTRODUCTION

Winter type pears usually require a period of cold storage in order to develop the ability to ripen and to produce ethylene. Summer pears such as 'Bartlett' have no chilling requirement even though they are harvested at greater firmness than winter pears (236). 'Anjou' pears normally require 50 to 60 days (51,49,52) at -1.1° C to ripen. However, depending on the fruit calcium concentration, and possibly other factors, they may require as

much as 90 days (51) 30 days (51) or even zero days (if calcium is low) (186) in cold storage at -1.1° C to begin to ripen at 20 C. 'Anjou' pears eventually ripen at 20° C even if they are not exposed to chilling (chapter III). Loss in firmness occurs first and climacteric ethylene follows. The two events are totally time separated when 'Anjou' pears are continuously held at high temperatures.

Propylene is a useful treatment for study of autocatalytic ethylene because it it mimies ethylene action and can be separated from ethylene by gas chromatography (34, 165). While propylene can mimic the activity of ethylene, much higher concentrations are required (typically 100 to 200 fold higher) in order to produce an equivalent response (1). Thus a 1.0 ul/l ethylene-induced response would require 130-150 ul/l propylene to evoke the same response (42). Exogenous ethylene applied to immature and mature 'Anjou' pears induced ripening and softening (186). Sensitivity of 'Anjou' pears to exogenous ethylene increases with time after anthesis (182). 'Anjou' pears treated at harvest with 500 ul/l propylene required about 9 days to produce autocatalytic (ie, greater than 1 ul/l) internal ethylene but required only 1 day after the fruits had satisfied the chilling requirements. Thus the sensitivity of fruit to propylene maximized after the chilling requirement was satisfied (86).

The purpose of this study was to investigate: 1. The time required for 'Anjou' pears to produce autocatalytic ethylene greater than 1 ul/l during storage at 20° C (not involving

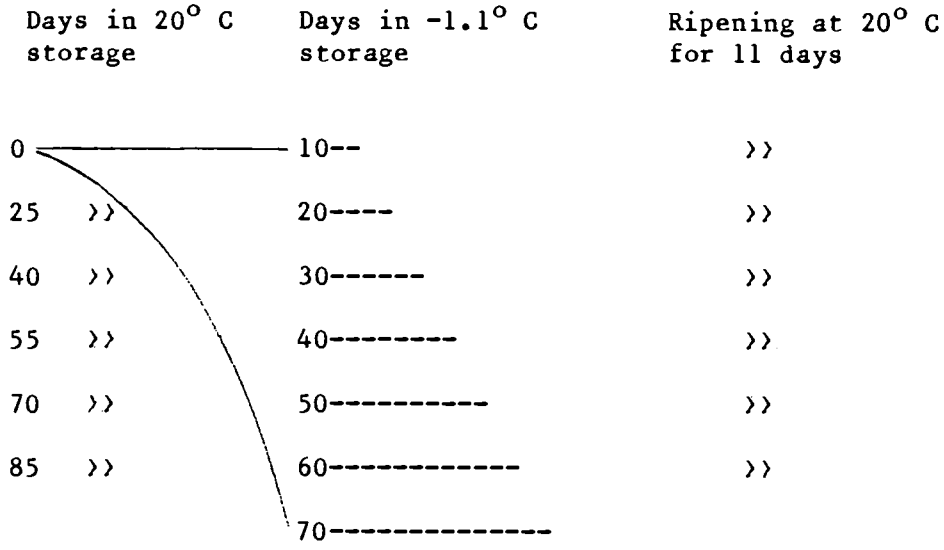
chilling) as compared to -1.1° storage (involving chilling). 2. The effects of -1.1° C and 20° C storage on the development of the sensitivity of the fruits to exogenous propylene.

MATERIALS AND METHODS

'Anjou' pear fruits were purchased from the Mid-Columbia Experiment Station, Hood River, Oregon in 1986. All fruits were harvested based on 147 days from full bloom and 70 N flesh firmness. After harvest the fruits were transferred into perforated polyethylene film-lined 20 kg cardboard cartons and placed into -1.1° C or 20° C storage. All the fruits were screened for cork spot. Only healthy fruits were used.

The time required by the fruits at harvest (0 days) or stored at -1.1° C for 25, 40, 55, 70, and 85 days and transferred to 20° C in order to ripen and the time to produce climacteric ethylene (as defined: greater than 1 ul/l) during ripening at 20° C, was then noted. Five replicates, three times per week, were used for internal ethylene determination (see methods chapter III).

In the reciprocal experiment, fruits were started intially at high temperature (20° C) for varying periods. This was followed by transfer to -1.1° C storage for progressive 10 day increments to see what effects chilling treatment would have on subsequent ethylene synthesis and ripening (at 20° C again). The design of this experiment is schematically presented below.



The amount of chilling (-1.1° C) required by the fruits after initial storage at 20° C for varying times in order to produce climacteric ethylene within 11 days at 20° C ripening temperature was also determined. After storage at 20° C for 0, 25, 40, 55, 70, or 85 days pears were transferred to -1.1° C. The fruits were then chilled (at -1.1° C) for 10, 20, 30, 40, 50, 60, or 70 days. The pears were again transferred to 20° C to ripen for 11 days. Internal ethylene was determined every other day on five replicates.

The sensitivity of the fruits to propylene was determined as follows: Forty fruits of each storage treatment (-1.1° or 20° C) stored for 0, 25, 40, 55, 70, and 85 days, then were placed in 20 1 jars to ripen for 14 days at 20° C. Humidified air flow containing 500 ul/l propylene at 1000 ml/min for each 20 1 jar was metered in by calibrated glass capillary tubes. This ensured that CO_2 accumulation did not exceed 0.5% in the atmosphere, and thus would not be inhibitory to ethylene synthesis. Internal ethylene

and flesh firmness were determined every other day on five replicates.

The ripening parameter determinations and the statistical analysis are described in the methods of chapter III.

RESULTS

A. CHILLING REQUIREMENTS

-1.1° C storage. Firmness of 'Anjou' pears only slightly decreased from 70 N to 65 N during 90 days at -1.1° C.

Internal ethylene during the same period at -1.1° C decreased from an initial value of 0.1 ul/l to about 0.01 at 14 days followed by a progressive increase to about 2 ul/l through 98 days. Thus 56 days was designated as the time to reach the 1 ul/l internal ethylene which is usually associated with the ability for continued ethylene production during ripening (Fig.VI.1.).

Fruits not subjected to cold storage but placed at 20° C to ripen at harvest showed a prolonged inability to produce ethylene more than 0.3 ul/l. It was only after 100 days at 20° C that internal ethylene exceeded 1 ul/l and then climacterically rose beyond 30 ul/l within the next 10 days (Fig.VI.2.).

Fruits held only 25 days at -1.1° C then moved to 20° C to ripen also showed a considerable delay (63 days) before 0.03 ul/l was exceeded. Within the next few days internal ethylene rapidly reached 1 ul/l and continued to rise to 30 ul/l by the 70th day (Fig.VI.2.).

Pears held 40 or 50 days at -1.1° C also had delayed

ethylene production, requiring 34 and 23 days, respectively, to attain 1 ul/l internally. Once 70 days or more of -1.1° C storage had been passed, there was essentially no delay in ability to initiate climacteric ethylene and maximum values above 40 ul/l were attained around the 14th day (Fig.VI.2.).

20° C storage. In contrast to the lack of change in firmness that occurred at -1.1° C storage, pears at 20° C showed a very slow decrease to 65 N at 21 days. Thereafter, softening rapidly progressed up to 42 days (about 35 N), followed by a slower loss in firmness to 10 N at 98 days (Fig.VI.3.).

Internal ethylene in 20° C storage remained well below 0.3 ul/l up to 80 days and at 90 days climacteric ethylene was initiated, surpassing 30 ul/l by day 98 (Fig.VI.3.).

20° C initial storage followed by -1.1° C storage. Pears held at -1.1° C for periods up to 60 days failed to synthesize internal ethylene above 0.4 ul/l during 12 days of ripening at 20° C. After 70 days in -1.1° C storage, then transfer to 20° C for ripening, internal ethylene exceeded 1 ul/l on the 7th day and increased to about 7 ul/l on the 12th day (Fig.VI.4).

Pears held at 20° C for 25 days required 70 days at -1.1° C in order to produce sustainable ethylene above 1 ul/l when placed back at 20° C for 11 days. Even though pears stored at -1.1° C for 50 or 60 days at -1.1° C had respectively, 1.5 and 10 ul/l internal ethylene on the first day of transfer to ripening, this was not sustainable, falling to values around 0.3 ul/l by day 7 of ripening. Evidence suggests (Ch.V, fig.3.) that the initial drop

ripening. Evidence suggests (Ch.V, fig.3.) that the initial drop in the 1st day ethylene, may have been due to accumulated ACC converting to ethylene (Fig.VI.5).

'Anjou' fruits held 40 days at 20° C required 60 or more additional days at -1.1° C storage to produce sustainable ethylene above 1 ul/l. Pears stored for 60 or 70 days at -1.1° C and also for 50 days had internal ethylene above 1 ul/l but the pears stored for 50 days at -1.1° C could not sustain ethylene above 0.3 ul/l in the ripening environment (Fig.VI.6.).

'Anjou' pears held 55 days at 20° C, required at least 50 days at -1.1° C storage to sustain ethylene upon ripening. Pears stored for either 50 or 40 days at -1.1° C had 1st day ripening ethylene above 1 ul/l (9 ul/l and 4 ul/l, respectively), but the pears stored for 40 days at -1.1° C stored could not further sustain ethylene, and it fell to 0.4 ul/l by day 5 (Fig.VI.7.).

Pears held 70 days at 20° C, followed by -1.1° C storage showed some interesting patterns. Despite the fact that -1.1° C fruits held for 20, 30, 40, and 50 days all had internal ethylene above 1 ul/l on day 1 of ripening, only the fruits stored for 40 or 50 days at -1.1° C had sustainable ethylene (Fig.VI.8.).

'Anjou' fruits held 85 days at 20° C, and then stored for 10 or 20 days at -1.1° C had more than 1 ul/l internal ethylene from the very first day of ripening and levels rapidly climbed to 100 ul/l by day 11 of ripening (Fig.VI.9.).

B. SENSITIVITY TO PROPYLENE

Fruit internal ethylene at harvest in response to 500 ul/l exogenous propylene at 20° C treatment, increased gradually from 0.08 ul/l to 15 ul/l in 14 days. The fruit internal ethylene had risen to 1 ul/l after the 8th day (Fig.VI.12, VI.13.).

-1.1° C storage. Fruits stored for 25, 40, 55, 70, and 85 days at -1.1° C required 6.3, 5.1, 4.3, 2.4, and 0 days in order to produce 1 ul/l in the presence of 500 ul/l propylene at 20° C (Figs.VI.12, VI.14.). All pears responded to propylene by generating ethylene and by softening rapidly.

20° C storage. Fruits stored at 20° C for 25, 40, 55, 70, and 85 days and then subjected to a 500 ul/l propylene environment at 20° C , required 9, 9, 8, 4, and 2 days in order to exceed 1 ul/l internal ethylene (Fig.VI.11, VI.14.).

DISCUSSION

For winter pears, the two parameters of ripening, loss of firmness and sustained ethylene synthesis (climacteric ethylene), show different responses to storage temperatures. Pears held at high (20° C) storage temperatures (Fig.VI.3 and Chapter III, Fig.III.5., III.10.) show a progressive loss in firmness without much increase in ethylene for up to 80 days. At low (-1.1° C) temperatures, although both softening and ethylene production are slowed, once chilling is satisfied upon ripening, ethylene production and softening develop concurrently. (Chapter III, Fig.III.5., III.10.). Then the dramatic effect of low temperature

is to retard softening as ethylene synthesis capability continues to develop.

As 'Anjou' pears are held progressively longer in -1.1° C storage, it is observed that when the pears are placed at 20° C to ripen, the time needed to produce climacteric ethylene becomes shorter and shorter (Fig.VI.10 and VI.11). Interestingly, when one adds the time at -1.1° C plus the time in 20° C ripening, one arrives at almost a constant number of days (about 90) in order to achieve climacteric ethylene. While this applies up to the time that chilling is satisfied, obviously it doesn't apply for storage beyond 90 days. Although this seems to hold for cold followed by warm ripening temperature, it doesn't hold as well for high temperature storage, followed by cold storage, and ripening.

The amount of time required by 'Anjou' pears subjected to -1.1° C storage in order to produce 1 ul/l sustainable internal ethylene at the ripening temperature (20° C) appears to decrease in a linear sense with increasing time in -1.1° C storage (Fig.VI.11, VI.13.). Alternatively, the sum of the amount of time that the fruit requires in low storage temperature (-1.1° C) and in ripening temperature (20° C) is about the same (Fig.VI.10.). However, this was not the case for fruits stored at 20° C and then chilled at -1.1° C (Fig.VI.13.). Although those fruits lose firmness during 20° C storage along with other changes, such as chlorophyll loss described in chapter V, this is not as strong a factor to cause a decrease in the amount of chilling needed to produce more than 1 ul/l internal ethylene upon ripening. The

chilling requirement of fruit held at 20° C dramatically decreases when firmness falls into the range of 25 to 15 N. As a result, the added times in warm storage temperature (20° C), and chilling temperature (-1.1° C), does not produce a constant figure (sum of days at 20° C plus days at -1.1° C) as for fruits stored at -1.1° C (Fig.VI.12.). This additive figure appears to increase with the loss in firmness and decline only when firmness is about 25 to 15 N (Fig.VI.12.).

Treatments of fruits treated with 500 ul/l propylene at harvest, resulted in internal ethylene above 1 ul/l after 8.3 days. 'Anjou' pears treated at harvest with 500 ul/l propylene in another study required about 9 days to produce internal ethylene of 1 ul/l (86).

The sensitivity of the pears to propylene increased the longer they were held in -1.1° C storage. After the 85th day of -1.1° C storage, the pears did not further respond to propylene, because the fruit were already able to produce 1 ul/l ethylene or more even without the stimulatory effect of propylene (Fig.VI.10, VI.2.). This maximization of the sensitivity of the fruit to propylene after the chilling requirement has been satisfied was previously reported (1,86)

Pears that have been stored at 20° C behave differently. About the same time (8.3 days) in 500 ul/l propylene environment is required for fruits stored at 20° C for 0, 25, 40 and 55 days to produce 1 ul/l internal ethylene. Only after 55 days (internal ethylene 0.18 ul/l, firmness 30 N), did the fruits

respond earlier than 8 days to propylene the longer they were held in 20° C storage (Fig.VI.14.).

A -1.1° C (or similar low temperature) storage the chilling environment appears to stimulate, probably through stress, the ripening mechanism, as expressed by the appearance of the climacteric over time and the development of ethylene autocatalysis, uniformly and proportionate to time in storage. The 20° C storage, however, alters the time sequence of the different components of the ripening mechanism. The loss in firmness (and chlorophyll) preceeds the rise in ethylene. The development of autocatalysis may indicate that ethylene production is part of the senescence process and not necessarily the initiating factor.

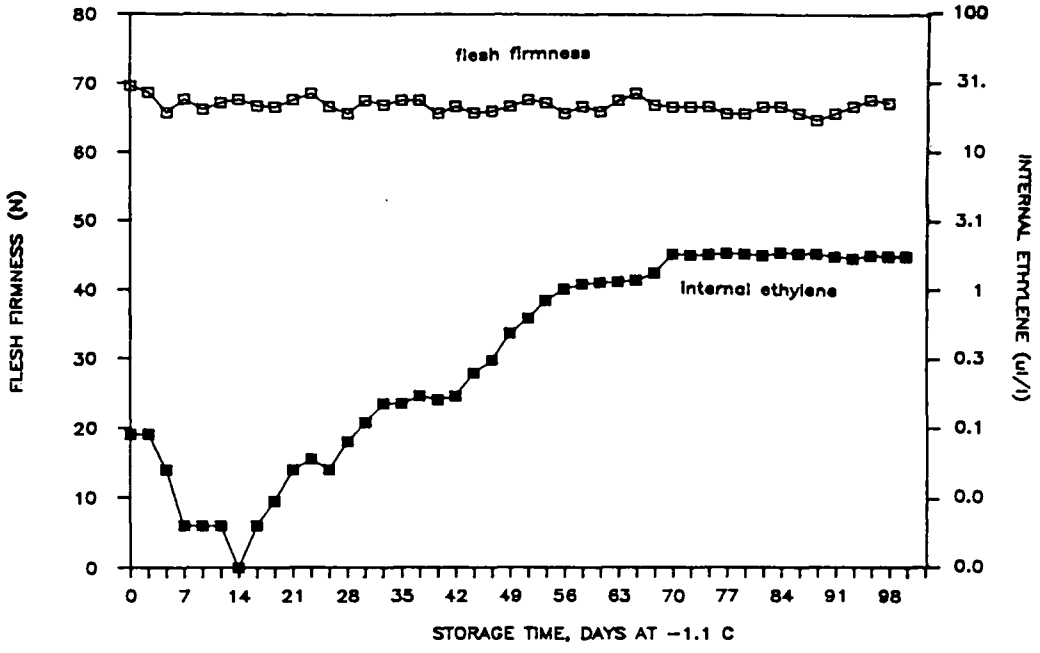


Fig.VI.1. Internal ethylene and flesh firmness of 'Anjou' pears during storage at -1.1°C.

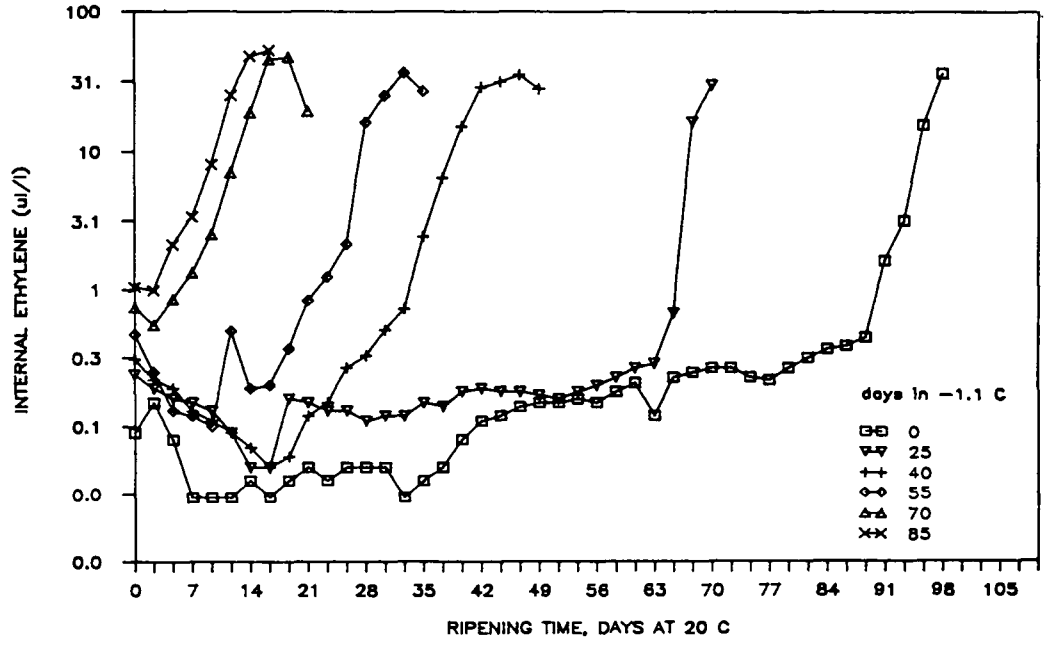


Fig.VI.2. Internal ethylene of 'Anjou' pears stored at -1.1° C storage for various durations of time, followed by ripening at 20° C. LSD.05: 4.6

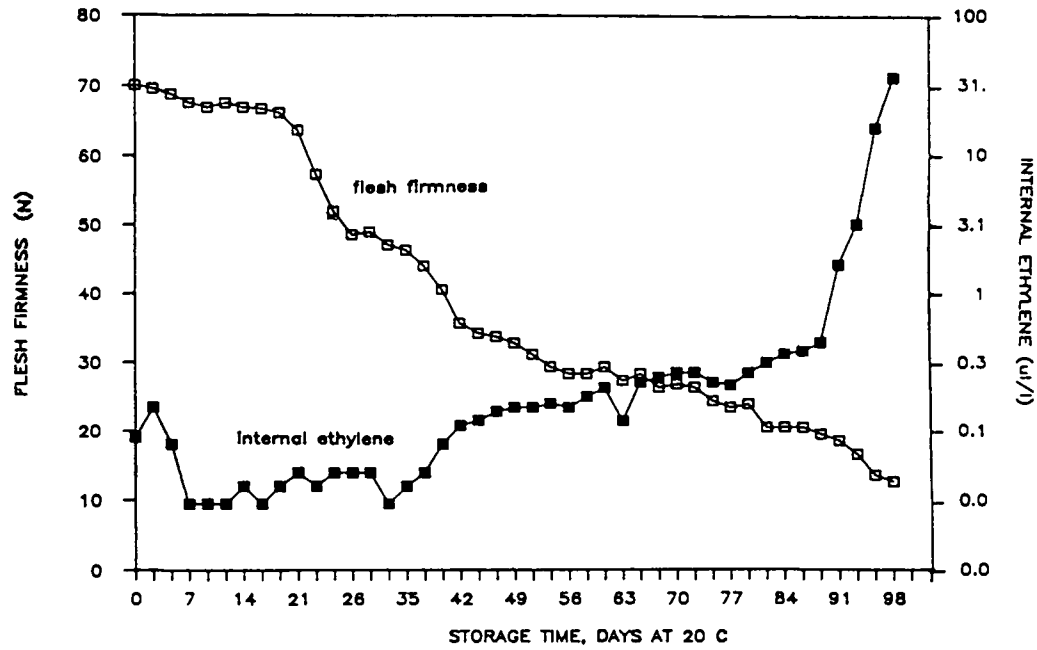


Fig.VI.3. Internal ethylene and flesh firmness of 'Anjou' pears during storage at 20°C.

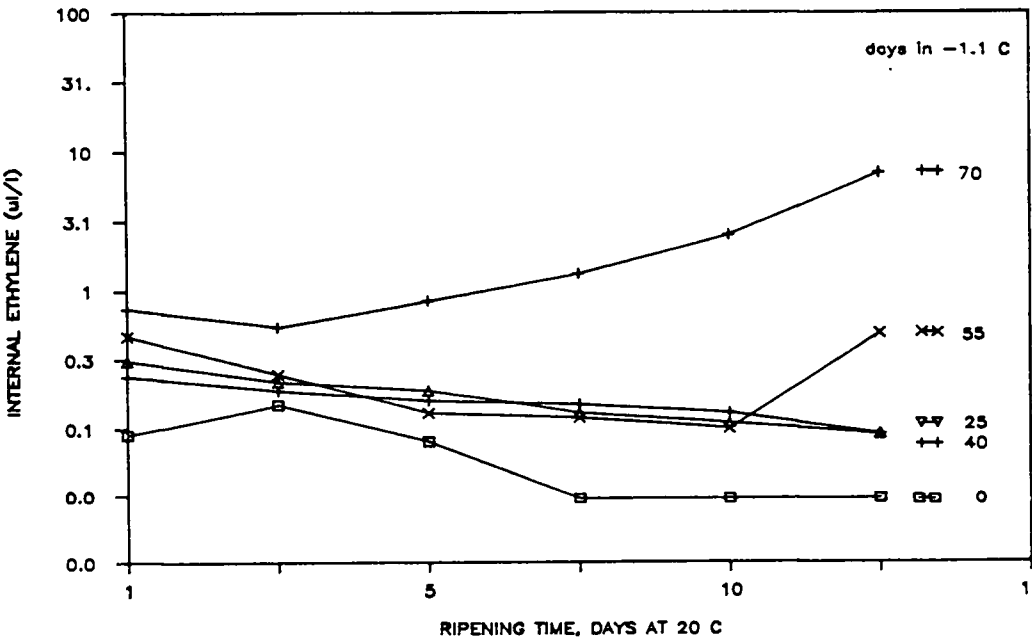


Fig.VI.4. Internal ethylene of 'Anjou' pears during ripening at 20°C, stored at -1.1°C for various amounts of time.
LSD.05: 4.6

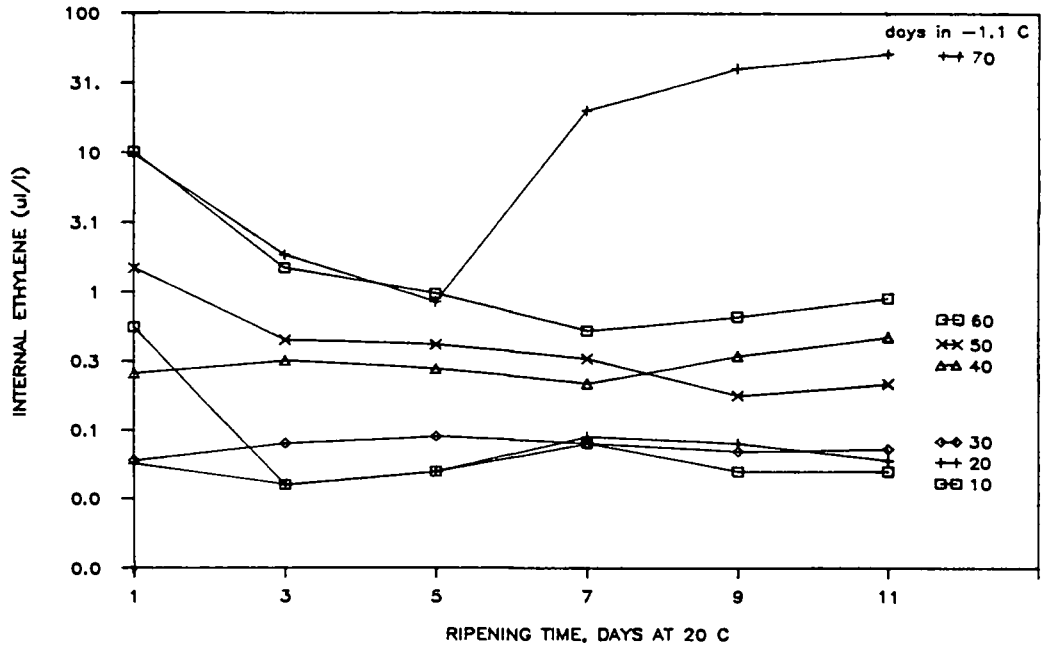


Fig.VI.5. Internal ethylene of 'Anjou' pears during ripening at 20°C, initially held for 25 days at 20°C, then stored at -1.1°C various amounts of time. LSD.05:5.7

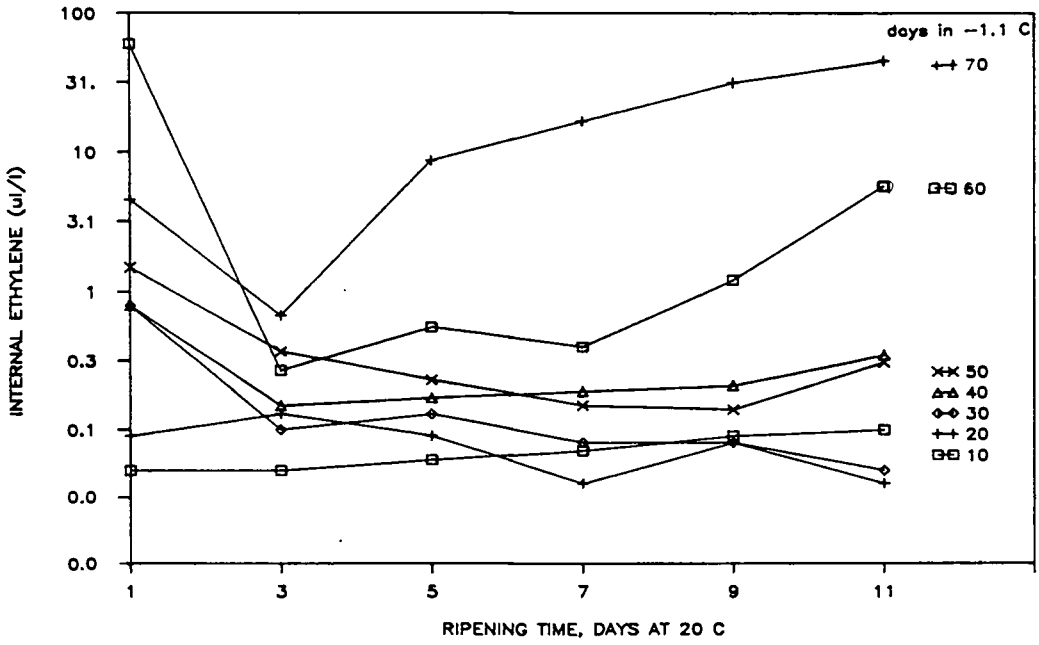


Fig.VI.6. Internal ethylene of 'Anjou' pears during ripening at 20°C, initially held for 40 days at 20°C, then stored at -1.1°C for various amounts of time. LSD.05: 4.7

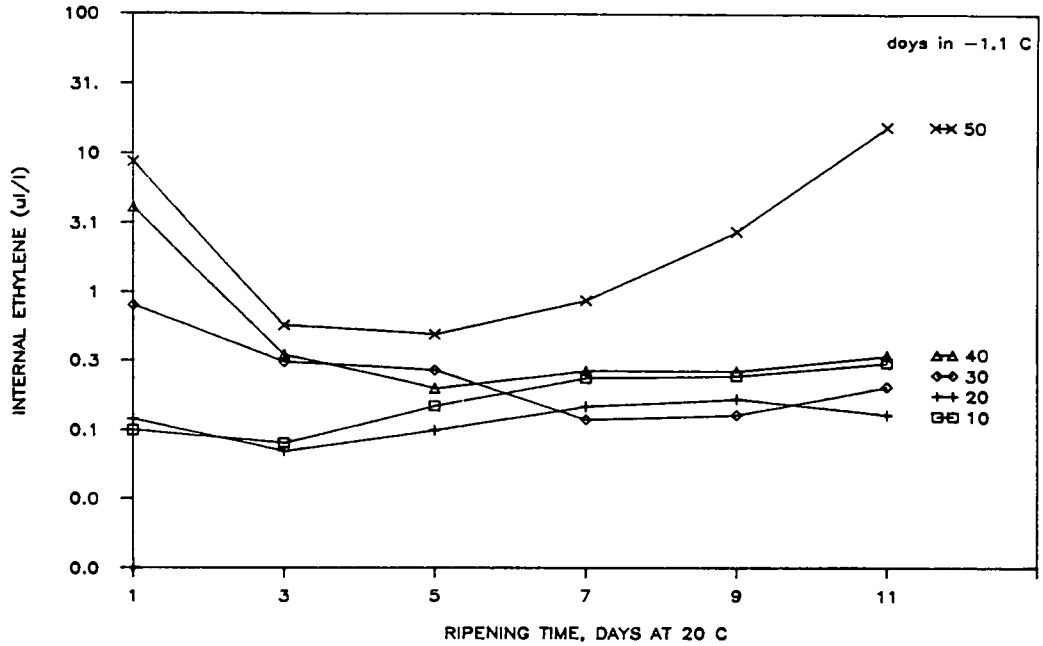


Fig.VI.7. Internal ethylene of 'Anjou' pears during ripening at 20°C, initially held for 55 days at 20°C, then stored at -1.1°C for various amounts of time. LSD.05: 5.3

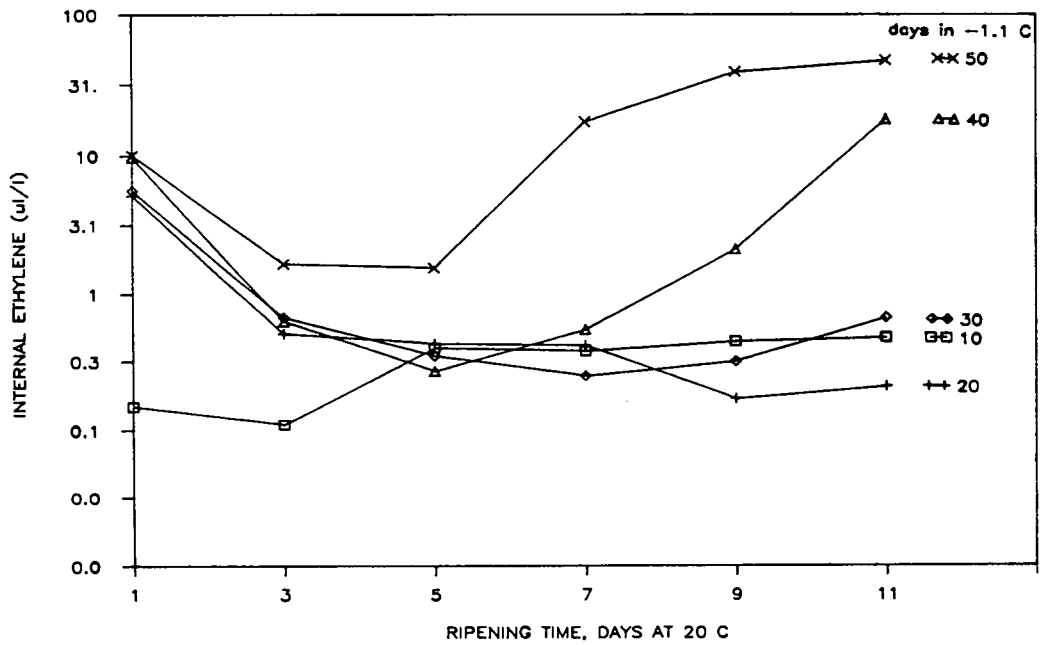


Fig.VI.8. Internal ethylene of 'Anjou' pears during ripening at 20°C, initially held for 70 days at 20°C, then stored at -1.1°C for various amounts of time. LSD.05: 5.1

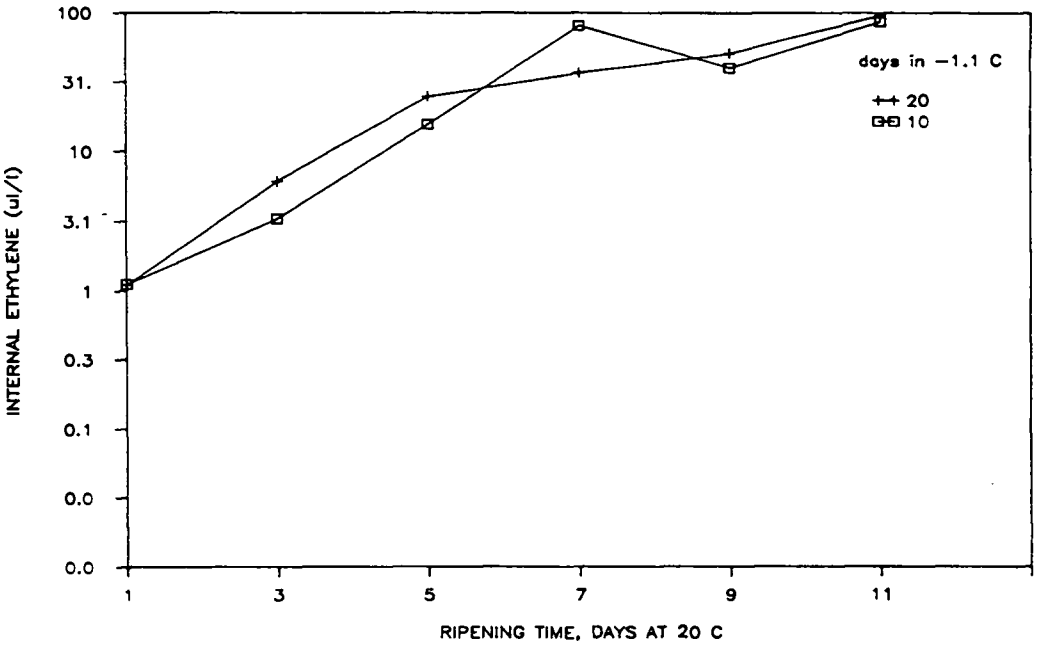


Fig.VI.9. Internal ethylene of 'Anjou' pears during ripening at 20°C, initially held for 85 days at 20°C, then stored at -1.1°C for various amounts of time. LSD.05: 5.8

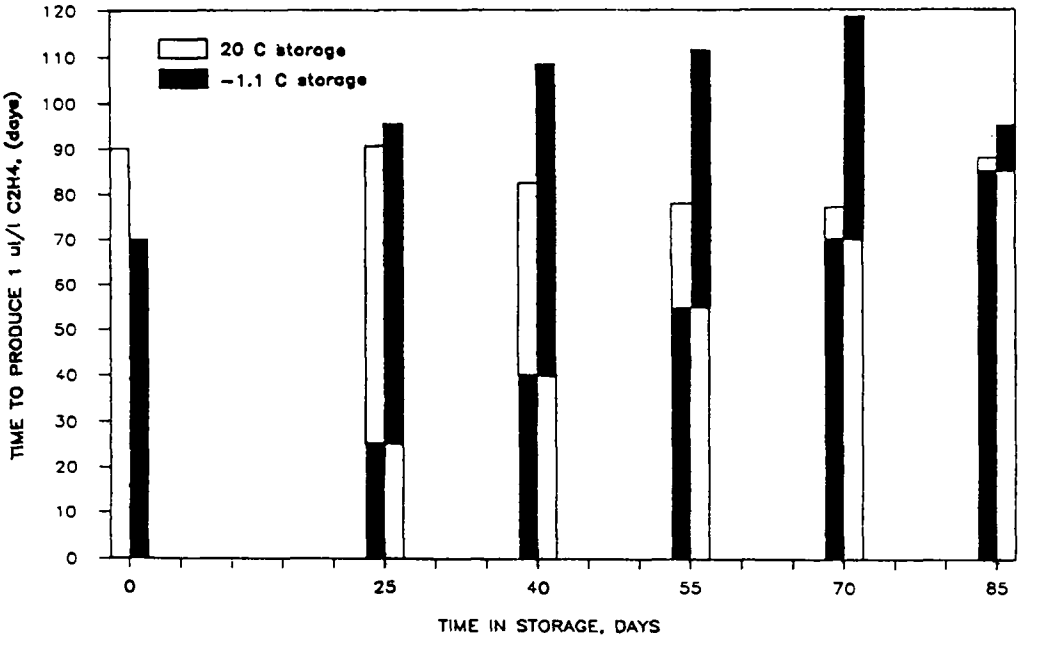


Fig.VI.10. Cumulative time in storage (-1.1°C and/or 20° C) for 'Anjou' pears to produce greater than 1 ul/l internal ethylene.

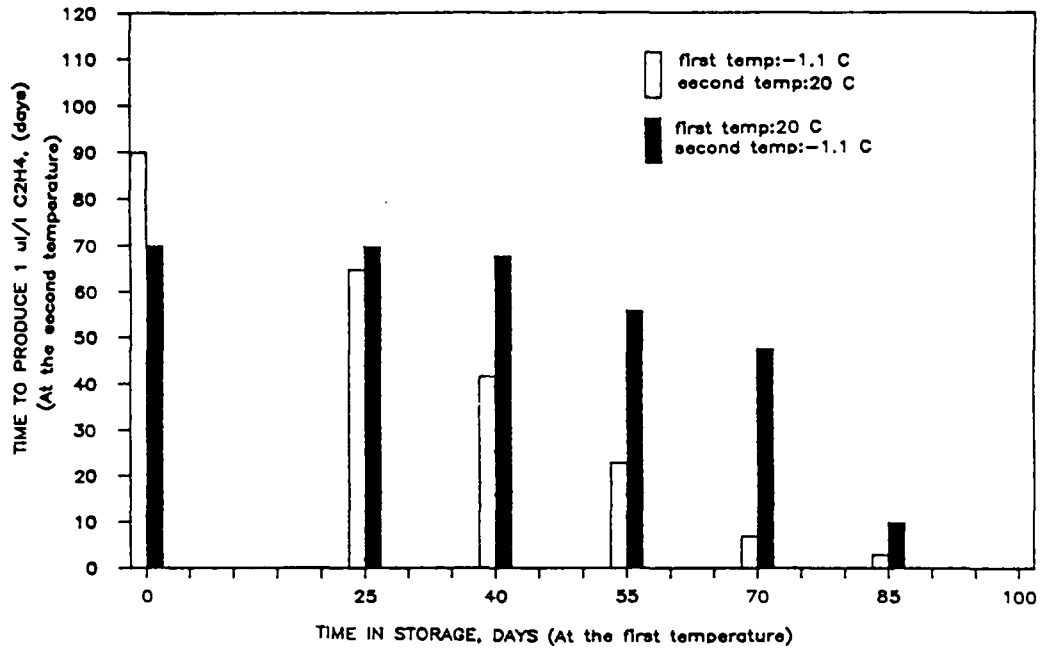


Fig.VI.11. Time for 'Anjou' pears to produce greater than 1 ul/l internal ethylene in the second storage temperature as a function of the time held in the first storage temperature.

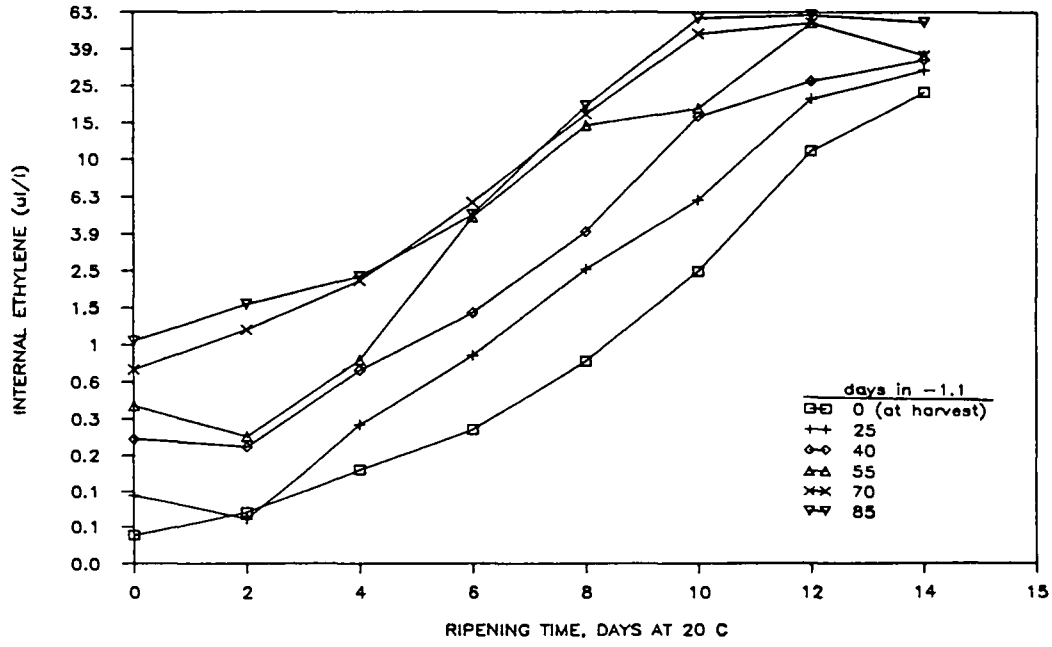


Fig.VI.12. Internal ethylene of 'Anjou' pears treated with 500 ul/l propylene at 20°C after storage at -1.1°C for varius amounts of time. LSD.05: 4.2

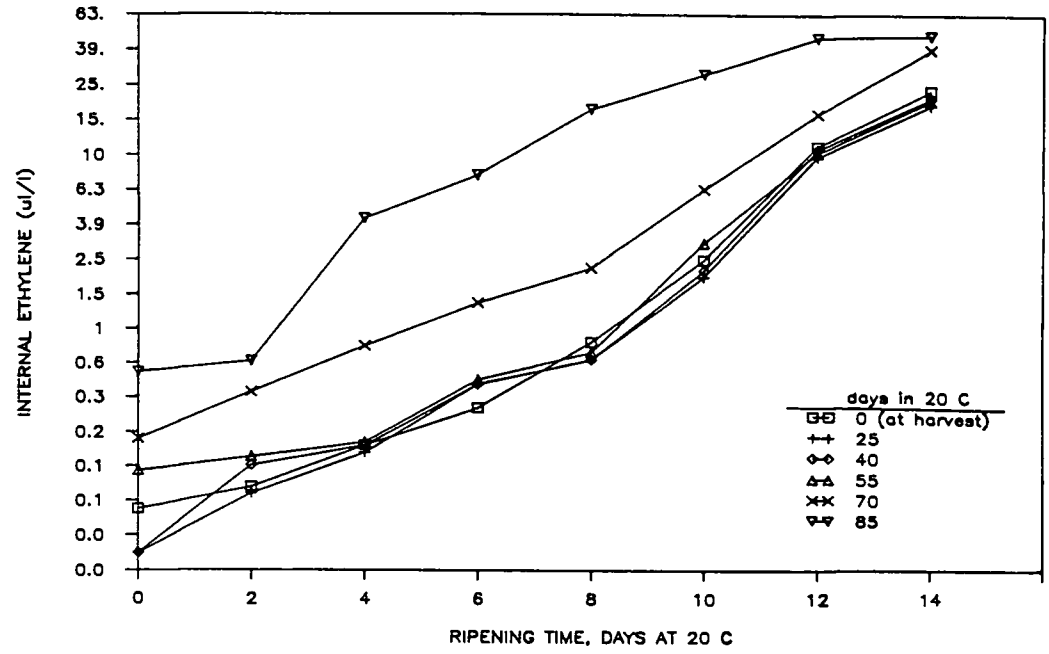


Fig.VI.13. Internal ethylene of 'Anjou' pears treated with 500 ul/l propylene after storage at 20°C for various amounts of time. LSD.05: 4.7

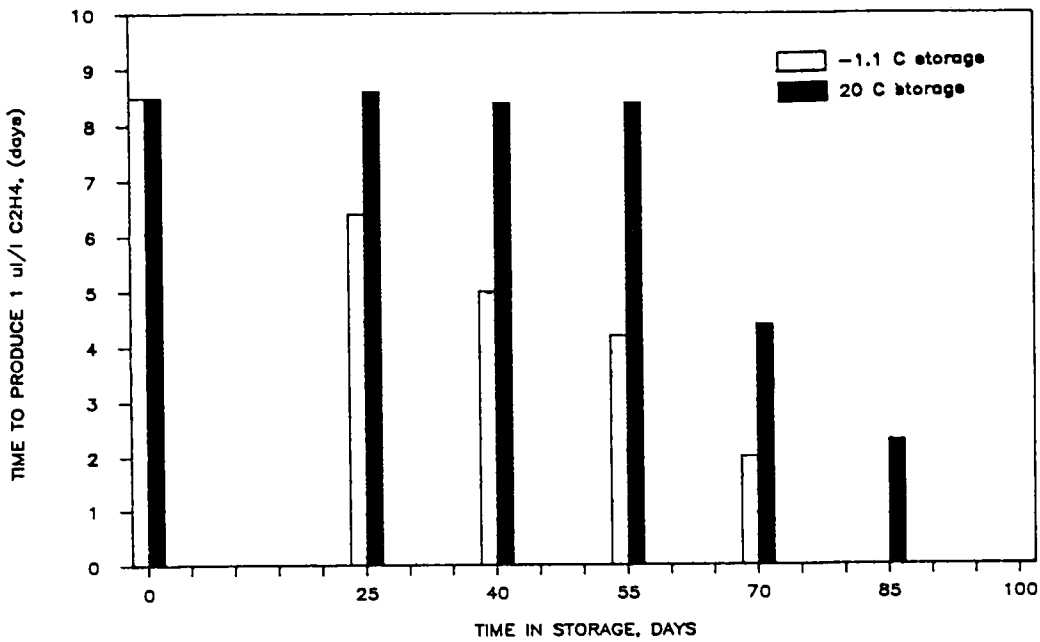


Fig.VI.14. Time required by 'Anjou' pears treated with 500 ul/l propylene at 20°C to produce 1 ul/l ethylene after storage at -1.1°C or 20°C for various periods of time.

CHAPTER VII

CONCLUSIONS

Mature 'Anjou' pear fruits in these experiments, required 55 to 70 days of -1.1° C storage in order to be able to produce sustainable internal ethylene greater than 1 ul/l, when transferred 20° C. This agrees with several previous studies during -1.1° C storage the pears did not lose firmness or chlorophyll and only slight increases in soluble polyuronides or total proteins were observed. However, after 55 or 70 days in -1.1° C storage, internal ethylene rose to 1.8 ul/l, ACC to 0.9 nmoles/g and titratable acidity decreased by 20%. Along with those changes at -1.1° C when the pears were warmed to 20° C, EFE activity peaked to 71 nl C_2H_4/g/h, the sensitivity of the fruit to exogenous propylene maximized, and linoleic acid had doubled. At this stage the fruits ripen very fast at 20° C, with losses in firmness synchronous with the rise in climacteric ethylene. The changes of the parameters of ethylene synthesis and other biochemical constituents associated with ripening may be typical of other ripening climacteric fruits after storage: depletion of the ACC pool, decreasing but enough EFE activity to sustain climacteric ethylene production, losses in amino acids and gains in protein and water soluble polyuronides.

It's somewhat surprising to observe EFE activity maximizing well in advance of both ACC concentration and internal ethylene.

Since ACC accummulates, clearly ACC synthase is not rate limiting, and it would seem that neither is EFE activity. Thus we are left with at least one rationalization in the concept that ACC may be sequestered or unavailable to EFE for convertion to ethylene. Whether this implies membrane barrier separation is an intriguing idea, especially in light of the changes in membrane lipid fatty acid unsaturation synchronous with chilling satisfaction. The lack of change in fatty acid unsaturation at 20° C storage casts a bit of shadow an that hypothesis, however.

Mature 'Anjou' pears held at 20° C after harvest, with no cold treatment are eventually capable of most of the ripening phenomena. They are unique in showing a much earlier loss in flesh firmness, 6 to 7 weeks ahead of the climacteric rise in ethylene. Climacteric ethylene production occured on the 70th to 90th week of 20° C storage, well after the fruit became eating ripe and soft to thumb pressure (about 20 N). Chlorophyll loss accompanied the loss in firmness and titratable acidity became half that at harvest. Free amino acid decreased and total protein decreased by about 20% when the climacteric ethylene initiated, along with the rise in ACC. EFE activity peaked about 25 days earlier. The sensitivity of the fruit to exogenous propylene increased and maximized and the requirements for chilling of fruit stored at 20° C decreased and minimized only after flesh firmness decreased to about 25 N. This pattern of ripening has not yet been observed in any other climacteric fruit and it may be a promising tool in order to investigate the role of softening in

the ripening process.

Fruits with partially satisfied chilling requirement ripened similarly to fruits that received no chilling. In all cases firmness decreased to about 25 N and preceded the climacteric rise in ethylene or increase in ACC levels. EFE activity reached a maximum well before ethylene, in all cases of partially chill-satisfied pears. The longer the duration in -1.1° C, the earlier was the onset of the parameters associated with ethylene synthesis. The changes were also more rapid for the other biochemical constituents associated with ripening.

Storage at 5° or 10° C resulted in a decrease of chilling requirements to 40 days for both storage temperatures. Linoleic acid increased about 50% on the 40th day of both 5° and 10° C storage. The pattern of 'Anjou' pear ripening after chilling at 5° or 10° C was intermediate between -1.1° and 20° C. Approximately 30% and 50% loss in firmness was observed during ripening at 20° C after 25 days of chilling at 5° and 10° C, respectively. Meanwhile internal ethylene only ranged up to 0.1 ul/l. Fifty percent loss in firmness is even observed in 'Anjou' pears stored at 10° C for 40 days. When followed by ripening at 20° C, this fruit readily produces ethylene and loses most of its remaining firmness. Storage at 10 ° C appears to stimulate ripening faster than all the other temperatures examined, probably because of the combined temperature effects warm enough for fruit softening and cool enough for the earlier ethylene synthesis.

Fruit sprayed with calcium during fruit development and then cold stored showed delayed softening, and required more time in storage (15 days) in all temperature treatments except in 10° C storage in order to produce 1 ul/l ethylene during ripening at 20° C compared to the water spayed controls. Calcium treatments had no effect on fatty acid profiles.

The practical implications from these studies are that by extending the chilling requirement such as under high calcium, one might expect substantially longer storability, yet have reasonably good ripening quality with strong capability to produce ethylene while fruits are still fairly firm quite late in storage. A further aspect is that delays in cooling such that 'Anjou' pears much turn right after harvest at temperatures in the 5° to 10° C range will have drastic effects both on fruit softening and shortening the time to initiate ethylene. Finally the 20° C data implies that 'Anjou' might be held up to 5 weeks without refrigeration with risk of some softening and certain chlorophyll loss, but without risk of producing ethylene. The added negative side of this is the problems of fungal pathogen growth and devastation at those warmer temperatures, and this was a difficulty in holding fruits experimentally at those higher temperatures. While one might expect the other varieties of winter pears to more or less behave as 'Anjou' it will remain to be demonstrated the extent to which these experimental parameters will follow the same patterns in apples, or other climacteric fruits. The separation of synchrony for softening and ethylene

pathways may provide some new tools to study reactions in fruit physiology without the interference or complication of one or the other factor.

CHAPTER VIII

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APPENDICES

Two-dimensional electroforesis procedures.

Two dimensional gel electrophoresis first indroduced by O'Farrell (6) has become very popular and is widely used for the analysis of complex protein patterns. The turnover, synthesis or modification of proteins during development or in response to environmental factors of embryonic nonchlorophyllous (6) and chlorophyllous (1) plants or cell cultures (3), results in changes in protein patterns that can be analyzed and compared by this technique.

Our intention was to investigate the changes in protein patterns during storage of 'Anjou' pears at -1.1° , and at 20° C. Different problems, however, such as perfection of the technique itself and the extraction procedures due to low protein content, interferences by phenolic compounds, and different tissue texture at different storage/ripening stages, affected the comparability of proteins and decreased the sensitivity of the method. Nevertheless the procedure for two dimensional gel electrophoresis is interesting enough to warrant consideration for future analysis of protein patterns of 'Anjou' pears or of fruits in general and it is hoped that the experience gained here, despite some disappointments, may be useful to others who follow.

Materials and methods. 'Anjou' pears sampled from the -1.1° or the 20° C storage temperature treatments were peeled, cored, quartered and frozen with liquid nitrogen. The frozen tissue was stored at -80° C until it was used. The frozen quarters then were ground under liquid nitrogen in a stainless steel Waring Blendor

until a fine white powder resulted. Twelve g of pear powder were combined with 20 ml of extraction buffer (0.2 M acetate/acetic acid, pH 5.6, containing 0.5% Triton X-100, and 5mM phenylmethylsulfonyl fluoride a protease inhibitor), 24 g insoluble PVPP, and 24 g XAD-4. PVPP and XAD-4 were prepared according to Loomis, et al (4,5) and equilibrated with the extraction buffer before use. The solution was stirred at 4° C for 60 min and filtered through glass wool. The insoluble material was then washed with additional 20 ml of buffer. The two filtrates were combined and centrifuged at 28,000xg for 20 min in a Sorvall RC-2B refrigerated centrifuge. Then the supernatant was made 15% TCA and the protein pellet was recovered by centrifugation at 10,000xg, washed five times with 0.1 M NH_4HCO_3 in methanol and dried under vacuum at 4° C.

The pellet was then solubilized (2) in SDS buffer: 4% (w/v) SDS, 2% (v/v) 2-mercaptoethanol, 10% (w/v) glycerol in 300 ul 0.5 M Tris, pH 8.5. The solution was heated for three min at 80° C and the insoluble material removed by centrifugation at 15,000xg for 20 min in an Eppendorf microfuge. Four volumes of cold (-20° C) 80% acetone were added and the solution was incubated overnight at -20° C. The pellet was then washed twice with 80% acetone and the pellet dried under nitrogen stream.

The pellet was then solubilized in buffer (9 M urea, 4% (v/v) NP-40, 2% (v/v) 2-mercaptoethanol, and 2% ampholytes pH 5-7, and 3-10) and the insoluble material was removed by centrifugation at 15,000xg for 20 min.

Two dimensional electrophoresis. The method described by O'Farrell (6) as modified by Hurkman and Tanaka (2) was used. Approximately 200 ug of protein were loaded at the acidic end of the focusing gels and overlaid with 10ul of 5 M urea. The upper (anode) buffer was 0.2% (v/v) H_2SO_4 and the lower (cathode) buffer was 0.5% (v/v) ethanolamine. The isoelectric focussing was conducted for 17 h at 250 V plus 1 h at 800 V. Following focussing the pH gradient was measured: The gels were cut into 0.5 cm pieces which were placed in test tubes containing 1 ml of freshly boiled 0.01 N NaCl and incubated overnight. The resulting solution was then measured with an Orion Research 901 pH meter.

The other gels were equilibrated for 45 min in an equilibration buffer (2.3% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (w/v) glycerol in 62.5 mM Tris-HCl, pH 6.8), frozen in 5 ml of the same buffer with dry ice/ethanol and stored at -80° C until used. The gels were warmed up and equilibrated once more in the same buffer for additional 45 min and used for the second dimension.

The second dimension SDS gels were 1.5 mm thick and consisted of a 14.8 cm 12% acrylamide separation gel overlaid with 1.2 cm stacking gel of 4% acrylamide. The focussing gel was sealed to the SDS gel with 5% agarose in equilibration buffer. Additional pieces of 5% agarose containing 0.1% bromophenol blue or molecular weight standards (rabbit muscle phosphorylase b; 97,400 KD, bovine serum albumin; 66,200 KD, hen egg white ovalbumin; 42,699 KD, bovine carbonic anhydrase; 31,000 KD, soybean trypsin

inhibitor; 21,500 KD, hen egg white lysozyme; 14,400 KD, Biorad). The running buffer was 25mM Tris, 195mM glycine, and 0.1% (w/v) SDS. The electrophoresis was run at 20 mamp/gel constant current at 12° C. The gels were stained with 0.01% Coomassie blue dye in 1:5:5 acetic acid: methanol:water.

Protein was determined by the method of Ramagli and Rodriguez (7).

One of the gels is shown in Plate.A.1.

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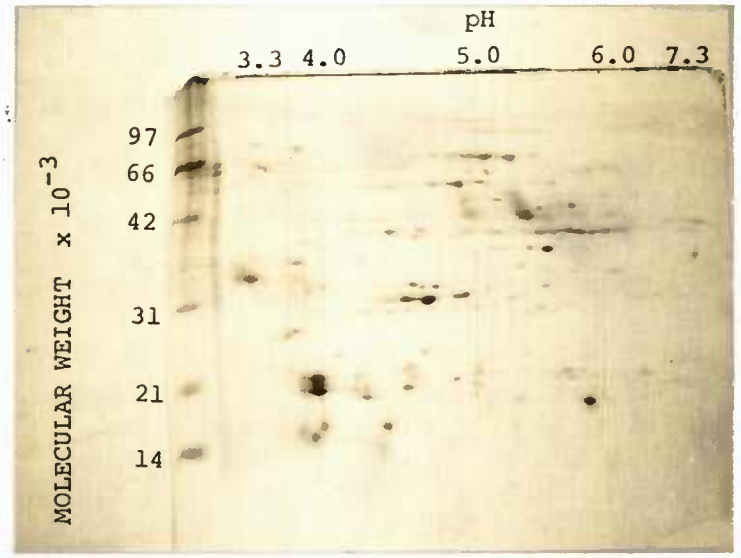

Plate.A.1. Two dimensional electroforesis protein pattern of 'Anjou' pears.

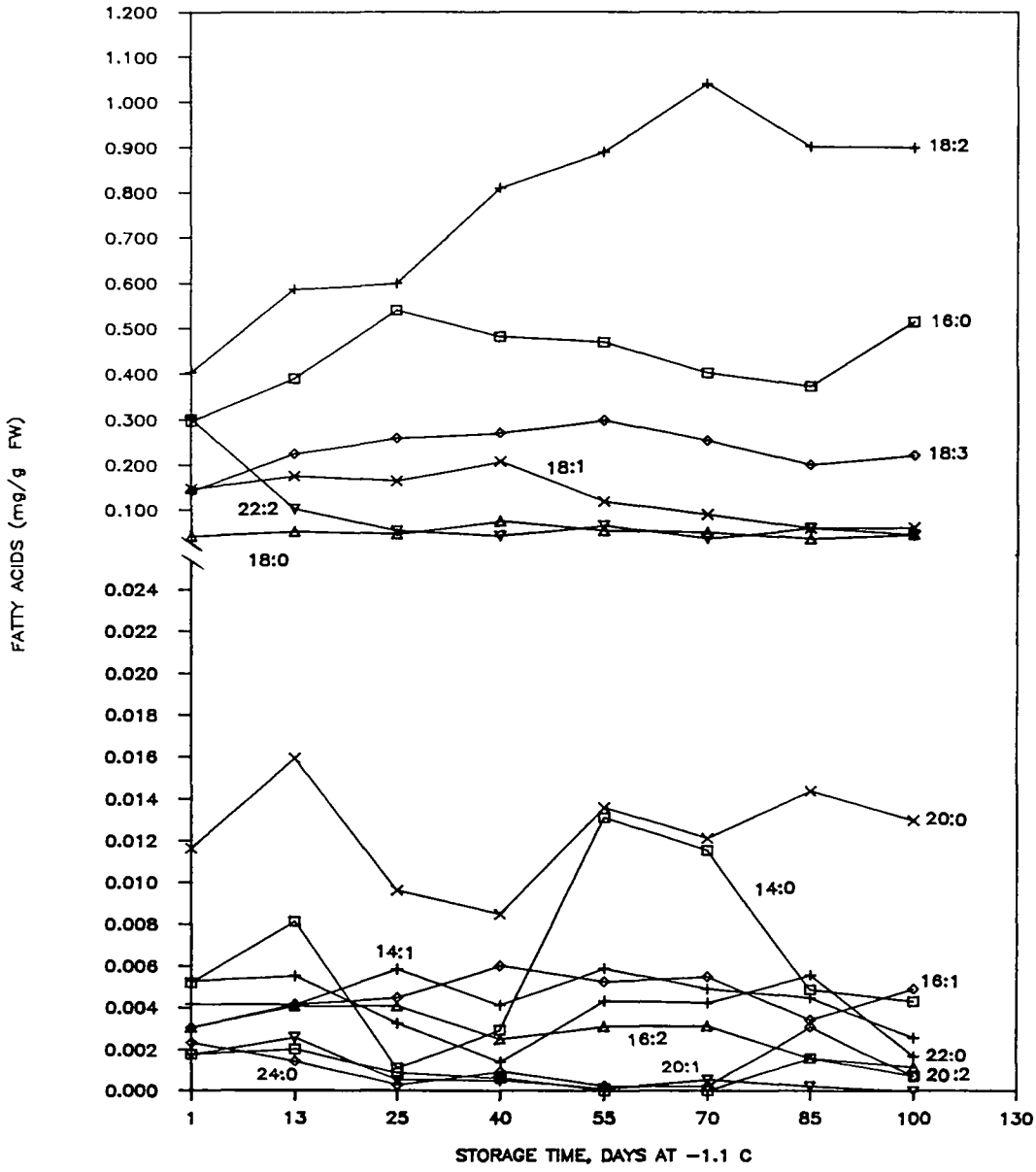

Fig.A.1. Fatty acid composition of calcium-treated 'Anjou' pears stored at -1.1'C.

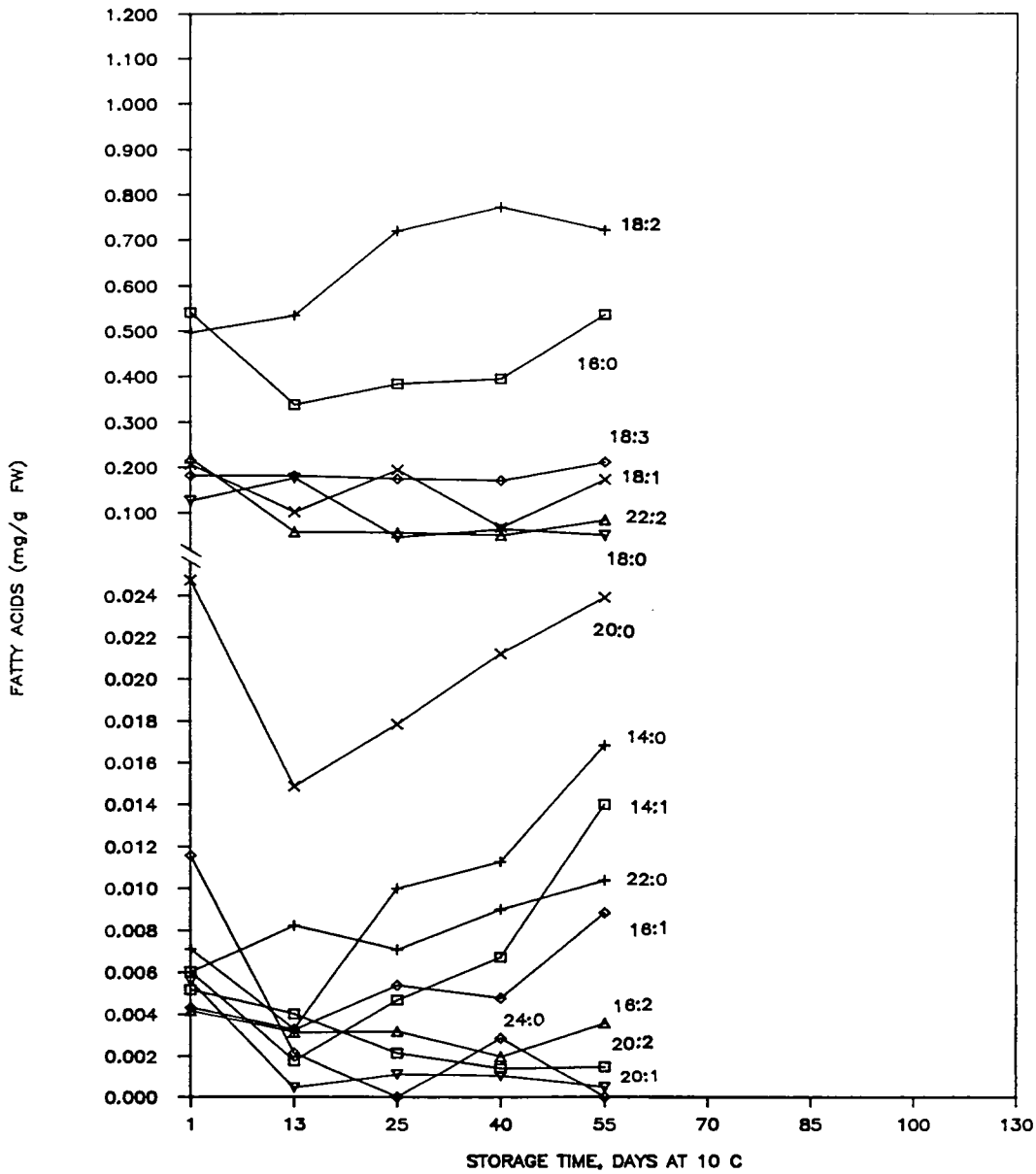

Fig.A.2. Fatty acid composition of 'Anjou' pears stored at 5°C.

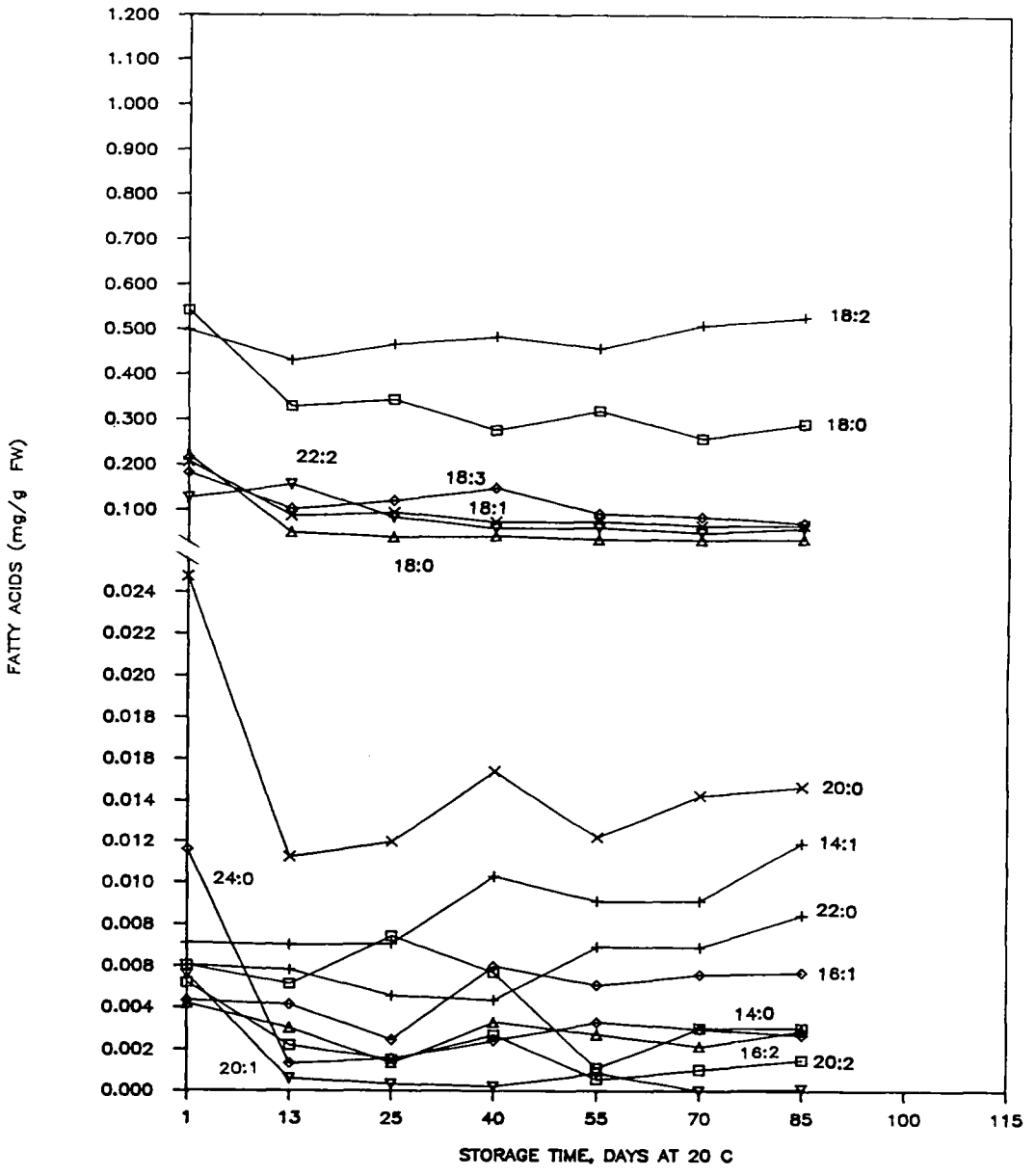

Fig.A.3. Fatty acid composition of 'Anjou' pears stored at 20°C.

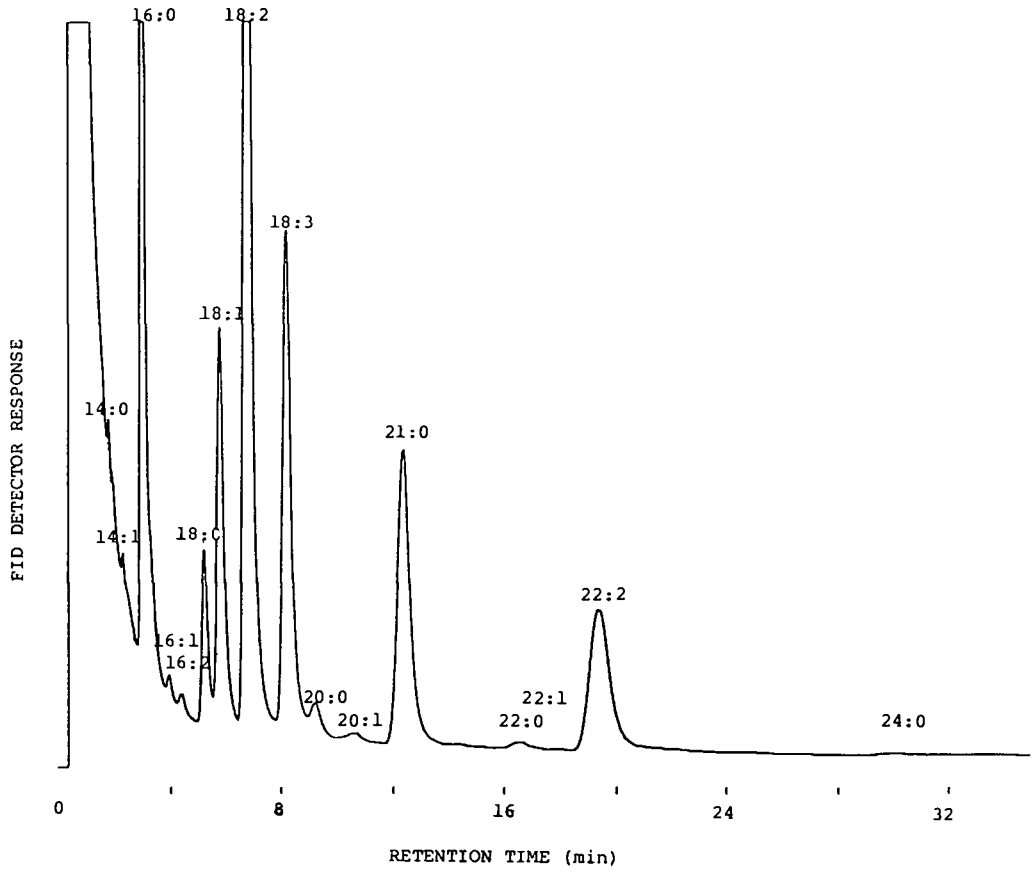

Fig.A.4. GLC chromatogram of typical 'Anjou' pear fruit total lipid fatty acid methyl esters. Column conditions: 3% SP-2310/ 2% SP-2300 on 100/200 mesh Cromosorb WAW (Supelco), 2 m x 4 mm O.D., column temperature 190° C, injector temperature 235° C, FID temperature 255° C, and N_2 carrier gas 30 ml/min.